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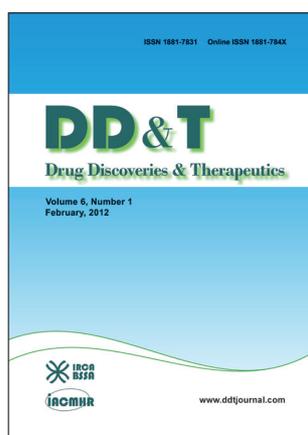
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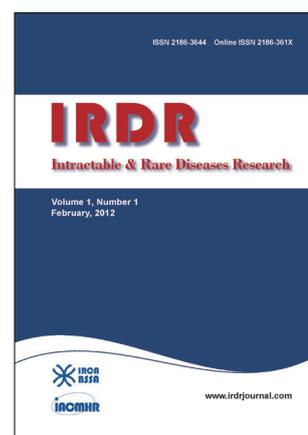
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Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: +81-3-5840-9968, Fax: +81-3-5840-9969
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Next generation sequencing: Coping with rare genetic diseases in China

David S Cram^{1,2,*}, Daixing Zhou¹

¹Berry Genomics Corporation, Beijing, China;

²Department of Anatomy and Developmental Biology, Monash University, Clayton, Australia.

Summary

With a population of 1.4 billion, China shares the largest burden of rare genetic diseases worldwide. Current estimates suggest that there are over ten million individuals afflicted with chromosome disease syndromes and well over one million individuals with monogenic disease. Care of patients with rare genetic diseases remains a largely unmet need due to the paucity of available and affordable treatments. Over recent years, there is increasing recognition of the need for affirmative action by government, health providers, clinicians and patients. The advent of new next generation sequencing (NGS) technologies such as whole genome/exome sequencing, offers an unprecedented opportunity to provide large-scale population screening of the Chinese population to identify the molecular causes of rare genetic diseases. As a surrogate for lack of effective treatments, recent development and implementation of noninvasive prenatal testing (NIPT) in China has the greatest potential, as a single technology, for reducing the number of children born with rare genetic diseases.

Keywords: Next generation sequencing (NGS), noninvasive prenatal testing (NIPT), whole exome sequencing (WES), chromosome disease, monogenic disease

1. Introduction

Genetic diseases, categorized as either chromosomal or monogenic diseases, affect approximately 1% of all individuals globally (1). There are over 100 different clinically recognized chromosome disease syndromes. Chromosome diseases are believed to be caused by de novo abnormal chromosomal rearrangements in the early preimplantation period of human development. The most prevalent is Down Syndrome (DS) with an incidence of 1 in 700 births. In contrast, around 7,000 distinct monogenic diseases have been described (2). These monogenic diseases originate from mutations carried in parental genomes and are inherited in an autosomal recessive, dominant or X-linked manner. In the Caucasian population, the most prevalent

monogenic disease is Cystic Fibrosis, with a carrier frequency of 1 in 25 and an incidence of 1 in 2,500 births. Based on the combined disease incidences of all chromosomal and monogenic diseases, genetic disease is considered relatively rare compared to more complex genetic diseases such as cancer. Being the most populated nation in the world, China has the largest share of patients with rare genetic diseases. It is generally believed that there are approximately 10 million such patients living among the 1.4 billion people (3), although this number could be grossly under-estimated due to patients with unrecognized or less severe disease.

Each year in China, we estimate that there are around 26,000 new DS patients added to the population. This estimate is based on an annual birth rate of 18 million newborn and a disease incidence of DS of 1 in 700 births. Factoring in the life expectancy of DS patients which today is generally over 50 years of age and, adjusting for population growth rate over the last 50 years, we estimate that DS patients alone currently account for around 1.4 million people. After DS, sex chromosomal diseases, such as Turner (45,X), Klinefelter (47,XXY), Triple X syndrome (47,XXX)

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Dr. David S Cram, Berry Genomics Corporation, Building 9, No. 6 Court, Jingshun East Rd, Chaoyang District, Beijing 100015, China.

E-mail: david.cram@berrygenomics.com

and Jacob (47,XYY) syndromes have a combined incidence of 1 in 1,000 births (4). With a near normal life expectancy, the number of Chinese individuals with sex chromosome disease syndromes is estimated to exceed 2 million. In a review of chromosome disease incidence in the United States (US), it has been estimated that the combined number of patients with other types of chromosome disease syndromes far exceeds that of DS and sex chromosome disease patients (5). On this basis, we estimate that the number of Chinese patients with rare genetic disease caused by chromosomal abnormalities alone is well over 10 million.

Although monogenic diseases are less prevalent than chromosome diseases, based on population size, they still represent a significant proportion of the overall genetic disease burden in China. On top of the list are blood disorders such as alpha- and beta- Thalassemia, Sickle Cell Anemia (SCA) and Hemophilia, the muscle disorders Duchenne Muscular Dystrophy (DMD) and Spinal Muscular Atrophy (SMA), the metabolic disease Phenylketonuria (PKU), the sensory disorder Hereditary Hearing Loss (HHL) and mental disabilities such as Fragile X Syndrome (FXS). In southern China, the incidence rates of alpha- and beta- thalassemia are among the highest in the world, with a combined carrier rate of 11% and 23% in Guangdong and Guangxi provinces respectively (6,7), making thalassemia the number one rare genetic disease in these regions. However, since the average life expectancy is less than 10 years, we estimate that the number of living thalassemia patients in these provinces would not exceed more than 25,000. For the other monogenic diseases mentioned, disease incidence is much lower, ranging from as high as 1 in 3,000 to as low as 1 in 10,000 individuals. Despite the low incidence rate of each individual single gene disease, their combined total still represents a large body of patients in China because of the sheer magnitude of the population size. We estimate that the number of existing patients afflicted with monogenic diseases exceeds well over a million in China.

2. Rare genetic disease management and treatment

Despite being increasingly recognized as an important health issue, management of rare genetic diseases in China still has long way to go. The main reason why China is currently lagging behind many other countries is primarily due to its large population size, with the majority of health service resources directed towards providing a good standard of general health care for all people. However, several other factors such as low economic status in the past, lack of ability to implement technological advances and a conservative cultural view of genetic disease have proven to be significant contributing factors that have impeded managing the

burden of genetic disease.

Even though the number of patients with genetic disease in China is very high, most stay confined in the family home and do not participate in daily life. Except in special events dedicated to a particular disease or disability, patients with genetic diseases are rarely seen in public. As in many other cultures, traditional Chinese values discourage a family from allowing the affected individual to go out. Without understanding the causes of the diseases, the society in the past tended to blame the family for the disability. Also, within the family, when a child was born with a genetic disease, there was a strong tendency for one of the parents to blame the other for bringing disrepute to the family, causing shame and guilt.

Armed with modern communication tools and internet, attitudes are rapidly changing. More than ever before, Chinese society as a whole is more likely to accept a person with a rare genetic disease. Families of rare genetic disease patients are openly sharing their experiences online. There are chat rooms created by internet companies to accommodate such needs though the regulations to protect patient privacy has not been put in place to safeguard information exchange. Recently, a public outcry against the Chinese internet giant Baidu's selling the hemophilia chat room information to a special interest group forced Baidu to abandon the practice.

This event also exposed lack of patient advocacy groups in China. During the event, it was the patient families, the public and the government agencies who were vocal and strongly demanded Baidu to make the correction, not only for the hemophilia chat room, but also for the chat rooms of the other rare genetic diseases. That being said, there is momentum in forming such advocacy groups. The Chinese Organization for Rare Diseases (CORD) represents one of such groups. Located in Beijing, CORD is visible in almost every major conference for genetic diseases. Further, since the first patient registry for genetic diseases was created in 2011 (8), registries are steadily growing.

In recent years, government sponsored programs for public awareness and screening of rare genetic diseases has played an important role in diseases prevention. In southern China, because of the prevalence of thalassemia, special educational programs were created to make the public aware of its existence, which led to a very high rate of acceptance for the thalassemia screening program. As a result, the birth rate of thalassemia patients has dropped substantially (9). To further such programs, in December of 2015, the National Health and Family Planning Commission (NHFPC) created a special committee consisting of eighteen prominent scientists and Chinese academy members, dedicated to research and advise the government in making policies for prevention and treatment of rare genetic diseases.

There is also a cultural shift in the way families

are coping with genetic disease in China. Now, more than ever, families and patients are presenting to clinics to seek the medical opinion of doctors regarding the disease and possible treatments to alleviate suffering. Medical doctors have become increasingly skilled at making the correct clinical diagnosis for most genetic diseases and some patients are willing to pay a small DNA testing fee to define the causative familial mutations. This is an important step if members of the families have married and are planning to have children, enabling the option of prenatal diagnosis. In cases where the type of disease is not obvious, sequencing based DNA testing of the likely genes can also be ordered to define the mutation(s) and provide a firm clinical diagnosis. Once parents have a better understanding of their child's disease and potential treatments, they are finding it much easier to come to terms with the disease in the family and cope better with the day to day impact of the disease burden.

For the vast majority of genetic diseases, there is no cure or effective treatments. Thus, once diagnosed, patients with rare genetic diseases have very limited treatment options. Patient care and support have improved substantially in recent years, but access to drugs is limited. As of 2014, the US FDA has approved seven enzyme replacement therapy drugs for lysosomal storage disorders, three drugs for hereditary angioedema, and one drug for cystic fibrosis (Kalydeco). The European Medicines Agency (EMA) has also approved one gene therapy drug (Glybera) for lipoprotein lipase deficiency. As listed in the clinical trial website (clinicaltrials.gov), some of these drugs are being evaluated under the guidelines of the Chinese Food and Drug Administration (CFDA). To receive treatments today, patients must be admitted to the trial cohorts.

Even if these drugs are approved by CFDA in the next few years, the cost of the drugs will prevent most patients from receiving treatment. On average, each drug costs about 300,000 US dollars per year per patient in the US and European Union (EU). The prices are set at such a high premium value because the number of patients in the US and EU is low, and because they are protected under the Orphan Drug Act. For some of these diseases, the number of patients in China is relatively high, making the pricing difficult to justify. The equivalent law underpinning the Orphan Drug Act has yet to be established in China. Such pricing will definitely render these drugs unaffordable for Chinese patients. The economy of rare genetic disease management in China will likely adopt a more cost-effective model for patients.

3. Next generation sequencing technologies for prevention of genetic disease

In China, prenatal diagnosis continues to play a small,

but significant role in prevention of chromosome disease, allowing couples the option of terminating an affected pregnancy. In all major hospitals in China, maternal serum screening and fetal ultrasound services are available to assist in the detection of fetuses with chromosome diseases. Confirmation of positive results is usually performed by amniocentesis and fetal karyotyping (10), although high-resolution array based chromosome analysis methods have been available in the last five years to detect more subtle chromosome disease syndromes. Nonetheless, detection of sex chromosome diseases still remains problematic because these syndromes cannot be detected by maternal serum screening and, in general, do not show any overt clinical signs on ultrasound (4).

Prevention of monogenic diseases has largely been ineffective because most couples are not aware of their carrier status prior to conceiving a child. Further, the vast majority of couples with a family history of genetic disease, and therefore at high risk, have not pursued testing due to the cost and time required to identify the causative mutations. Therefore, with traditional methods, the overall penetrance of diagnostic testing remains very low. Further, the situation has been confounded by other factors unique to China, including the fact that 50% of the population lives in rural areas and do not have good access to genetic services and, the cost of testing is not subsidized (11). Further, available genetic services are stretched to full capacity and thus it is logistically impossible with current laboratory infrastructure, staff expertise and equipment for public hospital diagnostic laboratories to cope, even if more patients presented for testing.

The advent of NGS technologies (12) such as noninvasive prenatal testing (NIPT) and whole exome sequencing (WES) offers new hope for disease prevention in China. In fact, China is one of the leading countries to develop and implement NIPT of maternal blood for fetal chromosome abnormalities (13,14). Several major companies including Berry Genomics and Beijing Genomics Institute have fundamentally driven this opportunity, making the test available for all pregnant women over the last five years. Furthermore, the CFDA took an unprecedented step to mandate that all sequencing platforms and technologies were accredited for clinical application, giving the doctors and patients more confidence with the test. NIPT has proven to be reliable and accurate, demonstrating very high sensitivity and specificity for detecting common aneuploidies such as T21 (DS), T18 (Edward syndrome) and T13 (Patau syndrome) as well as sex chromosome aneuploidies, with low false positive and negative rates (14).

The introduction of NIPT in China is beginning to have an impact on reducing the incidence of common chromosome diseases. For example, during 2015 where 18 million births were expected, commercial

companies performed approximately one million tests for pregnant women with borderline fetal risk. In this screened population, the detection rates for T21, T18 and T13 fetuses were approximately 1 in 200, 1 in 1,000 and 1 in 6,000, respectively. Since the vast majority of couples elected to terminate these affected pregnancies, the contribution of NIPT alone has already started a downward trend in prevention of chromosome disease in children, particularly DS. Therefore, with government support, expanding the availability of NIPT in China to the majority of pregnant women should be a key objective of commercial companies and hospital laboratories empowered with the technology. This will also require government subsidies for NIPT testing, further patient and doctor education and importantly, the development of a more cost-effective test to encourage higher uptake by pregnant women. If this can be achieved, in time, the balance will eventually be tipped in favor of preventing more babies born with chromosome diseases than babies born with these diseases.

There is also hope on the horizon for preventing single gene disease in China. Carrier testing before pregnancy is the key to success. Recently, WES technology has been developed and validated for identifying deleterious mutations associated with rare monogenic diseases (15). In the US, the uptake of WES has been high and is currently offered by several companies as a commercial service. China is heading in the same direction and within the next few years, it is anticipated that a cost effective WES test will be available to Chinese couples. To be quickly effective and provide the greatest impact, the testing strategy needs to be focused initially on the top 10 diseases and targeted to provinces or regions where the carrier frequency is known to be high. Thus couples identified at high genetic risk of having an affected child can have traditional invasive molecular testing of the fetus, which is widely available in most diagnostic laboratories. Alternatively, these couples could choose assisted reproduction and preimplantation genetic diagnosis (PGD) to select disease-free embryos for transfer and implantation (16). Using this approach couples can commence their pregnancy knowing that their fetus does not have the familial genetic disease. However, there are only around 20 fertility centers in China that have a license to perform PGD, and therefore access to this technology is currently limited.

Active research using the power of NGS technologies and effective clinical translation is central to further reducing the total burden of rare monogenic diseases in China (2,17-19). Currently, only half the genes and mutations associated with the 7,000 known monogenic diseases have been identified (2). With the increasing availability of whole genome sequencing (WGS), it has been predicted that within the next ten years, we will have a complete database of all genes and mutation types

causative of monogenic diseases. For the first time, this is bringing WGS closer to the clinic, providing valuable information to clinicians for making diagnoses of children with congenital conditions that were previously undiagnosable (17,18). One recent success story in China has been the application of NGS to unveil novel genes and mutations associated with hereditary hearing loss (20). This approach is now providing important information for genetic counseling and expanding the reproductive options for couples at genetic risk to prevent hearing loss in their offspring.

Current clinical research activities in China are now heavily focused on developing novel NIPT strategies for detecting the full spectrum of chromosome disease syndromes. NGS based NIPT methods for simultaneous detection of common aneuploidies as well as submicroscopic deletions and duplications are well advanced (21). At the clinical level, pilot studies are underway in several provinces to evaluate the reliability and accuracy of these new methodologies. In addition, promising NGS based technologies have been developed for NIPT of monogenic diseases. Haplotype based targeted sequencing of maternal plasma has been shown to be accurate for the diagnosis of HHL (22) and SMA (23). Further, an alternative NIPT method called circulating single molecule amplification and re-sequencing technology (cSMART) has also been demonstrated to accurately genotype the fetus in pregnancies at risk for Wilson Disease (24). In principle, even if there is no knowledge of the causative parental mutations, it is possible to develop these two technologies further for the diagnosis of a broader range of monogenic diseases. Thus, with a focused strategy to clinically implement these second-generation NIPT tests, it will be possible over time to substantially reduce the burden of chromosome and monogenic disease in China.

4. Conclusion

Management of the burden of rare genetic disease in China remains a challenging issue due to the sheer size of the population. With few treatment options, families cope remarkably well when caring for affected individuals. Government bodies, clinicians and patients are becoming increasingly educated about the causes of genetic disease. The way forward for China is active disease prevention, which will serve as a surrogate for the lack of effective and affordable treatments. A plethora of new NGS technologies are now widely available for detection of chromosome disease and uptake is rapidly growing. With the development of more cost effective tests combined with Government subsidies, increased numbers of pregnant women will more likely undertake NIPT for chromosome disease, and eventually when available, also embrace NIPT for monogenic diseases. If this can be achieved in the short

term, the number of Chinese children born annually with rare genetic diseases will rapidly decline and, over a sustained period of time, the number of existing individuals with rare genetic diseases will begin to steadily decline.

References

- Bankier A, Cram DS. Genetic testing: An informed choice. In: *The Sorting Society: Ethics of Genetic Testing and Therapy* (Thompson J, Skene L, eds.). Cambridge University Press, London, UK, 2008; pp.7-20.
- Boycott KM, Vanstone MR, Bulman DE, MacKenzie AE. Rare-disease genetics in the era of next-generation sequencing: Discovery to translation. *Nat Rev Genet.* 2013; 14:681-691.
- Wang JB, Guo JJ, Yang L, Zhang YD, Sun ZQ, Zhang YJ. Rare disease and legislation in China. *Lancet.* 2010; 375:716:708-709.
- Demaliaj E, Cerekja A, Piazzze J. Sex chromosome aneuploidies. aneuploidy in health and disease. In: *Aneuploidy in health and disease* (Storchova Z, eds.). InTech, Shanghai, China, 2012; pp.123-140.
- Wapner RJ, Martin CL, Levy B, *et al.* Chromosomal microarray versus karyotyping for prenatal diagnosis. *N Engl J Med.* 2012; 367:2175-2184.
- Xu XM, Zhou YQ, Luo GX, *et al.* The prevalence and spectrum of α and β thalassaemia in Guangdong Province: Implications for the future health burden and population screening. *J Clin Pathol.* 2004; 57:517-522.
- Xiong F, Sun M, Zhang X, Cai R, Zhou Y, Lou J, Zeng L, Sun Q, Xiao Q, Shang X, Wei X, Zhang T, Chen P, Xu X. Molecular epidemiological survey of haemoglobinopathies in the Guangxi Zhuang Autonomous Region of southern China. *Clin Genet.* 2010; 78:139-148.
- Zhang YJ, Wang YO, Li L, Guo JJ, Wang JB. China's first rare-disease registry is under development. *Lancet.* 2011; 378:769-770.
- Liao C, Mo QH, Li J, Li LY, Huang YN, Hua L, Li QM, Zhang JZ, Feng Q, Zeng R, Zhong HZ, Jia SQ, Cui YY, Xu XM. Carrier screening for alpha- and beta- thalassaemia in pregnancy: The results of an 11-year prospective program in Guangzhou Maternal and Neonatal hospital. *Prenat Diagn.* 2005; 25:163-171.
- An N, Li LL, Wang RX, Li LL, Yue JM, Liu RZ. Clinical and cytogenetic results of a series of amniocentesis cases from Northeast China: A report of 2500 cases. *Genet Mol Res.* 2015; 14:15660-15667.
- Chopra M, Duan T. Rare genetic disease in China: A call to improve clinical services. *Orphanet J Rare Dis.* 2015; 10:140.
- Lohmann K, Klein C. Next generation sequencing and the future of genetic diagnosis. *Neurotherapeutics.* 2014; 11:699-707.
- Liang D, Lv W, Wang H, Xu L, Liu J, Li H, Hu L, Peng Y, Wu L. Non-invasive prenatal testing of fetal whole chromosome aneuploidy by massively parallel sequencing. *Prenat Diagn.* 2013; 33:409-415.
- Song Y, Liu C, Qi H, Zhang Y, Bian X, Liu J. Noninvasive prenatal testing of fetal aneuploidies by massively parallel sequencing in a prospective Chinese population. *Prenat Diagn.* 2013; 33:700-706.
- Yang Y, Muzny DM, Reid JG, *et al.* Clinical whole-exome sequencing for the diagnosis of Mendelian disorders. *N Engl J Med.* 2013; 369:1502-1511.
- Berger VK, Baker VL. Preimplantation diagnosis for single gene disorders. *Semin Reprod Med.* 2014; 32:107-113.
- Madrigal I, Alvarez-Mora MI, Karlberg O, Rodríguez-Revenga L, Elurbe DM, Rabionet R, Mur A, Pie J, Ballesta F, Sauer S, Syvänen AC, Milà M. Efficient application of next-generation sequencing for the diagnosis of rare genetic syndromes. *J Clin Pathol.* 2014; 67:1099-1103.
- Grody WW, Thompson BH, Hudgins L. Whole-exome/genome sequencing and genomics. *Pediatrics.* 2013; 132(Suppl 3):S211-S215.
- Rabbani B, Mahdih N, Hosomichi K, Nakaoka H, Inoue I. Next-generation sequencing: Impact of exome sequencing in characterizing Mendelian disorders. *J Hum Genet.* 2012; 57:621-632.
- Wei Q, Zhu H, Qian X, Chen Z, Yao J, Lu Y, Cao X, Xing G. Targeted genomic capture and massively parallel sequencing to identify novel variants causing Chinese hereditary hearing loss. *J Transl Med.* 2014; 12:311.
- Yin AH, Peng CF, Zhao X, *et al.* Noninvasive detection of fetal submicroscopic abnormalities by semiconductor sequencing of maternal plasma DNA. *Proc Natl Acad Sci U S A.* 2015; 112:14670-14675.
- Meng M, Li X, Ge H, *et al.* Noninvasive prenatal testing for autosomal recessive conditions by maternal plasma sequencing in a case of congenital deafness. *Genet Med.* 2014; 16:972-976.
- Chen M, Lu S, Lai Z, Chen C, Luo K, Yuan Y, Wang Y, Li S, Gao Y, Chen F, Asan, Chen D. Targeted sequencing of maternal plasma for haplotype-based noninvasive prenatal diagnosis testing of spinal muscular atrophy. *Ultrasound Obstet Gynecol.* 2016. Doi: 10.1002/uog.15947. .
- Lv W, Wei X, Guo R, Liu Q, Zheng Y, Chang J, Bai T, Li H, Zhang J, Song Z, Cram DS, Liang D, Wu L. Noninvasive prenatal testing for Wilson disease by use of circulating single-molecule amplification and resequencing technology (cSMART). *Clin Chem.* 2015; 61:172-181.

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Fragile X syndrome: A review of clinical management

Reymundo Lozano^{1,2,*}, Atoosa Azarang^{1,2}, Tanaporn Wilaisakditipakorn^{1,2}, Randi J Hagerman^{1,2}

¹Medical Investigation of Neurodevelopmental Disorders MIND Institute, UC Davis, CA, USA;

²Department of Pediatrics, UC Davis, Sacramento, CA, USA.

Summary

The fragile X mental retardation 1 gene, which codes for the fragile X mental retardation 1 protein, usually has 5 to 40 CGG repeats in the 5' untranslated promoter. The full mutation is the almost always the cause of fragile X syndrome (FXS). The prevalence of FXS is about 1 in 4,000 to 1 in 7,000 in the general population although the prevalence varies in different regions of the world. FXS is the most common inherited cause of intellectual disability and autism. The understanding of the neurobiology of FXS has led to many targeted treatments, but none have cured this disorder. The treatment of the medical problems and associated behaviors remain the most useful intervention for children with FXS. In this review, we focus on the non-pharmacological and pharmacological management of medical and behavioral problems associated with FXS as well as current recommendations for follow-up and surveillance.

Keywords: Fragile X syndrome, Autism Spectrum Disorder, Intellectual Disability, developmental delay, premutation, clinical management, clinical guidelines, treatment, medical problems, *FMRI*, *FMRP*

1. Introduction

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability (ID) and the most common monogenetic cause of autism (ASD) that is known. FXS was initially described in 1969 by Lubs and colleagues (1) and the first fragile X-linked pattern of inheritance was reported by Martin and Bell in 1949 (2,3). In FXS there is a mutation in the Fragile X Mental Retardation 1 (*FMRI*) gene which involves an expansion of more than 200 CGG repeats. Individuals in the normal population have approximately 5 to 40 CGG repeats within *FMRI* and individuals who are carriers for FXS (premutation) have 55 to 200 CGG repeats (4-6). The molecular basis of FXS is characterized by the CGG full mutation and methylation of the cytosine bases, which leads to silencing of transcription and

deficiency or absence of the encoded protein, Fragile X mental retardation protein (FMRP). FMRP is a major protein regulator of the translation of many mRNAs involved in synaptic plasticity (7). Therefore, in FXS the lack of FMRP causes significant intellectual deficits. Usually, expansions occur between generations when passed on by a female with the premutation into a full mutation in the offspring (8). Women, who are known carriers of the *FMRI* gene mutation, can obtain prenatal diagnosis including chorionic villus sampling (CVS) and/or amniocentesis studies as recommended by the American College of Obstetricians and Gynecologist (9). Preimplantation diagnostic services and *in vitro* fertilization are also available (10-12). Individuals with FXS present with a wide range of learning disabilities ranging from normal functioning to borderline cognition or mild to severe ID. The average Intelligence Quotient (IQ) of males with the full mutation is 40 (13). Intellectual and developmental disability occurs in 85% of males and 25% of females. Furthermore, FXS also accounts for approximately 2 to 5% of all individuals diagnosed with ASD. In FXS about 60% of males have ASD (14). Physical manifestations are subtle in infants and young boys. These include: midface hypoplasia with sunken eyes, arched palate, macroorchidism, and large cupped ears among others (Figure 1).

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*Address correspondence to:

Dr. Reymundo Lozano, Medical Investigation of Neurodevelopmental Disorders MIND Institute, UC Davis, CA, USA; Department of Pediatrics, UC Davis, Sacramento, CA, USA.

E-mail: Reymundo.lozano@mssm.edu



Figure 1. Young boy with fragile X syndrome and his Go Talk device. Note prominent ears with cupping of the pinnae. The participant's family provided informed consent for the use of this picture.

Medical problems associated with FXS include mitral valve prolapse, otitis media, seizures, strabismus, joint laxity, sleep disturbances, and gastrointestinal problems. In this review, we provided a summary of the prevalence and clinical management of medical problems associated with FXS other than ID and ASD (Table 1).

2. Fragile X syndrome associated medical problems

2.1. After birth problems

Boys with FXS are slightly larger than average in weight at birth. The mean birth weight from earlier studies ranges from 3,490 gms. to 4,046 gms. in white male infants (15). The mean birth weight of boys with the FXS was in the 70th percentile, they also had a higher birth weight than their siblings when this was corrected for gestational age and sex (16). The mean birth weight in FXS was increased and the average linear growth was also above the mean for typically developed boys with the greatest increase after the second year of life. In contrast, the weight measurements were on average below the mean until two years of age. It is suggested that in FXS there is a disturbance of early infantile growth (17); however, the overall proportion of infants with low birth weight was similar to that in the general population (18). After birth, the head circumference tends to rise above the 50th percentile and continues to be larger than those without FXS. Jacobs *et al.* noted that in six of nine affected men, the head circumference was greater than the 90th percentile (18), but other studies have shown that the mean head circumference (19-21) and the mean birth length are not different of those of control population (21). Hagerman and colleagues found no difference in the height, weight or head circumference of girls with FXS compared with those without the full mutation (22).

Some studies reported that the height of males with FXS is greater than the 50th percentile and height curves for FXS were higher at nearly every point in

the prepubertal section of the curves, but height was lower at postpubertal ages (23,24). A subset of children with FXS can be misdiagnosed as having Sotos syndrome or Prader-Willi syndrome (25). The Prader-Willi phenotype (PWP) can be observed in FXS and it consists of extreme obesity, hyperphagia, lack of satiation after meals, small genitalia, delayed puberty, sometimes short stature and stubby hands and feet (26-28). Sotos-like syndrome was reported in 1986 in two boys with FXS featuring large size at birth, unusual length, large head circumference and minor facial abnormality (29).

Structural longitudinal magnetic resonance imaging (MRI) study of preschoolers with FXS observed generalized brain overgrowth compared to controls, evident at age two and maintained across ages 4-5 (30). The molecular biology of FXS suggests a possible mechanism for brain growth patterns. Harlow and colleagues have demonstrated that FMRP inhibits the generation of progenitor neurons from glia cells but enhances the glial cell number in mouse cerebral cortex, suggesting that the lack of FMRP, as seen in FXS might result in an increased proliferation of progenitor glial cells and subsequent cerebral cortical overgrowth (31). The presence of early brain differences in young children with FXS points to aberrant early brain development in this condition (31).

FMRP also regulates the phosphatase and tensin homolog (*PTEN*) gene translation that in turn regulates growth. The results of genetic and regression analysis showed that in both boys and girls, total pubertal height gain is impaired, whereas the rate of growth during the preadolescent period is increased, compared with the growth rate of subjects without FXS. The study demonstrates the linear effect of progressively reduced levels of FMRP on a number of physical measurements (32). This effect is predictably less strong in females than in the males because of the presence of the second unaffected X chromosome. The inverse relationship of height and limb length with FMRP deficit supports a possible role of hypothalamic dysfunction in growth disturbances in FXS that may be more severe in those with the PWP (33). This dysfunction may cause a premature increase in the pulsating secretion of high doses of estrogen, thus leading to earlier epiphyseal maturation (34). The hypothesis of premature activation of the hypothalamic-pituitary-gonadal axis may explain the cause of growth impairment in FXS and occasional precocious puberty in females with FXS, a few cases have been reported (35,36).

2.2. Otitis media (OM)

OM is one of the most frequent medical problems associated with FXS. Even when children with FXS have a high pain threshold and may not specifically complain about ear pain, 85% of children with FXS

Table 1. Summary of medical problems and management

Medical problem	Prevalence	Age of presentation	Severity	Complications	Recommendations	Follow up
Growth	Low birth weight - FXCRC: 9% - Other studies: 8% Preterm - FXCRC: 16% - Other studies: 12%	- Brain overgrowth 2-5 years - Height deceleration: after puberty	Moderate	- Overweight	- Lifestyle changes including healthy diet and exercise to minimize problems associated with increased weight	- Monitor patient's weight, height and head circumference closely in each visit
Otitis media	- FXCRC: 55% - Other studies: 45-85%	Childhood	Mild	- Acute sinusitis - Recurrent otitis media - Exacerbate the cognitive and language deficits - Conductive hearing loss	- Pneumococcal and influenza vaccines - Breastfeeding for at least 4-6 months - Eliminate passive exposure to tobacco smoke - Reduce pacifier and bottle usage - Antibiotic therapy - Ear tubes placement	- Surveillance of the potential adverse effects of antibiotic prophylaxis including hypersensitivity reaction and gastrointestinal problems
Seizures	- FXCRC: 10 % - Other studies: 12-18%	Early childhood	Mild to severe	- Developmental and behavioral morbidity	- Educate parents and follow up patients with history of seizures - Carbamazepine - Valproic acid	- EEG - Drug-specific blood test - Discontinue medication after patient is seizure-free for 2 years unless EEG is abnormal
Mitral valve prolapse	- FXCRC: 0.8% - Other studies: 50% of males and 20% of females	Childhood to adolescence	Mostly asymptomatic	- Rarely mitral regurgitation, congestive heart failure and endocarditis	- Mitral valve repair or replacement rarely required	- Surveillance cardiac evaluation
Gastrointestinal problems	- FXCRC: 2%-8% - Other studies: 11%	Childhood to adolescence	Mild	- Failure to thrive - Irritability - Behavioral problems	- Thickening agents - Antacids - Histamine-2 blockers - Proton pump inhibitors	- Surveillance on height and weight
Sleep problems	- FXCRC: 26.9% - Other studies: 32%-47%	Infancy and childhood	Mild to moderate	- Disturbance in daytime performance - Behavioral problems	- Behavioral intervention - Melatonin - Clonidine	- Monitor the side effects of sleep medications - Careful history of sleep habits
Obstructive sleep apnea	- FXCRC: 7% - Other studies: 21-32%	Childhood	Moderate to severe	- Vigilance impairment and neuropsychological deficit - Decrease in day time performance - Increase in behavioral problems	- Steroids to reduce tonsillar hypertrophy - Continuous positive airway pressure - Surgical intervention including tonsillectomy - Behavioral therapy	- Monitoring and managing obstructive sleep apnea in every child visit - Refer to sleep specialist
Strabismus	- FXCRC: 17% - Other studies: 8%	Early childhood	Mild to moderate	- Amblyopia	- Corrective eyeglasses - Visual training exercise - Surgery	- Comprehensive ophthalmologic examination of every child with FXS by age 4 or sooner if strabismus is detected
Tic disorder	- FXCRC: 6% - Other studies: 15%	Before 18 years of age	Mild	- Usually uncomplicated	- Educational and supportive approach - Medications rarely necessary	- Long term relationships to improve patients self-esteem and coping with their tics
Toileting issues	49%	Delayed several years	Mild to moderate	- Enuresis - Encopresis	- Behavioral therapy - Desmopressin - Fiber supplements in case of constipation	- Toileting counselling by 1 year of age
Sensory integration problem	20%-50%	Early childhood	Moderate to severe	- Lack of participation in activities that are necessary for brain development	- Medications related to inattention, anxiety, aggression and autonomic symptoms - OT therapy	- Assessment tools such as sensory profile questionnaire, etc.

FXCRC: Fragile X Clinical and Research Consortium Study; FXS: Fragile X syndrome.

have at least one diagnosed episode of OM (37). An ear examination is warranted for any change of behavior and sleep patterns as well other symptoms including fever, vomiting, and headache. Children with FXS commonly develop OM complications including decreased hearing acutely and at least one-fourth develop acute sinusitis. Furthermore, OM recurs in about 50% of children with FXS recurrent 5 years of age (37). There is not data reported about the rates of chronic otitis. Recurrent otitis media may cause conductive hearing deficits and exacerbate the cognitive, language, and behavior problems that exist in this syndrome (38); therefore, the treatment of OM should be aggressive. The American Academy of Pediatrics initial recommendation for uncomplicated OM is an observation period for children 6 months to 2 years with unilateral OM without otorrhea and for children older than 2 years with bilateral OM without otorrhea; however, we recommend to consider skipping the observation period and using antibiotic therapy in children with FXS (39). The craniofacial changes in FXS including a long face and collapsible Eustachian tubes predispose children to OM infections. Signs of slight redness, mobility impairments and abnormal positioning of tympanic membrane (TM) such as retraction or bulging, should be carefully assessed. Initial antibiotic therapy for 10 days includes a high dose of amoxicillin (80-90 mg/kg per day in 2-3 divided doses). If not improvement after 48-72 hour, Amoxicillin-clavulanate (same dose of amoxicillin + 6.4 mg/kg per day of clavulanate (amoxicillin to clavulanate ratio, 14:1) in 2 divided doses) or Ceftriaxone (50 mg IM or IV for 3 day) are recommended. A low threshold for early tympanostomy tube placement and antibiotic prophylaxis (amoxicillin low dose) is also advised. The potential adverse effects of antibiotics, principally allergic reaction and gastrointestinal tract consequences, such as diarrhea are important considerations for tympanostomy tubes over prophylaxis.

Clinicians should stress the recommendations of pneumococcal conjugate and influenza vaccine to all children, according to the schedule of the Advisory Committee on Immunization Practices, American Academy of Pediatrics (AAP), and American Academy of Family Physicians (AAFP). Multiple studies provide evidence that breastfeeding for at least 4 to 6 months reduces episodes of OM and recurrent OM (40-43). Eliminating passive exposure to tobacco smoke could also reduce the incidence of OM in infancy. In addition, bottles and pacifiers have been also associated with OM (44-49). Finally, Xylitol syrup, chemically a pentitol or 5-carbon polyol sugar alcohol, has shown a statistically significant reduction (25%) in the risk of occurrence of OM among healthy children (50).

2.3. Seizures

Seizure prevalence studies in FXS have shown

discordant results; a study conducted in neurology clinics reported a broad prevalence range of 14% to 44%, while studies that focus on FXS patients in community hospitals or FXS clinics reported lower ranges of 12-18% (51-53). Typically, males have a higher prevalence when compare to females. In the national survey of caregivers of individuals with FXS, from 1,394 individuals, 14% of males and 6% of females were reported to have seizure (54,55). Studies in the *Fmr1* knockout (KO) mouse shows immature dendritic connections, increased number of long and thin spines which point to the deficiency in the normal selection or pruning of the synaptic contacts that occurs in neuronal development (56,57). These results demonstrate that FMRP is important in the maturation of adult dendritic spine morphology (58). Immature dendritic connections can predispose the KO mouse to audiogenic seizures, although deficits in gamma amino butyric acid (GABA) inhibition are also related to the seizures in FXS (59,60). Similar abnormal dendritic formations are also observed in the brain of humans with FXS and may explain the higher frequency of seizures. In addition to structural changes, the absence or deficiency of FMRP leads to increased neuronal excitability and susceptibility to seizure (61). Other studies hypothesize that the pathophysiology of seizures in those with FXS can be related to the imbalance of the excitatory and inhibitory neurotransmitter systems (60,61).

It is important to consider that many children with FXS have abnormal electroencephalogram (EEG) without overt seizures (62,63). In those with overt seizures, all types of seizures can occur. Some studies have shown a predominance of generalized seizures (64), secondary generalized seizure and status epilepticus seizure (64). Seizures in FXS may also resemble benign focal epilepsy in childhood with centro-temporal spikes (65,66). In general, complex partial seizures are the major type of seizure in FXS. The observed seizures are – typically- not severe and mostly limited to childhood (66); however, the presence of seizures at an early age appears to be associated with developmental and behavioral morbidity that can impact brain function. Remarkably, those patients with FXS and seizures are more likely to have ASD (67). The current practice is to educate parents and follow-up patients closely for any possible episodes of seizure: starring spells, unexplained behavior, atypical facial gestures, vomiting at night, regression of development, language or behavior changes, as well as, significant sleep disturbance. If seizures are suspected, then it is recommended to obtain an EEG in both the waking and sleeping states (68). It is also important to tell families to avoid soy formulas in young children with FXS because of the recent report of soy formula intake increasing the prevalence of seizures in those with ASD and FXS (69).

Seizures are usually easily managed on

monotherapy with anticonvulsants. Historically, most individuals with FXS have experienced good control with carbamazepine or valproic acid, with fairly limited adverse effects (69). Carbamazepine stabilizes the inactivated state of voltage-gate sodium channels. Its action leaves the affected neuronal cells less excitable. Carbamazepine has also $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits containing GABA receptor agonist actions. Carbamazepine-gene testing, pharmacogenomics or pharmacogenetics, to look for the human leukocyte antigen B 1502 (HLA-B*1502), the variant may determine whether carbamazepine could be an effective treatment or whether side effects may develop. The United States Food and Drug Administration (FDA) recommends that patients with Asian ancestry should be tested for the *HLA-B*1502* gene variant before treatment. Testing individuals of other ancestries is not typically performed (70-72). The carbamazepine label contains warning for blood dyscrasia and common side effects are drowsiness, dizziness, headaches and migraines, motor coordination impairment, nausea, vomiting, and/or constipation. Carbamazepine has also the advantage that can be used as a mood stabilizer at a typical dosage (73). The valproic acid mechanism of action is not fully understood, but the reduction of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), as well as, the blockade of voltage-dependent sodium channels may protect against seizures; the increased brain levels of GABA may contribute to its mood stabilizer properties as well as its antiepileptic mechanism of action. The most common adverse effects of valproic acid are digestive complaints (diarrhea, nausea, vomiting and indigestion), vision problems (double vision or lazy eye), hormonal disturbances (increased testosterone production in females and menstrual irregularities), hair loss, memory problems, weight gain, infections, low platelet count, dizziness, drowsiness, tremor and headache (74,75). The FDA recommends patient testing on the Valproate (VP) drug label to avoid prescribing the drug to individuals with urea cycle disorders, the information is lacking about what type of genetic testing and how it should be carried out. Newer studies correlating genotype-phenotype associations with the clinical response will be helpful to increase drug efficacy and to reduce drug-related toxicity (76).

For those who failed carbamazepine or valproic acid, lamotrigine can be used as a fairly effective second line. Phenytoin has the adverse effects of gum hypertrophy and can interfere with dental hygiene. Phenobarbital and gabapentin also should be avoided because they exacerbate behavioral problems including hyperactivity (76). Drug-specific blood level testing, liver function studies, electrolytes, complete blood count (CBC) and general health monitoring should be considered for any child taking anticonvulsant medications (76).

2.4. Mitral valve prolapse

Mitral Valve Prolapse (MVP, floppy mitral valve) is a valvular heart condition that is characterized by the displacement of an abnormally thickened mitral valve leaflet into the left atrium during systole (77). The prevalence of MVP in the general population is estimated at 2-3% (77); however, MVP is observed in 7% of autopsies in the United States (78). Studies of individuals with FXS have shown that MVP occurs in approximately 50% of males and 20% females with echocardiogram confirmation (79,80). However, a recent Fragile X Clinical Research Center (FXCRC) database study using only clinical reports showed a prevalence of only 0.8%. Perhaps this relates to the fact that MVP is more common in adults than children and often cannot be diagnosed by just auscultation. Careful cardiac auscultation is recommended during every annual physical examination and if a systolic murmur or the classical MVP murmur is detected (a mid-systolic click, followed by a late systolic murmur heard best at the apex), then it is recommended to request a cardiology evaluation which should include an echocardiogram (81). Individuals with MVP, particularly those without symptoms, often require no treatment (82). Those rare cases of MVP and symptoms of arrhythmias or dysautonomia may benefit from beta-blockers. Individuals with MVP are at higher risk of infective endocarditis, approximately three- to eightfold the risk of the general population (82). Before 2007, the American Heart Association recommended prophylaxis for dental surgery and other invasive procedures that could introduce bacteria into the blood stream. Thereafter, the association determined that individuals with MVP should not receive prophylaxis routinely; prophylaxis for dental procedures should be recommended only for patients with underlying cardiac conditions associated with the highest risk of adverse outcome from infective endocarditis (83).

Surveillance cardiac evaluations are necessary for those with moderate MVP, in order to evaluate the degree of regurgitation. In very rare instances when MVP is associated with severe mitral regurgitation, mitral valve repair or surgical replacement may be necessary. In the general population, MVP is observed in individuals who tend to have low body mass index (BMI), it is unknown if MVP in FXS is associated with lower BMI. Abnormal elastin fibers have been detected in the cardiac valves and in the skin of individuals with FXS so MVP is thought to be related to the connective tissue problems seen in FXS and are related to abnormalities of the elastin fibers (84). Dilation of the aortic root is also seen in many individuals with FXS in both childhood and adulthood and this is also associated with abnormal elastin fibers (35,58); Typically, this is not progressive nor have significant aneurisms been reported. In summary, MVP carries a very low risk of complications, but in rare severe cases complications may include mitral

regurgitation, infective endocarditis and congestive heart failure. Further, larger longitudinal studies that described the prevalence and MVP and its complications are necessary.

2.5. Gastrointestinal problems

The frequency of gastro intestinal (GI) problems in FXS remains to be determined, but initial and current studies showed a similar proportion (prevalence ~11%) of children suffering from diarrhea and gastro-esophageal reflux disease (GERD) (85,86). Interestingly GI problems have been described to be quite common in other connective tissue disorders, such as Ehlers-Danlos syndrome (EDS) and Marfan syndrome; such problems include GERD, irritable bowel syndrome, and diarrhea (87-90). Even more intriguing is the association of the premutation and irritable bowel syndrome and the fact that developmental disorders and autism are usually associated with constipation rather than diarrhea as observed in FXS (91). General recommendations should be provided and medication management, such as, thickening agents, antacids, histamine-2 (H-2) blockers and proton-pump inhibitors, should be prescribed if necessary. Individuals with FXS have higher pain threshold which along with the communication deficits can mask the frequency of abdominal pain and other gastrointestinal symptoms. Surveillance on height and weight are appropriate to determine a failure to thrive (FTT) and referral to gastroenterologist specialist and nutritionist are recommended in the presence of FTT or poor weight gain. It is likely that the frequent loose stools in FXS are related to autonomic dysregulation including sympathetic hyperarousal and chronic anxiety (92).

2.6. Sleep

Sleep problems are very common in the general population and even more common in young children with FXS. There are many issues that disturb normal sleeping patterns such as problems falling asleep, frequent nighttime awakenings, waking up too early, and parasomnias. In children with FXS the prevalence of sleep problems was reported to be 26-47% (93,94) which is higher than the prevalence observed in typical children (10-25%) (95,96). A recent study showed that the prevalence did not have gender or demographic differences and that the severity of sleep disturbance in FXS children was more pronounced when compared to typically developing children. The most frequent problems reported were difficulty falling asleep and frequent nighttime awakening (97). In addition, altered sleep patterns and dysregulated melatonin profiles have been observed in adolescents with FXS as well as greater variability in total sleep time, difficulty in sleep maintenance, and significantly greater nocturnal melatonin production in the boys with FXS (98). Children

with FXS are at a higher risk for sleep problems at very young age (~3 years of age) and the sleep problems may not resolve with age. Therefore; it is recommended that a careful history of sleep habits must be included in every clinical visit (99), starting at a young age and continue throughout their life. The physician may simply ask the parents if they have any concerns about their child's sleep or if their child takes more than 30 minutes to fall asleep at bedtime. Standardized parent questionnaires, such as the Child's Sleep Habit's Questionnaire or a two-week sleep diary are good tools to assess sleep problems (99).

Treatment of sleep problems in FXS includes behavioral interventions and medications. Behavioral intervention should include bedtime routines, positive reinforcement, effective instructions and parental support. An example is "extinction" (removing reinforcement to reduce a behavior) which can effectively reduce the falling to sleep period and increase overall sleep time. The medications used to treat this medical problem include melatonin and if needed, clonidine (99). Melatonin can effectively improve total night sleep duration, sleep latency time, and sleep-onset time (100). A study in the *Fmr1* KO mouse showed that the therapeutic effects of melatonin may be due to its antioxidant effects and ability to normalize synaptic connections (101,102). Other studies of antioxidants in the KO mouse include alpha-tocopherol (vitamin E) and N-acetyl-cysteine (NAC) (103) and omega-3 therapy (104) with improvement in the maturity of dendritic spines and enhanced Brain Derived Neurotrophic Factor (BDNF) levels in the hippocampus respectively. However, these antioxidants have not been studied for improvement in sleep in FXS. Melatonin should be given 1 hour before bedtime. The dose recommended for children with FXS ranges 0.5-5 mg. It is recommended to start with the lowest dose of 0.5 mg then adjust the dose with the response (105,106). No significant adverse effects of melatonin have been reported in those with FXS although in some patients it can cause agitation (106,107). Another study reported increased seizures in children with neurologic disabilities treated with melatonin but this has not been seen in FXS (108). Clonidine is alpha-agonist with off-labeled use for insomnia in the pediatric population. It is also used to treat attention deficit hyperactive disorder (ADHD) symptoms because it can decrease motor activity (109). Clonidine has an overall calming effect for the treatment of ADHD in FXS, but clonidine can cause significant sedation at higher doses so it is helpful for facilitating sleep. Dangerous side effects can occur in overdose so its use must be carefully monitored. The clonidine patch or catapres transdermal therapeutic system (Catapres-TTS1, 2 and 3) should not be used in young children who might pull it off and eat it because this leads to a significant overdose. Clonidine should not be used in the patients with a history of cardiovascular disease or depression (109).

2.7. Obstructive sleep apnea

Obstructive sleep apnea (OSA) is characterized by repeat brief episodes of airflow obstruction in the oral-nasal airway that occurs during sleep (110). These episodes of complete airflow cessation (apnea) or partial airflow obstruction (hypopnea) result in both frequent and transient reduction of brain oxygen levels (111). It occurs more often during rapid eye movement (REM) sleep and is rarely preceded by body movements (112). The prevalence of OSA among normal children is about 0.8% to 2.8% (113); however, it can be higher among children with neurodevelopment problems including FXS (114,115). OSA-related symptoms included loud snoring, apnea, awakening with gasping breaths, enuresis and daytime sleepiness (116,117). OSA in children is associated with concentration deficits, reduce learning ability, lower cognitive function, and school difficulties. Vigilance impairments and neuropsychological deficits are among the main symptoms seen in OSA (118). Some studies suggest that vigilance impairment is attributed mostly to nocturnal hypoxemia (118). In addition to cognitive issues, a large number of studies found that OSA is associated with medical problems such as cardiac tissue changes as well as systolic and diastolic blood pressure changes. Previous reports suggest that children with OSA and hypertrophied tonsils tend to aspirate oropharyngeal secretion which can lead to pneumonia (119). The association of GERD with OSA has been documented previously, possible due to higher esophageal negative pressure which is generated by increased respiratory efforts (120). Studies suggest that in typically developing children, early diagnosis, and treatment of pediatric OSA may improve the child's long-term cognitive, social potential and school performance. The standard diagnostic procedure for establishing the presence of OSA is the overnight polysomnography (PSG) (120). Although overnight PSG can be very effective in diagnosing OSA, for some patients the test is labor-intensive. The management of OSA has three main aspects. The first step is drug therapy, which may alleviate adenoidal and tonsillar hypertrophy. The second is drainage of nasal secretions, and the third step is surgery. Adenoidectomy with or without tonsillectomy is the primary treatments for OSA and it is usually very effective for those with FXS (121). Continuous positive airway pressure (CPAP) is a feasible therapeutic intervention in children with neurodevelopment deficits including FXS, although it is reported that patients have a low compliance to this therapy (121).

2.8. Strabismus

Strabismus is one of the phenotypic characteristics in FXS and it is an abnormality of the ocular motility and deviation of the eyes away from binocular vision. Strabismus is better defined as exotropia, esotropia,

hypotropia, and hypertropia which describe the orientation of the eyes. Exotropia is the most common type of strabismus found in FXS and it is thought to be caused by an asymmetrical tone of the extraocular muscles (122). Early studies reported the prevalence of strabismus in FXS ranging from 28 to 57% (123-125), however later studies found that the prevalence was only 4.4-8% (126,127) and a recent FXCRC study reported a prevalence of 17.5%. The initial higher rates are thought to be related to selection bias in the earlier studies. Nevertheless, the prevalence was significantly higher than the prevalence in typical children (2.6% vs. 4%) (128,129).

It is crucial to detect strabismus early in life because if left untreated strabismus may progress to amblyopia, a permanent decrease in visual acuity due to the disuse of the abnormal eye during visual development. Tests used to detect the strabismus are; corneal light reflex, cover/uncover test and simultaneous red reflex test. Once strabismus is detected, the child should be referred to a pediatric ophthalmologist for further evaluation and management. It is recommended that every child with FXS have a comprehensive ophthalmologic examination by age 4 or sooner if an abnormality is detected (130). The treatment of strabismus should improve vision impairment and alignment abnormalities. The vision impairment leading to amblyopia can be treated by occluding the preferred eye and correcting the refractive errors of the affected eye with eyeglasses. The ocular alignment can be corrected by visual training exercises, but surgery is needed in many cases (131).

2.9. Tic disorder

Tics disorders are generally classified according to the age of onset, duration, and severity of symptoms and the presence of vocal and/or motor tics (132). The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) lists three types of tic disorders: Tourette syndrome (TS), chronic motor or vocal tic disorder, and provisional (transient) tic disorder (133). The prevalence of transient tic disorders in children aged 13-14 years is from 3-15% and chronic motor tics ranges from 2-5% (134-136); however, tic disorders may be more frequent than the prevalence reported because many patients with tics do not seek medical attention (136).

The prevalence of tics in FXS differs among studies. Tics were reported in 16% of 152 people with FXS in a cohort study in the United States. Tic disorders are characterized by involuntary or semi-voluntary, sudden, brief and rapid, recurrent, repetitive, non-rhythmic, unpredictable, meaningless and stereotyped motor movements, such as, eye blinking, shoulder shrugging and throat clearing or sounds produced by moving air through the nose, mouth or throat (phonic or vocal tics)

including swearing and complex expressions (136). Tics are not constant and appear in the background of normal motor activity, except for extremely severe cases (137). Tics disorders appear before 18 years of age and occur before taking stimulant medications in those FXS, however, tics may be unmasked or worsened by stimulants. An important step in the approach to the patient with tics is to rule out secondary causes of tics disorders. Drugs including stimulants, antidepressants, antihistamine and antiepileptic's may cause tics; tics disappear with the interruption of these medications (137). Treatment is not always necessary and only severe cases should be treated preferably with monotherapy at low doses (138,139). Usually clonidine or guanfacine use in FXS is effective for tics in FXS; however, most of the time tics are not severe and do not need medication treatment. Aripiprazole or risperidone may sometimes be helpful to treat tics in FXS (139).

2.10. Other Problems

Toileting issues are one of the most challenging problems for patients and their families. The problems include bowel and bladder control, washing and wiping abilities, and inclination to be toilet trained. Nearly half (48.8%) (140) of children with FXS had toileting problems and the time they were toilet-trained was delayed compared to the normal population. A study of functional skills of individuals with FXS showed that the majority of females with FXS could demonstrate toileting skills by age 11 to 15 years, while males by age 15 to 20 (141).

We must take into consideration that toilet training is a challenging task for parents even in typically developed children. The guidelines for toilet training for children with FXS are not different from those of typical children. The most important step is to start the training when the child is ready; the appropriate time to initiate the training should be based on developmental and behavioral milestones achievements rather than chronological age (142). Physicians should initiate conversations about this issue with the parents at a young age (~1 year of age), it is also important to discuss with the family how to assess the child's readiness for toilet training in order to avoid maladaptive behaviors among other psychological problems associated with failed toileting training. Special concerns for children with FXS may be due to their increased anxiety, slow learning skills, sensory sensitivity and defensiveness (142). The steps for toilet training are deciding what words to use, picking a potty-chair, helping the child recognize signs of needing to use the potty-chair, making trips to the potty-chair as a routine, and encouraging the use of the potty-chair (143). Positive reinforcement, extinction, and a star-chart can be used as strategies in the training. During the training accidents should be expected, the parents should address these events lightly and avoid upsetting comments and negative reinforcement. Punishment and scolding will

only make the training harder and may increase the time needed for toilet training. Creating a routine pattern and patience are keys to success in the training.

Other common problems in children with FXS mentioned by caregivers and physicians are sensory processing and integration issues. Sensory processing and integration have major roles in human development (144). Individuals with FXS have an enhanced sympathetic response to sensory stimuli (145), and the feel of a potty-chair. The sensation of evacuation is often anxiety provoking to children with FXS such that they may avoid these stimuli.

The sensory process has two important components which are sensory discrimination and sensory modulation. Sensory discrimination is the process in which sensory stimuli are distinguished, given their meaning and use. Problems with sensory discrimination can cause poor recognition and interpretation of sensory stimuli, which in turn may result in difficulties in sensory-motor skill development, such as, brushing teeth, climbing or riding a bike, being a picky eater, etc. Sensory modulation is how the sensory stimuli are used and responded to. Problems with this process can cause hyper-response, over-activity, poor attention and poor coping. The most common sensory modulation difficulty reported in FXS is hyperarousal. Examples of the processing problem are difficulty tolerating bright lights and loud noises, crowded places overstimulation, difficulty making good eye contact, and trouble tolerating certain clothes. These problems are related to a lack of normal habituation to a sensory stimulus seen in both electrodermal studies (145) and even on Functional Magnetic Resonance Imaging (fMRI) studies to recurrent direct or indirect eye contact (146).

To attain full assessment and treatment plans, a team approach is needed. The team usually includes occupational therapists, physical therapists, speech therapists, educators, psychologists, and physicians. The team can be adjusted for each individual's problems. There are many tools that have been proven useful and reliable for assessing an individual's condition such as the Sensory Profile questionnaire, the Sensory Processing Measure questionnaire, the Movement Assessment Battery for Children, the Quick Neurological Screening Test and the Berg Balance Scale (146). It is recommended that children with FXS should receive routine assessments from occupational therapists and receive occupational therapy at least twice a week during early development (66). The treatments are individualized for each patient's medical problem.

3. Discussion

Clinicians need to know that those with FXS are at risk for a wide range of medical problems other than ID, ADHD, and ASD that are so common in FXS. The diagnosis and treatment of the medical problems in

FXS are described here and the treatment of behavioral problems are described elsewhere including the use of targeted treatments to reverse the cognitive and behavioral problems (147,148). Many of the medical problems in FXS, such as OM, MVP, GERD, hernias, joint dislocation, and flat feet are related to the connective tissue problems inherent in the syndrome. These connective tissue problems are related to the lack of FMRP on the structure of the elastin fibrils in the skin, heart, vessels and organs (149). These changes also relate to the soft and velvet like skin seen in FXS. Improvements in the looseness of connective tissue in FXS have been reported with the use of minocycline, a targeted treatment that lowers Matrix Metalloproteinase 9 (MMP9) levels. In FXS minocycline has been shown to be efficacious for behavior in children. Minocycline has also been used to treat aortic aneurysms because of the effects of pulling together connective tissue in cardiology studies so it may be helpful for dilated aortas in FXS, although this is rarely a problem. Most of these problems are treated symptomatically as described above and the response is usually good to such treatment (Table 1). It is likely that the most severe medical problem in FXS, seizures, will also improve with targeted treatments, although the response to standard anticonvulsants is good as described above. The key to this treatment is early and aggressive intervention because ongoing seizures will further exacerbate ID and ASD severity. The future looks bright for not only reversing the cognitive and behavioral problems but also many of the medical problems of FXS with targeted treatments (150).

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References

1. Lubs HA. A marker X chromosome. *Am J Hum Genet.* 1969; 21:231-244.
2. Verkerk AJ, Pieretti M, Sutcliffe JS, *et al.* Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell.* 1991; 65:905-914.
3. Oberle I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boué J, Bertheas MF, Mandel JL. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science.* 1991; 252:1097-1102.
4. de Vries BB, Wiegers AM, Smits AP, Mohkamsing S, Duivendoorn HJ, Fryns JP, Curfs LM, Halley DJ, Oostra BA, van den Ouweland AM, Niermeijer MF. Mental status of females with an *FMR1* gene full mutation. *Am J Hum Genet.* 1996; 58:1025-1032.
5. Hantash FM, Goos DG, Tsao D, Quan F, Buller-Burckle A, Peng M, Jarvis M, Sun W, Strom CM. Qualitative assessment of *FMR1* (CGG)_n triplet repeat status in normal, intermediate, premutation, full mutation, and mosaic carriers in both sexes: Implications for fragile X syndrome carrier and newborn screening. *Genet Med.* 2010; 12:162-173.
6. Maddalena A, Richards CS, McGinniss MJ, Brothman A, Desnick RJ, Grier RE, Hirsch B, Jacky P, McDowell GA, Popovich B, Watson M, Wolff DJ. Technical standards and guidelines for fragile X: The first of a series of disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics. Quality Assurance Subcommittee of the Laboratory Practice Committee. *Genet Med.* 2001; 3:200-205.
7. Darnell JC, Richter JD. Cytoplasmic RNA-binding proteins and the control of complex brain function. *Cold Spring Harb Perspect Biol.* 2012; 4:a012344.
8. Yrigollen CM, Martorell L, Durbin-Johnson B, Naudo M, Genoves J, Murgia A, Polli R, Zhou L, Barbouth D, Rupchock A, Finucane B, Latham GJ, Hadd A, Berry-Kravis E, Tassone F. AGG interruptions and maternal age affect *FMR1* CGG repeat allele stability during transmission. *J Neurodev Disord.* 2014; 6:24.
9. American College of Obstetricians and Gynecologists Committee on Genetics . ACOG Committee Opinion No. 469: Carrier screening for fragile X syndrome. *Obstet Gynecol.* 2010; 116:1008-1010.
10. Reches A, Malcov M, Ben-Yosef D, Azem F, Amit A, Yaron Y. Preimplantation genetic diagnosis for fragile X syndrome: Is there increased transmission of abnormal *FMR1* alleles among female heterozygotes? *Prenat Diagn.* 2009; 29:57-61.
11. Malcov M, Naiman T, Yosef DB, Carmon A, Mey-Raz N, Amit A, Vagman I, Yaron Y. Preimplantation genetic diagnosis for fragile X syndrome using multiplex nested PCR. *Reprod Biomed Online.* 2007; 14:515-521.
12. Black SH. Preimplantation genetic diagnosis. *Curr Opin Pediatr.* 1994; 6:712-716.
13. Garber KB, Visootsak J, Warren ST. Fragile X syndrome. *Eur J Hum Genet.* 2008; 16:666-672.
14. Schaefer GB, Mendelsohn NJ. Genetics evaluation for the etiologic diagnosis of autism spectrum disorders. *Genet Med.* 2008; 10:4-12.
15. Partington MW. The fragile X syndrome II: Preliminary data on growth and development in males. *Am J Med Genet.* 1984; 17:175-94.
16. Turner G, Daniel A, Frost M. X-linked mental retardation, macro-orchidism, and the Xq27 fragile site. *J Pediatr.* 1980; 96:837-841.
17. Brondum Nielsen K. Growth pattern in boys with fragile X. *Am J Med Genet.* 1988; 30:143-147.
18. Jacobs PA, Glover TW, Mayer M, Fox P, Gerrard JW, Dunn HG, Herbst DS. X-linked mental retardation: A study of 7 families. *Am J Med Genet.* 1980; 7:471-489.
19. Prouty LA, Rogers RC, Stevenson RE, Dean JH, Palmer KK, Simensen RJ, Coston GN, Schwartz CE. Fragile X syndrome: Growth, development, and intellectual

- function. *Am J Med Genet.* 1988; 30:123-142.
20. Thake A, Todd J, Bunday S, Webb T. Is it possible to make a clinical diagnosis of the fragile X syndrome in a boy? *Arch Dis Child.* 1985; 60:1001-1007.
 21. Wilson MG, Marchese CA. Prenatal diagnosis of fragile X in a heterozygous female fetus and postnatal follow-up. *Prenat Diagn.* 1984; 4:61-66.
 22. Hagerman RJ, Jackson C, Amiri K, Silverman AC, O'Connor R, Sobesky W. Girls with fragile X syndrome: Physical and neurocognitive status and outcome. *Pediatrics.* 1992; 89:395-400.
 23. Kidd SA, Lachiewicz A, Barbooth D, Blitz RK, Delahunty C, McBrien D, Visootsak J, Berry-Kravis E. Fragile X syndrome: A review of associated medical problems. *Pediatrics.* 2014; 134:995-1005.
 24. Butler MG, Brunschwig A, Miller LK, Hagerman RJ. Standards for selected anthropometric measurements in males with the fragile X syndrome. *Pediatrics.* 1992; 89(6 Pt 1):1059-1062.
 25. de Vries BB, Robinson H, Stolte-Dijkstra I, Tjon Pian Gi CV, Dijkstra PF, van Doorn J, Halley DJ, Oostra BA, Turner G, Niermeijer MF. General overgrowth in the fragile X syndrome: Variability in the phenotypic expression of the *FMR1* gene mutation. *J Med Genet.* 1995; 32:764-769.
 26. Nowicki ST, Tassone F, Ono MY, Ferranti J, Croquette MF, Goodlin-Jones B, Hagerman RJ. The Prader-Willi phenotype of fragile X syndrome. *J Dev Behav Pediatr.* 2007; 28:133-138.
 27. McLennan Y, Polussa J, Tassone F, Hagerman R. Fragile X syndrome. *Curr Genomics.* 2011; 12:216-224.
 28. Jacobs PA, Mayer M, Matsuura J, Rhoads F, Yee SC. A cytogenetic study of a population of mentally retarded males with special reference to the marker (X) syndrome. *Hum Genet.* 1983; 63:139-148.
 29. Mattei JF, Mattei MG, Aumeras C, Auger M, Giraud F. X-linked mental retardation with the fragile X. A study of 15 families. *Hum Genet.* 1981; 59:281-289.
 30. Hazlett HC, Poe MD, Lightbody AA, Styner M, MacFall JR, Reiss AL, Piven J. Trajectories of early brain volume development in fragile X syndrome and autism. *J Am Acad Child Adolesc Psychiatry.* 2012; 51:921-933.
 31. Harlow EG, Till SM, Russell TA, Wijetunge LS, Kind P, Contractor A. Critical period plasticity is disrupted in the barrel cortex of *FMR1* knockout mice. *Neuron.* 2010; 65:385-398.
 32. Loesch DZ, Huggins RM, Bui QM, Taylor AK, Hagerman RJ. Relationship of deficits of *FMR1* gene specific protein with physical phenotype of fragile X males and females in pedigrees: A new perspective. *Am J Med Genet A.* 2003; 118a:127-134.
 33. Loesch DZ, Huggins RM, Hoang NH. Growth in stature in fragile X families: A mixed longitudinal study. *Am J Med Genet.* 1995; 58:249-256.
 34. Reiss AL, Eliez S, Schmitt JE, Patwardhan A, Haberecht M. Brain imaging in neurogenetic conditions: Realizing the potential of behavioral neurogenetics research. *Ment Retard Dev Disabil Res Rev.* 2000; 6:186-197.
 35. Hagerman RJ. *Fragile X Syndrome: Diagnosis, Treatment, and Research.* 3rd ed. Baltimore, MD, John Hopkins University Press, MD, USA, 2002; pp:69-85
 36. Hagerman RJ. *Treatment of Neurodevelopmental Disorders: Targeting Neurobiological Mechanisms.* Oxford University Press, NY, USA, 2014; pp:63-89
 37. Hoffman HJ, Daly KA, Bainbridge KE, Casselbrant ML, Homøe P, Kvestad E, Kvaerner KJ, Vernacchio L. Panel 1: Epidemiology, natural history, and risk factors. *Otolaryngol Head Neck Surg.* 2013; 148(4 Suppl):e1-e 25.
 38. Hagerman RJ, Altshul-Stark D, McBogg P. Recurrent otitis media in the fragile X syndrome. *Am J Dis Child.* 1987; 141:184-187.
 39. Lieberthal AS, Carroll AE, Chonmaitree T, Ganiats TG, Hoberman A, Jackson MA, Joffe MD, Miller DT, Rosenfeld RM, Sevilla XD, Schwartz RH, Thomas PA, Tunkel DE. The diagnosis and management of acute otitis media. *Pediatrics.* 2013; 131:e964-99.
 40. Daly KA, Giebink GS. Clinical epidemiology of otitis media. *Pediatr Infect Dis J.* 2000; 19(5 Suppl):S31-6.
 41. Duffy LC, Faden H, Wasielewski R, Wolf J, Krystofik D. Exclusive breastfeeding protects against bacterial colonization and day care exposure to otitis media. *Pediatrics.* 1997; 100:E7.
 42. Duncan, B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breast-feeding for at least 4 months protects against otitis media. *Pediatrics.* 1993; 91:867-872.
 43. Scariati PD, Grummer-Strawn LM, Fein SB. A longitudinal analysis of infant morbidity and the extent of breastfeeding in the United States. *Pediatrics.* 1997; 99:E5.
 44. Etzel RA, Pattishall EN, Haley NJ, Fletcher RH, Henderson FW. Passive smoking and middle ear effusion among children in day care. *Pediatrics.* 1992; 90(2 Pt 1):228-232.
 45. Ilicali OC, Keleş N, Değer K, Savaş I. Relationship of passive cigarette smoking to otitis media. *Arch Otolaryngol Head Neck Surg.* 1999; 125:758-762.
 46. Wellington M, Hall CB. Pacifier as a risk factor for acute otitis media. *Pediatrics.* 2002; 109:351-352.
 47. Kerstein R. Otitis media: Prevention instead of prescription. *Br J Gen Pract.* 2008; 58:364-365.
 48. Brown CE, Magnuson B. On the physics of the infant feeding bottle and middle ear sequela: Ear disease in infants can be associated with bottle feeding. *Int J Pediatr Otorhinolaryngol.* 2000; 54:13-20.
 49. Niemela M, Pihakari O, Pokka T, Uhari M. Pacifier as a risk factor for acute otitis media: A randomized, controlled trial of parental counseling. *Pediatrics.* 2000; 106:483-488.
 50. Azarpazhooh A, Limeback H, Lawrence HP, Shah PS. Xylitol for preventing acute otitis media in children up to 12 years of age. *Cochrane Database Syst Rev.* 2011; 11:Cd007095.
 51. Berry-Kravis E. Epilepsy in fragile X syndrome. *Dev Med Child Neurol.* 2002; 44:724-728.
 52. Berry-Kravis E, Knox A, Hervey C. Targeted treatments for fragile X syndrome. *J Neurodev Disord.* 2011; 3:193-210.
 53. Musumeci SA, Hagerman RJ, Ferri R, Bosco P, Dalla Bernardina B, Tassinari CA, De Sarro GB, Elia M. Epilepsy and EEG findings in males with fragile X syndrome. *Epilepsia.* 1999; 40:1092-1099.
 54. Berry-Kravis E, Raspa M, Loggin-Hester L, Bishop E, Holiday D, Bailey DB. Seizures in fragile X syndrome: Characteristics and comorbid diagnoses. *Am J Intellect Dev Disabil.* 2010; 115:461-472.
 55. Lozano R, Rosero CA, Hagerman RJ. Fragile X spectrum disorders. *Intractable Rare Dis Res.* 2014; 3:134-146.
 56. Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, Weiler IJ, Greenough WT. Abnormal dendritic spines in

- fragile X knockout mice: Maturation and pruning deficits. *Proc Natl Acad Sci U S A*. 1997; 94:5401-5404.
57. Musumeci SA, Bosco P, Calabrese G, Bakker C, De Sarro GB, Elia M, Ferri R, Oostra BA. Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. *Epilepsia*. 2000; 41:19-23.
 58. Kidd SA, Lachiewicz A, Barbooth D, Blitz RK, Delahunty C, McBrien D, Visootsak J, Berry-Kravis E. Fragile X syndrome: A review of associated medical problems. *Pediatrics*. 2014; 134:995-1005.
 59. D'Hulst C, Kooy RF. The GABAA receptor: A novel target for treatment of fragile X? *Trends Neurosci*. 2007; 30:425-431.
 60. Lozano R, Hare EB, Hagerman RJ. Modulation of the GABAergic pathway for the treatment of fragile X syndrome. *Neuropsychiatr Dis Treat*. 2014; 10:1769-1779.
 61. Chonchaiya W, Tassone F, Ashwood P, Hessel D, Schneider A, Campos L, Nguyen DV, Hagerman RJ. Autoimmune disease in mothers with the FMR1 premutation is associated with seizures in their children with fragile X syndrome. *Hum Genet*. 2010; 128:539-548.
 62. Kluger G, Böhm I, Laub MC, Waldenmaier C. Epilepsy and fragile X gene mutations. *Pediatr Neurol*. 1996; 15:358-360.
 63. Musumeci SA, Ferri R, Colognola RM, Neri G, Sanfilippo S, Bergonzi P. Prevalence of a novel epileptogenic EEG pattern in the Martin-Bell syndrome. *Am J Med Genet*. 1988; 30:207-212.
 64. Finelli PF, Pueschel SM, Padre-Mendoza T, O'Brien MM. Neurological findings in patients with the fragile-X syndrome. *J Neurol Neurosurg Psychiatry*. 1985; 48:150-153.
 65. Hagerman PJ, Stafstrom CE. Origins of epilepsy in fragile X syndrome. *Epilepsy Curr*. 2009; 9(4):108-12.
 66. Hagerman RJ, Berry-Kravis E, Kaufmann WE, Ono MY, Tartaglia N, Lachiewicz A, Kronk R, Delahunty C, Hessel D, Visootsak J, Picker J, Gane L, Tranfaglia M. Advances in the treatment of fragile X syndrome. *Pediatrics*. 2009; 123:378-390.
 67. Garcia-Nonell C, Ratera ER, Harris S, Hessel D, Ono MY, Tartaglia N, Marvin E, Tassone F, Hagerman RJ. Secondary medical diagnosis in fragile X syndrome with and without autism spectrum disorder. *Am J Med Genet A*. 2008; 146a:1911-1916.
 68. Incorpora G, Sorge G, Sorge A, Pavone L. Epilepsy in fragile X syndrome. *Brain Dev*. 2002; 24:766-769.
 69. Westmark CJ. Soy infant formula and seizures in children with autism: A retrospective study. *PLoS One*. 2014; 9:e80488.
 70. Alrashood ST. Carbamazepine. *Profiles Drug Subst Excip Relat Methodol*. 2016; 41:133-321
 71. Amstutz, U, Shear NH, Rieder MJ, Hwang S, Fung V, Nakamura H, Connolly MB, Ito S, Carleton BC. Recommendations for HLA-B*15:02 and HLA-A*31:01 genetic testing to reduce the risk of carbamazepine-induced hypersensitivity reactions. *Epilepsia*. 2014; 55:496-506.
 72. Kaniwa N, Saito Y. Pharmacogenomics of severe cutaneous adverse reactions and drug-induced liver injury. *J Hum Genet*. 2013; 58:317-326.
 73. Barde M, Bellivier F. Treatment of bipolar disorder. *Rev Prat*. 2014; 64:1195-1202.
 74. Rossi S. Australian Medicines Handbook in Adelaide. The Australian Medicines Handbook Unit Trust. 2013; 1190-1193
 75. Torrioli M, Vernacotola S, Setini C, Bevilacqua F, Martinelli D, Snape M, Hutchison JA, Di Raimo FR, Tabolacci E, Neri G. Treatment with valproic acid ameliorates ADHD symptoms in fragile X syndrome boys. *Am J Med Genet A*. 2010; 152A:1420-1427
 76. Ghodke-Puranik Y, Thorn CF, Lamba JK, Leeder JS, Song W, Birnbaum AK, Altman RB, Klein TE. Valproic acid pathway: Pharmacokinetics and pharmacodynamics. *Pharmacogenet Genomics*. 2013; 23:236-241.
 77. Hayek E, Gring CN, Griffin BP. Mitral valve prolapse. *Lancet*. 2005; 365:507-518.
 78. Sternbach G, Varon J. John Barlow: Mitral valve prolapse. *J Emerg Med*. 1993; 11:475-478.
 79. Sreeram N, Wren C, Bhate M, Robertson P, Hunter S. Cardiac abnormalities in the fragile X syndrome. *Br Heart J*. 1989; 61:289-291.
 80. Crabbe LS, Bensky AS, Hornstein L, Schwartz DC. Cardiovascular abnormalities in children with fragile X syndrome. *Pediatrics*. 1993; 91:714-715.
 81. Hagerman RJ, Synhorst DP. Mitral valve prolapse and aortic dilatation in the fragile X syndrome. *Am J Med Genet*. 1984; 17:123-131.
 82. Loehr JP, Synhorst DP, Wolfe RR, Hagerman RJ. Aortic root dilatation and mitral valve prolapse in the fragile X syndrome. *Am J Med Genet*. 1986; 23:189-194.
 83. Wilson W, Taubert KA, Gewitz M, *et al*. Prevention of infective endocarditis: Guidelines from the American Heart Association: A guideline from the American Heart Association Rheumatic Fever, Endocarditis and Kawasaki Disease Committee, Council on Cardiovascular Disease in the Young, and the Council on Clinical Cardiology, Council on Cardiovascular Surgery and Anesthesia, and the Quality of Care and Outcomes Research Interdisciplinary Working Group. *J Am Dent Assoc*. 2007; 138:739-745, 747-760.
 84. Waldstein G, Mierau G, Ahmad R, Thibodeau SN, Hagerman RJ, Caldwell S. Fragile X syndrome: Skin elastin abnormalities. *Birth Defects Orig Artic Ser*. 1987; 23:103-114.
 85. Goldson E, Hagerman RJ. Fragile X syndrome and failure to thrive. *Am J Dis Child*. 1993; 147:605-607.
 86. Utari A, Adams E, Berry-Kravis E, Chavez A, Scaggs F, Ngotran L, Boyd A, Hessel D, Gane LW, Tassone F, Tartaglia N, Leehey MA, Hagerman RJ. Aging in fragile X syndrome. *J Neurodev Disord*. 2010; 2:70-76.
 87. Castori M, Morlino S, Celletti C, Ghibellini G, Bruschini M, Grammatico P, Blundo C, Camerota F. Re-writing the natural history of pain and related symptoms in the joint hypermobility syndrome/Ehlers-Danlos syndrome, hypermobility type. *Am J Med Genet A*. 2013; 161a:2989-3004.
 88. Kumar A, Agarwal S. Marfan syndrome: An eyesight of syndrome. *Meta Gene*. 2014; 2:96-105.
 89. Parida SK, Kriss VM, Hall BD. Hiatus/paraesophageal hernias in neonatal Marfan syndrome. *Am J Med Genet*. 1997; 72:156-158.
 90. Winarni TI, Chonchaiya W, Sumekar TA, Ashwood P, Morales GM, Tassone F, Nguyen DV, Faradz SM, Van de Water J, Cook K, Hamlin A, Mu Y, Hagerman PJ, Hagerman RJ. Immune-mediated disorders among women carriers of fragile X premutation alleles. *Am J Med Genet A*. 2012; 158a:2473-2481.
 91. Pang KH, Croaker GD. Constipation in children with autism and autistic spectrum disorder. *Pediatr Surg Int*. 2011; 27:353-358.

92. Cordeiro L, Ballinger E, Hagerman R, Hessler D. Clinical assessment of DSM-IV anxiety disorders in fragile X syndrome: Prevalence and characterization. *J Neurodev Disord.* 2011; 3:57-67.
93. Kronk R, Bishop EE, Raspa M, Bickel JO, Mandel DA, Bailey DB Jr. Prevalence, nature, and correlates of sleep problems among children with fragile X syndrome based on a large scale parent survey. *Sleep.* 2010; 33:679-687.
94. Kronk R, Dahl R, Noll R. Caregiver reports of sleep problems on a convenience sample of children with fragile X syndrome. *Am J Intellect Dev Disabil.* 2009; 114:383-392.
95. Mindell JA, Meltzer LJ, Carskadon MA, Chervin RD. Developmental aspects of sleep hygiene: Findings from the 2004 National Sleep Foundation Sleep in America Poll. *Sleep Med.* 2009; 10:771-779.
96. Owens JA, Spirito A, McGuinn M. The Children's Sleep Habits Questionnaire (CSHQ): Psychometric properties of a survey instrument for school-aged children. *Sleep.* 2000; 23:1043-1051.
97. Richdale AL. A descriptive analysis of sleep behaviour in children with Fragile X. *Journal of Intellectual and Developmental Disability.* 2003; 28:135-144.
98. Gould EL, Loesch DZ, Martin MJ, Hagerman RJ, Armstrong SM, Huggins RM. Melatonin profiles and sleep characteristics in boys with fragile X syndrome: A preliminary study. *Am J Med Genet.* 2000; 95:307-315.
99. Consensus of the Fragile X Clinical & Research Consortium on Clinical Practices. Consensus of the Fragile X Clinical & Research Consortium on Clinical Practices: Sleep in Children with Fragile X Syndrome. 2012. <https://fragilex.org/treatment-intervention/consensus-on-clinical-practices/> (accessed June 17, 2016)
100. Wirojanan J, Jacquemont S, Diaz R, Bacalman S, Anders TF, Hagerman RJ, Goodlin-Jones BL. The efficacy of melatonin for sleep problems in children with autism, fragile X syndrome, or autism and fragile X syndrome. *J Clin Sleep Med.* 2009; 5:145-150.
101. Hagerman R, Lauterborn J, Au J, Berry-Kravis E. Fragile X syndrome and targeted treatment trials. *Results Probl Cell Differ.* 2012; 54:297-335.
102. Romero-Zerbo Y, Decara J, el Bekay R, Sanchez-Salido L, Del Arco-Herrera I, de Fonseca FR, de Diego-Otero Y. Protective effects of melatonin against oxidative stress in *Fmr1* knockout mice: A therapeutic research model for the fragile X syndrome. *J Pineal Res.* 2009; 46:224-234.
103. el Bekay R, Romero-Zerbo Y, Decara J, Sanchez-Salido L, Del Arco-Herrera I, Rodríguez-de Fonseca F, de Diego-Otero Y. Enhanced markers of oxidative stress, altered antioxidants and NADPH-oxidase activation in brains from Fragile X mental retardation 1-deficient mice, a pathological model for Fragile X syndrome. *Eur J Neurosci.* 2007; 26:3169-3180.
104. Pietropaolo S, Goubran MG, Joffre C, Aubert A, Lemaire-Mayo V, Crusio WE, Layé S. Dietary supplementation of omega-3 fatty acids rescues fragile X phenotypes in *Fmr1-Ko* mice. *Psychoneuroendocrinology.* 2014; 49:119-129.
105. Jan JE, Freeman RD. Melatonin therapy for circadian rhythm sleep disorders in children with multiple disabilities: What have we learned in the last decade? *Dev Med Child Neurol.* 2004; 46:776-782.
106. Jan JE, Freeman RD, Fast DK. Melatonin treatment of sleep-wake cycle disorders in children and adolescents. *Dev Med Child Neurol.* 1999; 41:491-500.
107. Dodge NN, Wilson GA. Melatonin for treatment of sleep disorders in children with developmental disabilities. *J Child Neurol.* 2001; 16:581-584.
108. Sheldon SH. Pro-convulsant effects of oral melatonin in neurologically disabled children. *Lancet.* 1998; 351:1254.
109. Wrenn CC, Heitzer AM, Roth AK, Nawrocki L, Valdovinos MG. Effects of clonidine and methylphenidate on motor activity in *Fmr1* knockout mice. *Neurosci Lett.* 2015; 585:109-113.
110. Tirosh E, Borochowitz Z. Sleep apnea in fragile X syndrome. *Am J Med Genet.* 1992; 43:124-127.
111. Hamlin A, Liu Y, Nguyen DV, Tassone F, Zhang L, Hagerman RJ. Sleep apnea in fragile X premutation carriers with and without FXTAS. *Am J Med Genet B Neuropsychiatr Genet.* 2011; 156b:923-928.
112. Ramirez A, Khirani S, Aloui S, Delord V, Borel JC, Pépin JL, Fauroux B. Continuous positive airway pressure and noninvasive ventilation adherence in children. *Sleep Med.* 2013; 14:1290-1294.
113. Marcus CL, Brooks LJ, Draper KA, Gozal D, Halbower AC, Jones J, Schechter MS, Ward SD, Sheldon SH, Shiffman RN, Lehmann C, Spruyt K; American Academy of Pediatrics. Diagnosis and management of childhood obstructive sleep apnea syndrome. *Pediatrics.* 2012; 130:e714-755.
114. Tirosh E, Tal Y, Jaffe M. CPAP treatment of obstructive sleep apnoea and neurodevelopmental deficits. *Acta Paediatr.* 1995; 84:791-794.
115. De Luca Canto G, Pachêco-Pereira C, Aydinoz S, Major PW, Flores-Mir C, Gozal D. Biomarkers associated with obstructive sleep apnea: A scoping review. *Sleep Med Rev.* 2014; 23c:28-45.
116. Brietzke SE, Gallagher D. The effectiveness of tonsillectomy and adenoidectomy in the treatment of pediatric obstructive sleep apnea/hypopnea syndrome: A meta-analysis. *Otolaryngol Head Neck Surg.* 2006; 134:979-984.
117. Walsh JK, Farrell MK, Keenan WJ, Lucas M, Kramer M. Gastroesophageal reflux in infants: Relation to apnea. *J Pediatr.* 1981; 99:197-201.
118. Montplaisir J, Bédard MA, Richer F, Rouleau I. Neurobehavioral manifestations in obstructive sleep apnea syndrome before and after treatment with continuous positive airway pressure. *Sleep.* 1992; 15(6 Suppl):S17-9.
119. Konno A, Hoshino T, Togawa K. Influence of upper airway obstruction by enlarged tonsils and adenoids upon recurrent infection of the lower airway in childhood. *Laryngoscope.* 1980; 90 (10 Pt 1):1709-1176.
120. Arad-Cohen N, Cohen A, Tirosh E. The relationship between gastroesophageal reflux and apnea in infants. *J Pediatr.* 2000; 137:321-326.
121. Southall DP, Stebbens VA, Mirza R, Lang MH, Croft CB, Shinebourne EA. Upper airway obstruction with hypoxaemia and sleep disruption in Down syndrome. *Dev Med Child Neurol.* 1987; 29:734-742.
122. Hatton DD, Buckley E, Lachiewicz A, Roberts J. Ocular status of boys with fragile X syndrome: A prospective study. *J AAPOS.* 1998; 2:298-302.
123. Maino DM, Schlange D, Maino JH, Caden B. Ocular anomalies in fragile X syndrome. *J Am Optom Assoc.* 1990; 61:316-323.
124. Van Splunder J, Stilma JS, Evenhuis HM. Visual performance in specific syndromes associated with intellectual disability. *Eur J Ophthalmol.* 2003; 13:566-574.
125. Maino DM, Wesson M, Schlange D, Cibis G, Maino JH.

- Optometric findings in the fragile X syndrome. *Optom Vis Sci.* 1991; 68:634-640.
126. Hatton DD, Buckley E, Lachiewicz A, Roberts J. Ocular status of boys with fragile X syndrome: A prospective study. *J aapos.* 1998; 2:298-302.
 127. Alanay Y, Unal F, Turanli G, *et al.* A multidisciplinary approach to the management of individuals with fragile X syndrome. *J Intellect Disabil Res.* 2007; 51(Pt 2):151-161.
 128. Repka MX, Friedman DS, Katz J, Ibranke J, Giordano L, Tielsch JM. The prevalence of ocular structural disorders and nystagmus among preschool-aged children. *J aapos.* 2012; 16:182-184.
 129. Saldarriaga W, Tassone F, González-Teshima LY, Forero-Forero JV, Ayala-Zapata S, Hagerman R. Fragile X syndrome. *Colomb Med (Cali).* 2014; 45:190-198.
 130. Consensus of the Fragile X Clinical & Research Consortium on Clinical Practices: Physical Problems in Fragile X Syndrome. 2012. <https://fragilex.org/treatment-intervention/consensus-on-clinical-practices/> (accessed June 17, 2016)
 131. Wang X, Gao X, Xiao M, Tang L, Wei X, Zeng J, Li Y. Effectiveness of strabismus surgery on the health-related quality of life assessment of children with intermittent exotropia and their parents: A randomized clinical trial. *J AAPOS.* 2015; 19:298-303.
 132. Rampello L, Alvano A, Battaglia G, Bruno V, Raffaele R, Nicoletti F. Tic disorders: From pathophysiology to treatment. *J Neurol.* 2006; 253:1-15.
 133. Modafferi S, Stornelli M, Chiarotti F, Cardona F, Bruni O. Sleep, anxiety and psychiatric symptoms in children with Tourette syndrome and tic disorders. *Eur J Paediatr Neurol.* 2016; 1090-3798:30026-5.
 134. Robertson MM, Stern JS. Tic disorders: New developments in Tourette syndrome and related disorders. *Curr Opin Neurol.* 1998; 11:373-380.
 135. Swain JE, Scahill L, Lombroso PJ, King RA, Leckman JF. Tourette syndrome and tic disorders: A decade of progress. *J Am Acad Child Adolesc Psychiatry.* 2007; 46:947-968.
 136. Jankovic J, Rohaidy H. Motor, behavioral and pharmacologic findings in Tourette's syndrome. *Can J Neurol Sci.* 1987; 14 (3 Suppl):541-546.
 137. Jankovic J. Tourette syndrome. Phenomenology and classification of tics. *Neurol Clin.* 1997; 15:267-275.
 138. Bagheri MM, Kerbeshian J, Burd L. Recognition and management of Tourette's syndrome and tic disorders. *Am Fam Physician.* 1999; 59:2263-2272, 2274.
 139. Jimenez-Jimenez FJ, Garcia-Ruiz PJ. Pharmacological options for the treatment of Tourette's disorder. *Drugs.* 2001; 61:2207-2220.
 140. Equit M, Piro-Hussong A, Niemczyk J, Curfs L, von Gontard A. Elimination disorders in persons with Prader-Willi and Fragile-X syndromes. *Neurol Urodyn.* 2013; 32:986-992.
 141. Bailey DB, Raspa M, Holiday D, Bishop E, Olmsted M. Functional skills of individuals with fragile X syndrome: A lifespan cross-sectional analysis. *Am J Intellect Dev Disabil.* 2009; 114:289-303.
 142. Stadtler AC, Gorski PA, Brazelton TB. Toilet training methods, clinical interventions, and recommendations, American Academy of Pediatrics. *Pediatrics.* 1999; 103(6 Pt 2):1359-1368.
 143. Mota DM, Barros AJ. Toilet training: Methods, parental expectations and associated dysfunctions. *J Pediatr (Rio J).* 2008; 84:9-17.
 144. Cascio CJ. Somatosensory processing in neurodevelopmental disorders. *J Neurodev Disord.* 2010; 2:62-69.
 145. Miller LJ, McIntosh DN, McGrath J, Shyu V, Lampe M, Taylor AK, Tassone F, Neitzel K, Stackhouse T, Hagerman RJ. Electrodermal responses to sensory stimuli in individuals with fragile X syndrome: A preliminary report. *Am J Med Genet.* 1999; 83:268-279.
 146. Bruno JL, Garrett AS, Quintin EM, Mazaika PK, Reiss AL. Aberrant face and gaze habituation in fragile X syndrome. *Am J Psychiatry.* 2014; 171:1099-1106.
 147. Hare EB, Hagerman RJ, Lozano R. Targeted treatments in fragile X syndrome. *Expert Opinion on Orphan Drugs.* 2014; 2:531-541.
 148. Gross C, Hoffmann A, Bassell GJ, Berry-Kravis EM. Therapeutic Strategies in Fragile X syndrome: From bench to bedside and back. *Neurotherapeutics.* 2015; 12:584-608.
 149. Waldstein G, Hagerman R. Aortic hypoplasia and cardiac valvular abnormalities in a boy with fragile X syndrome. *Am J Med Genet.* 1988; 30:83-98.
 150. Hagerman RJ, Des-Portes V, Gasparini F, Jacquemont S, Gomez-Mancilla B. Translating molecular advances in fragile X syndrome into therapy: A review. *J Clin Psychiatry.* 2014; 75:e294-307.

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Review of targeted treatments in fragile X syndrome

Andrew Ligsay^{1,2}, Randi J Hagerman^{1,3,*}

¹ Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, University of California, Davis Medical Center, Sacramento, CA, USA;

² University of California, Davis School of Medicine, Sacramento, CA, USA;

³ Department of Pediatrics, University of California, Davis Medical Center, Sacramento, CA, USA.

Summary

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability, and is the leading single-gene cause of autism spectrum disorders. It is due to a loss of the fragile X mental retardation protein, which leads to molecular, behavioral, and cognitive deficits in these patients. Improvements in our understanding of its pathophysiology have led to the development of numerous targeted treatments in FXS as highlighted by metabotropic glutamate receptor antagonists and gamma-Aminobutyric acid receptor modulators. This review will summarize relevant pre-clinical data and results from clinical trials in human subjects with FXS. It will also highlight upcoming studies and future directions for clinical trials as well.

Keywords: Fragile X syndrome, targeted treatments, clinical trials

1. Introduction

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability with prevalence rates estimated at 1:5,000 males and 1:8,000 females (1). Its etiology is due to a cytosine-guanine-guanine (CGG) repeat expansion mutation in the *FMR1* gene located on the long arm of the X chromosome. The normal range for individuals is up to 44 CGG repeats, whereas patients with FXS have >200. Above this threshold, the gene becomes methylated and silenced, resulting in significantly reduced or absent levels of the *FMR1* gene product (FMRP). FMRP is a RNA-binding protein that is heavily expressed in neurons (2,3), and functions in the stability, localization, and translation of select mRNAs (4).

FXS has a unique neuropsychiatric phenotype consisting of activating symptoms such as hyperactivity, anxiety, attention deficits, mood lability, sleep disturbances and increased susceptibility to seizures

(5). FXS is also the leading single-gene cause of autism spectrum disorder (ASD), which is prevalent in approximately 60% of patients (6,7). Many of the genes associated with idiopathic ASD may be regulated by FMRP as well (8). Recent studies have shown a positive correlation between FMRP expression and cognition in individuals with FXS (9-11). As a whole, female patients are typically less affected (lower incidences of ASD, higher intelligence quotient (IQ) scores, less severe IQ declines in longitudinal studies) compared to males due to compensation of FMRP from their second X chromosome (12).

At the cellular level, FMRP plays an important role in the development and maintenance of dendritic spines, and its absence causes the spines to appear long, thin and tortuous with filopodia-like projections (13-15). Lack of FMRP has been associated with defects in synaptic pruning, and imbalances between long-term depression (LTD) and long-term potentiation (LTP) (16). A study by Pan *et al.* (2010) also showed an increase in turnover of these spines (17). These processes lead to abnormalities such as immature synaptic connections, alterations in synaptic plasticity, and impaired memory formation.

Increased understanding of the neurobiology and pathophysiology, coupled with advances in animal models, has paved a way for the development of numerous targeted treatments for FXS. These have

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*Address correspondence to:

Dr. Randi J Hagerman, Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, University of California, Davis Medical Center, 2825 50th Street, Sacramento, CA 95817, USA.
E-mail: rjhagerman@ucdavis.edu

already rescued cellular and behavioral defects in both the *dfmr* mutant fly and *Fmr1* knock out (KO) mouse. Research also suggests treatment at younger ages is most optimal to improve developmental trajectories for patients (18). Once clinical trials show safety and efficacy in adult populations, there is hope that studying outcomes in children will reveal even more positive results. The purpose of this review is to discuss the findings of major randomized clinical trials, as well as highlight upcoming studies in the field of targeted treatments in FXS.

2. Metabotropic glutamate receptor 5 (mGluR) antagonists

Perhaps the most prominent theory regarding the pathophysiology of FXS involves upregulation of mGluR-mediated processes (19). Normally, stimulation of mGluRs in dendrites triggers increased local protein synthesis, ultimately resulting in AMPA receptor internalization and slowing of net synaptic maturation through mGluR-mediated LTD. This is important in memory formation and brain development. Concurrently, mGluRs increase FMRP synthesis, which serves as a braking mechanism that negatively regulates mGluR activity. The mGluR theory of FXS states that lack of FMRP allows mGluR-mediated LTD to become hyperactive, and thus lead to features associated with FXS. This theory has been validated through observed defects at the cellular level and correlating these with behavioral abnormalities across multiple animal models (4). Moreover, studies in *Fmr1* KO mice with partial reduction of mGluR expression showed correction of many of these abnormalities further demonstrating mGluRs play a significant role in the pathophysiology of FXS (20).

Translating these to human trials has not shown the dramatic benefits seen in the animal models. In an open label, single-dose trial of fenobam in 12 adults with FXS (6 males and 6 females), half of the subjects showed at least 20% improvement in prepulse inhibition (PPI) – a measure of sensory gating – within 1 hour of dosing, and most subjects showed clinical improvement in areas of hyperactivity and anxiety (21). It was also well tolerated in these participants and no serious adverse events were reported. However, the company failed financially because of an unrelated trial in ASD and therefore further trials in FXS could not be carried out.

In a double-blind, placebo-controlled crossover trial of AFQ056 (mavoglurant) in 30 adult males with FXS, no significant improvements were observed in the primary or secondary endpoints in the overall population while taking study drug compared to placebo (22). However, a small subgroup of participants with fully methylated *FMR1* promoter regions showed significant improvements in stereotypic behavior,

hyperactivity, and inappropriate speech as measured by the Aberrant Behavior Checklist-Community Edition (ABC-C) as well as in the overall ABC-C total score. These results encouraged two larger, multinational double-blind, placebo-controlled and parallel group trials of mavoglurant: one in adolescents (Phase III trial) and one in adults (Phase II trial) (23). In each study, participants were stratified into partial or full *FMR1* methylation groups and then randomized to placebo or one of three doses of mavoglurant: 25 mg BID, 50 mg BID, or 100 mg BID. Over 170 adults and 130 adolescents were randomized in these studies, but neither showed significant improvement on any test measures regardless of dose or methylation status. However, many families saw improvement in behavior and cognition particularly in follow-up open label continuation of mavoglurant that for some lasted longer than a year. The benefits of the open label study however were not controlled and could not be accurately captured by outcome measures. A multicentered controlled trial of mavoglurant will be studied in children with FXS ages 3 to 6 yo and is planned in combination with parent implemented language intervention (PILI) carried out through skype. It is likely that a younger age combined with an intensive learning program and outcome measures that assess cognition through language will demonstrate improvement with this mGluR5 antagonist.

The mGluR5 antagonist, basimglurant, was studied in two multinational, double-blind placebo-controlled trials. One trial was designed for adolescents and adults ages 14 to 50 years (24), and the other was designed for children ages 5 to 13 years (25). Subjects in the older study were randomized to placebo, basimglurant 0.5 mg daily, or basimglurant 1.5 mg daily; whereas age-equivalent dosages to match the adult steady state exposure levels was used for the child study (0.5 mg equivalent = 0.2 mg daily in 5-8 yo and 0.3 mg daily in 9-13 yo; 1.5 mg equivalent = 0.6 mg daily in 5-8 yo and 0.9 mg daily in 9-13 yo). In total, 122 adults, 63 adolescents, and 47 children were randomized. Neither study showed significant improvement on primary or secondary test measures in favor of basimglurant. However, post-hoc analysis showed males with low *FMR1* methylation and subjects who were not taking concomitant antipsychotic medication had slightly improved performances on select test measures while taking basimglurant. Again, many families found this medication also beneficial to their children with FXS but the benefit could not be precisely captured on the outcome measures.

Fenobam, mavoglurant, and basimglurant were all generally well tolerated by participants and showed good safety profiles in their respective trials. However, they have failed to show efficacy on designated test measures, with positive results being largely limited to those found in post-hoc analyses. The achievements of

mGluR5 NAMs from preclinical work have not fully translated to successes in human patients yet, and it is likely that the pathophysiology of FXS in humans is more complex than projected by animal models.

3. γ -Aminobutyric acid (GABA) modulators

The GABA system is one of the main inhibitory components of the central nervous system (CNS), and recent evidence shows GABAergic dysfunction in FXS animal models (26-30). This is believed to contribute to activating behavioral symptoms described previously, and has been implicated in fragile X-associated cellular abnormalities as well (31-33).

The GABA system functions through two major receptor subtypes: a GABA_A ion channel and a metabotropic G-protein coupled GABA_B receptor. GABA_A receptors are reduced in the neocortex, cerebellum, and hippocampus of the *Fmr1* KO mouse with deficits more pronounced at younger ages (27,34,35). These receptors consist of multiple subunits, and those containing an extrasynaptic δ -subunit are diminished by 50% in certain areas of the KO mouse brain (29). FMRP has been shown to directly bind to mRNA encoding for GABA_A subunits serving as a possible stabilization factor, and reintroduction of FMRP can correct δ -subunit mRNA to normal levels (34). Further, PET imaging in human subjects with FXS indicate significant reductions in GABA_A receptor availability throughout the CNS (36), which suggests the GABA_A deficit is not simply an artifact of the KO mouse. Trials of GABA_A agonists in animal models have shown positive results by restoring neuronal excitability in the amygdala to normal levels, mitigating anxiety and hyperactive behaviors, and rescuing the audiogenic seizure phenotype (30,33,34). Neuroactive steroids, in particular, could be well suited to treat FXS because they potentiate the effects of GABA_A receptors containing the δ -subunit.

Ganaxolone is a synthetic analog of the neuroactive steroid allopregnanolone, and has been previously used in the treatment of epilepsy and post-traumatic stress disorder. It is well tolerated in both pediatric and adult populations, and initial trials in the KO mouse have specifically improved seizures and showed a dose-dependent reduction in stereotypic and repetitive behaviors (30,34). Moreover, unlike benzodiazepines which also target GABA receptors, ganaxolone does not show tolerance allowing potential for long-term use (37). There is currently a double-blind, placebo-controlled crossover trial of ganaxolone in children with FXS being conducted at the UC Davis MIND Institute (NCT01725152). The study is now closed to enrollment, and safety and efficacy results will be available later this year.

On the other hand, GABA_B receptors have been shown to lower presynaptic glutamate release (38);

therefore, targeting GABA_B in FXS may both increase the inhibitory effect of the GABAergic system and decrease input from excess mGluR activation. Treatment with racemic baclofen, a GABA_B receptor agonist, rescued overactive protein synthesis and AMPA receptor internalization in FXS animal models, and reduced audiogenic seizures and abnormal dendritic spine density as well (39). These results spurred a phase II double-blind, placebo-controlled crossover trial with 4-week treatment periods separated by a washout (40). Sixty-three subjects with the FXS full mutation were randomized, and the drug was flexibly titrated in each treatment period and continued at optimal dose for 4 weeks total. Multiple behavioral and cognitive assessments were performed throughout each arm of the study, but failed to show significant improvement in the primary endpoint of the study, the ABC-C Irritability Subscale. However, there was significant improvement on the Visual Analogue Scale (VAS) and on the ABC-C_{FX} (41) Social Avoidance (SA) subscale ($p = 0.008$). Blinded treatment preference by clinicians ($p = 0.05$) and parents ($p = 0.09$) showed trends in favor of arbaclofen, as well as improvements on the CGI-S ($p = 0.09$) and CGI-I ($p = 0.15$). Furthermore, post hoc analysis revealed subjects that were more socially impaired (as designated by baseline ABC-LSW scores) showed significant improvements in favor for arbaclofen in multiple assessments including the CGI-S ($p = 0.009$), CGI-I ($p = 0.02$), the Vineland Adaptive Behavior Scale (VABS) Socialization Subscale ($p = 0.03$), ABC-C SW subscale ($p = 0.07$), and ABC-C_{FX} SA subscale ($p = 0.04$). Arbaclofen showed no safety issues as well.

Two large 8-week placebo-controlled trials of arbaclofen were subsequently conducted: a flexible dose trial in adolescents and adults (ages 12-50 years; $n = 125$, 119 completed) and a fixed dose (3 doses and placebo groups) trial in children (ages 5-11 years; $n = 172$, 159 completed). The older trial failed to show efficacy over placebo in the primary outcome measure, the ABC-C_{FX} SA or any secondary measures (42). However, the trial in children showed a positive trend on the ABC-C_{FX} in the highest dose group compared to placebo, and found significant improvement in several key secondary outcomes as well. Unfortunately, the development of arbaclofen was terminated due to financial constraints.

Finally, acamprosate is a GABA agonist that has properties at both GABA_A and GABA_B receptors. It is FDA approved to treat alcohol withdrawal, and showed positive results in an initial open-label trial of 3 adults with FXS on the CGI-I and in areas of communication (43). In a subsequent 10-week trial in 12 children with FXS (44), nine subjects met criteria for treatment response (CGI-I score of 1 or 2 and a $\geq 30\%$ improvement on the ABC-SW). There was also significant improvement relative to baseline in the ABC-Hyperactivity subscale ($p = 0.04$), Social

Responsiveness Scale (SRS, $p = 0.005$), the ADHD-Rating Scale ($p < 0.0001$), and in the communication domain on the VABS ($p = 0.03$). Acamprosate was also found to be safe in these participants. The study also looked at levels of soluble amyloid precursor protein (sAPP) and sAPPalpha in blood samples (45) as these are known to be elevated in ASD (46-48). Treatment with acamprosate was able to normalize elevated levels of sAPP and sAPPalpha in these patients (45), and the authors suggest sAPP and sAPPalpha may be viable biomarkers to assess treatment response in future studies. Acamprosate is currently being tested to determine whether effects on hyperactivity and social functioning observed in the open-label studies can be verified in a small placebo-controlled trial in FXS (NCT01911455).

4. Minocycline

In addition to its involvement in both mGluR and GABA pathways, FMRP negatively regulates the translation of the protein matrix metalloproteinase 9 (MMP-9), with lack of FMRP leading to elevated MMP-9 activity in FXS (49). This dysregulation has also been associated with immature dendritic spine morphology (50,51). However, novel research in *Fmr1/Mmp-9* double KO mice revealed dendritic spines were similar to those in wild-type (wt) mice (52) suggesting MMP-9 is integral to the pathophysiology of FXS-associated defects at the neuronal level.

Minocycline is an antibiotic of the tetracycline class typically used to treat acne, but is known to decrease MMP-9 activity as well. Preclinical trials of minocycline in the *Fmr1* KO mouse have induced mature dendritic spine morphology and improved anxiety and cognitive measures within one month of use (50,51). However, while positive results in behavior have been seen in both young and adult *Fmr1* KO mice, research suggests younger mice have longer-lasting benefits, whereas improvements in adult mice disappear soon after cessation of treatment (53).

Initial open label studies of minocycline in both pediatric and adult populations showed positive results in areas of language, attention, anxiety, hyperactivity, and overall improvement (54,55). These data spurred a 6-month double-blind, placebo-controlled crossover trial in children with FXS (56). Sixty-six participants were randomized in the study with 55 children completing at least one arm and 48 finishing both arms of the study. Significant improvements were observed in one of the primary outcome measures, the Clinical Global Impression-Improvement scale (CGI-I), as well as in areas of mood and anxiety on the VAS. Overall, minocycline was safe and well-tolerated as most reported AEs were mild in nature. No significant differences in AEs were found between treatment and placebo groups, although one patient experienced a

seizure while on placebo. Continued research should be conducted to confirm its safety profile because long-term treatment with minocycline may darken the skin, gums, and dentition of permanent teeth. Minocycline has also been associated with a rare lupus-like syndrome, and thus an antinuclear antibody (ANA) titer should be checked within the first 6 months of treatment and subsequently annually if stable or more frequently if elevated. Severe chronic headache, rash, or swollen joints should necessitate immediate cessation of treatment. Occasionally, loose stools can occur with minocycline treatment, so use of a probiotic daily while on minocycline will be beneficial to replenish normal flora in the intestine. Also, minocycline should not be taken at the same time as milk because they interfere with absorption when given together. One should wait at least 30 minutes before taking milk after the minocycline dose.

A subgroup of these study patients treated with minocycline (8 males and 4 females) also underwent an event related potential (ERP) study (57). Patients with FXS have exaggerated EEG amplitudes to auditory stimuli and lack a habituation response after repeated stimulation (58-61). In this trial, treatment with minocycline showed statistically significant reductions in temporal activation due to auditory stimuli, as well as improvements in habituation (57). There was also a significantly increased ERP response in the central P2 component, which correlated with improvements on the CGI-I. Despite the low sample size, this data suggests ERP measures may be a possible objective measure to detect treatment response in the FXS population. Additional studies are needed to evaluate ERP paradigms as an outcome measure that correlates with clinical improvement. In addition, MMP-9 levels in FXS are elevated in blood samples and minocycline lowered these levels in the minocycline trial (50). This measure also appears to be a good biomarker in FXS that can be used to monitor treatment response in future trials of minocycline and perhaps in the use of other targeted treatments for FXS.

5. Selective Serotonin Reuptake Inhibitors (SSRIs)

Serotonin dysfunction has been linked to some behaviors associated with FXS and ASD. A study in patients with FXS found that those with a 5-HT transporter polymorphism causing hyperactive serotonin reuptake receptors – and thus lower synaptic serotonin availability – had increased aggression and destructive behaviors (62). Metabolomic and PET imaging studies also demonstrate decreased serotonin production in patients with ASD as well (63,64). These deficits appear to be more pronounced in young children (< 5 years) (63), and are especially deficient in the frontal lobe in children with ASD, which has been associated with language impairment (65). Targeted

treatments toward serotonin not only provide a potential avenue to correct these behaviors in FXS, but serotonin also increases LTP, which could enhance learning and cognitive function (66,67). SSRIs, in particular, provide additional benefit as they stimulate Brain-Derived Neurotrophic Factor (BDNF) production (68), which could help ameliorate cellular abnormalities in the FXS brain.

An initial retrospective study of young children (12 to 50 months) with FXS who were treated with low-dose sertraline (2.5 to 5 mg/day) revealed significant improvement in the developmental trajectory of expressive and receptive language as measured by the Mullen Scales of Early Learning (MSEL) compared to those not treated with sertraline (18). These results spurred a 6-month randomized, placebo-controlled trial of low-dose sertraline (2.5 to 5.0 mg/day) in children ages 2-6 years with FXS (Hess *et al*, unpublished data). Fifty-seven participants were randomized in the study: 27 to sertraline and 30 to placebo. Fifty-two participants completed the study. Primary outcome measures including the CGI-I and the receptive and expressive language scales on the MSEL were not significantly improved compared to placebo; however, areas of fine motor, visual perception, and the Cognitive T score sum did show improvement. A subset of patients with ASD also demonstrated significant improvements in expressive language on the MSEL when treated with sertraline. The dosages used in this study were safe and well-tolerated, and all families opted to continue sertraline after the conclusion of the study.

This trial of low-dose sertraline in young children with FXS revealed significant benefits in areas of cognition and behavior, and was especially notable due to sertraline's positive effect on language. Additional trials should be conducted to replicate these results, and there is also a trial of sertraline in children with idiopathic ASD being conducted at the University of California, Davis MIND Institute (NCT02385799).

6. Lovastatin

Lovastatin is an HMG-CoA reductase inhibitor used in the treatment of hyperlipidemia and hypercholesterolemia. It has a well-known safety profile, and has been approved by the United States Food and Drug Administration to treat familial hypercholesterolemia in children as young as 10 years (69). Preclinical studies have also shown lovastatin inhibits RAS-MAPK-ERK1/2 activation (70), and treatment with lovastatin significantly improved cognitive deficits in the neurofibromatosis type 1 (NF1) mouse model through inhibition of these pathways (71).

Many of the proteins upregulated in FXS are believed to be downstream consequences of increased ERK1/2 activity (72), and trials of lovastatin in the *Fmr1* KO mouse have shown numerous benefits such as decreasing extracellular receptor kinase-mediated protein synthesis,

correcting exaggerated mGluR-mediated LTD, blocking mGluR5-mediated epileptiform bursting hippocampal neurons, dampening hyperexcitability in the visual cortex, and reducing the incidence and severity of seizures (73,74). Recently, a 12-week open label trial of lovastatin in patients with FXS was completed (75); sixteen participants were initially enrolled (mean age = 18 ± 5 years), and 15 subjects finished the study. Results showed significant benefit on the ABC-C after 4 weeks of treatment, and further improvements were observed with continued use throughout the study specifically in areas of hyperactivity, lethargy, social avoidance, and stereotypy as determined by the ABC-C_{FX} (41). Participants also experienced significant improvements in communication, daily living skills, and coping skills on the VABS. Lovastatin was well-tolerated in these participants, and all AEs were transient and mild in intensity.

Lovastatin appears to be a promising therapy for patients with FXS, and future studies should continue to assess its effects. There are two clinical trials currently underway: one is a phase 4 trial looking at combined lovastatin and parent-implemented language intervention (PILI; NCT02642653) and another is a phase II trial evaluating combined minocycline and lovastatin (NCT02680379).

7. Additional trials

An open label trial of lithium in 15 patients with FXS demonstrated significant benefits in several behavioral measures and perhaps most importantly lithium treatment lead to normalization of ERK phosphorylation rates which is a quantitative measure of biological changes in the targeted pathway with lithium (76). Further controlled trials are warranted for lithium, although kidney toxicity after long term use is worrisome for pediatric patients with FXS, which may require prolonged treatment with lithium.

A small randomized, double-blind, placebo-controlled, single-dose trial of intranasal oxytocin was recently completed in adolescent and adult males with FXS (77). Oxytocin acts as both a hormone and neuropeptide, and has been shown to have anxiolytic and pro-social qualities. Eight subjects completed the study, and oxytocin was found to ameliorate behaviors such as anxiety and hyperarousal as suggested through improvements in eye gaze frequency and decreases in salivary cortisol levels. Additional studies are being conducted, although predominantly in ASD populations.

Another promising medication in FXS is trofinetide, a synthetic analogue of the terminal tripeptide tail of Insulin Growth Factor-1 (IGF-1) made by Neuren. Trofinetide demonstrated promising results in the KO mouse with improvement in behavior and also normalization of ERK and Akt levels (78). This led to a multicenter controlled trial in adolescents and adults with

Table 1. Overview of Clinical Trials in fragile X syndrome

Clinical Trial Registration Number	Compound (Drug Class)	Clinical Trial Phase	Target Population	Principal Investigator	Status/Results
<i>Metabotropic glutamate receptor 5 (mGluR5) antagonists</i>					
NCT01357239/ NCT01253629	Mavoglurant (AFQ056)	Phase IIb/Phase IIb	Adolescents/Adults	Novartis Pharmaceuticals	Completed (23)
NCT00718341	Mavoglurant (AFQ056)	Phase IIa	Adults	Novartis Pharmaceuticals	Completed (22)
NCT01517698/ NCT01750957	Basimglurant (RO4917523)	Phase IIb (adult/adolescents), Phase IIa (children)	Adults and adolescent/Children	Hoffmann-La Roche	Completed (24,25)
<i>γ-aminobutyric acid (GABA) modulators</i>					
NCT01282268/ NCT00788073	Arbaclofen (GABA _B agonist)	Phase III	Adults and adolescents/Children	Seaside Therapeutics	Completed (81)
NCT01013480	Arbaclofen (GABA _B agonist)	Phase II	Adults, adolescents and children	E. Berry-Kravis, MD, PhD	Completed (40)
NCT01911455	Acamprosate (GABA agonist)	Phase II	Adults, adolescents and children	E. Berry-Kravis, MD, PhD & C. Erickson, MD	Recruiting
NCT01725152	Ganaxolone (GABA _A agonist)	Phase II	Adolescents and children	R. Hagerman, MD	Completed/Pending
NCT02126995	Metadoxine (ion-pair salt of pyridoxine, GABA activator)	Phase II	Adults and adolescents	E. Berry-Kravis, MD, PhD	Completed (80)
<i>Minocycline</i>					
NCT01053156	Minocycline (Tetracycline)	Phase II	Adolescents and children	R. Hagerman, MD	Completed (56-57)
<i>Selective Serotonin Reuptake Inhibitors (SSRI)</i>					
NCT01474746	Sertraline	Phase II	Children	R. Hagerman, MD	Completed (Hess <i>et al</i> , unpublished data)
<i>Additional Trials</i>					
NCT00054730	CX516 (Ampakine)	Phase II	Adults	E. Berry-Kravis, MD, PhD	Completed (82)
NCT01894958	Trofnetide (NNZ-2566; neurotrophic peptide)	Phase II	Adult and adolescent males	E. Berry-Kravis, MD, PhD	Completed/Pending
NCT01254045	Oxytocin (neuropeptide)	Phase II	Adults and adolescent	A. Reiss, MD	Completed (77)
NCT01329770	Ascorbic acid and α-tocopherol	Phase II	Adolescents and children	Y. de Diego-Otero, PhD & L. Pérez Costillas, MD, PhD	Completed (83)
NCT01120626	Donepezil (cholinergic drug)	Phase II	Adults and adolescents	A. Reiss, MD	Completed (84)
<i>Combined Trials</i>					
NCT02642653	Combined Lovastatin (HMG-CoA reductase inhibitor) and PILI	Phase IV	Children	R. Hagerman, MD	Open for recruitment
NCT02680379	Combined Minocycline (Tetracycline) and Lovastatin (HMG-CoA reductase inhibitor)	Phase II	Adults and adolescents	F. Corbin, MD, PhD	Not yet open for Recruitment

HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A, PILI = Parent-Implemented Language Intervention.

FXS. Although the initial study has not been published at this time, preliminary results look promising (79), and additional studies are planned for the future.

Metadoxine, a combination of vitamin B6 and a 2-pyrrolidone-5-carboxylate ring, is a medication

developed for alcohol toxicity with GABA agonist effects. Metadoxine has been beneficial in the KO mouse by lowering ERK and Akt and has been studied in adolescents and adults with FXS in a multicenter controlled trial (80). The preliminary results were

promising so it is likely to be further assessed in children with FXS (Table 1).

8. Conclusions and future directions

Although significant improvements outside of subgroup and *post hoc* analyses have yet to be reproduced, these clinical trials highlight many salient points as we work toward future trials in FXS. While the outcomes measures utilized in the aforementioned studies have been validated in multiple populations, our toolbox of assessments is largely limited to questionnaires, which are often subjective. Moreover, patients with FXS may bottom out on rating scales depending on the severity of their phenotype. There has already been progress in developing questionnaires specifically graded to monitor changes in patients with FXS (41), and there is a movement toward objective measures to monitor treatment response such as studying ERPs as was carried out in the minocycline trial (57) or MMP-9 levels that were lowered in the minocycline trial (50). Upcoming trials are also studying combination therapy, as this may be another avenue toward unveiling beneficial effects of targeted treatments as well (NCT02642653, NCT02680379). Nevertheless, the positive results found in previous studies should not be diminished, but rather should serve as a sign of progress toward a better understanding of the pathology and clinical treatment of patients with FXS.

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Conflict of Interest: RH has received funding from Roche, Novartis, Neuren and Alcobra for carrying out treatment studies in patients with fragile X syndrome. She has also consulted with Roche, Novartis, Alcobra and Zynerba regarding treatment studies in individuals with fragile X syndrome.

References

1. Tassone F, Iong KP, Tong TH, Lo J, Gane LW, Berry-Kravis E, Nguyen D, Mu LY, Laffin J, Bailey DB, Hagerman RJ. *FMR1* CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome Med.* 2012; 4:100.
2. Weiler IJ, Irwin SA, Klintsova AY, Spencer CM, Brazelton AD, Miyashiro K, Comery TA, Patel B, Eberwine J, Greenough WT. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc Natl Acad Sci U S A.* 1997; 94:5395-5400.
3. Feng Y, Gutekunst CA, Eberhart DE, Yi H, Warren ST, Hersch SM. Fragile X mental retardation protein: Nucleocytoplasmic shuttling and association with somatodendritic ribosomes. *J Neurosci.* 1997; 17:1539-1547.
4. Gross C, Hoffmann A, Bassell GJ, Berry-Kravis EM. Therapeutic Strategies in Fragile X Syndrome: From Bench to Bedside and Back. *Neurotherapeutics.* 2015; 12:584-608.
5. Saldarriaga W, Tassone F, Gonzalez-Teshima LY, Forero-Forero JV, Ayala-Zapata S, Hagerman R. Fragile X syndrome. *Colomb Med (Cali).* 2014; 45:190-198.
6. Hagerman RJ, Ono MY, Hagerman PJ. Recent advances in fragile X: A model for autism and neurodegeneration. *Curr Opin Psychiatry.* 2005; 18:490-496.
7. Harris SW, Hessler D, Goodlin-Jones B, Ferranti J, Bacalman S, Barabato I, Tassone F, Hagerman PJ, Herman H, Hagerman RJ. Autism profiles of males with fragile X syndrome. *Am J Ment Retard.* 2008; 113:427-438.
8. Steinberg J, Webber C. The roles of FMRP-regulated genes in autism spectrum disorder: Single- and multiple-hit genetic etiologies. *Am J Hum Genet.* 2013; 93:825-839.
9. Pretto D, Yrigollen CM, Tang HT, Williamson J, Espinal G, Iwahashi CK, Durbin-Johnson B, Hagerman RJ, Hagerman PJ, Tassone F. Clinical and molecular implications of mosaicism in *FMR1* full mutations. *Front Genet.* 2014; 5:318.
10. Loesch DZ, Huggins RM, Bui QM, Epstein JL, Taylor AK, Hagerman RJ. Effect of the deficits of fragile X mental retardation protein on cognitive status of fragile X males and females assessed by robust pedigree analysis. *J Dev Behav Pediatr.* 2002; 23:416-423.
11. Loesch DZ, Bui QM, Grigsby J, Butler E, Epstein J, Huggins RM, Taylor AK, Hagerman RJ. Effect of the fragile X status categories and the fragile X mental retardation protein levels on executive functioning in males and females with fragile X. *Neuropsychology.* 2003; 17:646-657.
12. Klaiman C, Quintin EM, Jo B, Lightbody AA, Hazlett HC, Piven J, Hall SS, Chromik LC, Reiss AL. Longitudinal profiles of adaptive behavior in fragile X syndrome. *Pediatrics.* 2014; 134:315-324.
13. Hinton VJ, Brown WT, Wisniewski K, Rudelli RD. Analysis of neocortex in three males with the fragile X syndrome. *Am J Med Genet.* 1991; 41:289-294.
14. Rudelli RD, Brown WT, Wisniewski K, Jenkins EC, Laure-Kamionowska M, Connell F, Wisniewski HM. Adult fragile X syndrome. Clinico-neuropathologic findings. *Acta Neuropathol.* 1985; 67:289-295.
15. Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA, Larsen BP, Kooy F, Willems PJ, Cras P, Kozlowski PB, Swain RA, Weiler IJ, Greenough WT. Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination. *Am J Med Genet.* 2001; 98:161-167.
16. Pfeiffer BE, Huber KM. The state of synapses in fragile X syndrome. *Neuroscientist.* 2009; 15:549-567.
17. Pan F, Aldridge GM, Greenough WT, Gan WB. Dendritic spine instability and insensitivity to modulation by sensory experience in a mouse model of fragile X

- syndrome. Proc Natl Acad Sci U S A. 2010; 107:17768-17773.
18. Winarni TI, Schneider A, Borodyanskara M, Hagerman RJ. Early intervention combined with targeted treatment promotes cognitive and behavioral improvements in young children with fragile x syndrome. Case Rep Genet. 2012; 2012:280813.
 19. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. Trends Neurosci. 2004; 27:370-377.
 20. Dolen G, Osterweil E, Rao BS, Smith GB, Auerbach BD, Chattarji S, Bear MF. Correction of fragile X syndrome in mice. Neuron. 2007; 56:955-962.
 21. Berry-Kravis E, Hessel D, Coffey S, Hervey C, Schneider A, Yuhas J, Hutchison J, Snape M, Tranfaglia M, Nguyen DV, Hagerman R. A pilot open label, single dose trial of fenobam in adults with fragile X syndrome. J Med Genet. 2009; 46:266-271.
 22. Jacquemont S, Curie A, des Portes V, *et al.* Epigenetic modification of the *FMRI* gene in fragile X syndrome is associated with differential response to the mGluR5 antagonist AFQ056. Sci Transl Med. 2011; 3:64ra1.
 23. Berry-Kravis E, Des Portes V, Hagerman R, Jacquemont S, Charles P, Visootsak J, Brinkman M, Rerat K, Koumaras B, Zhu L, Barth GM, Jaecklin T, Apostol G, von Raison F. Mavoglurant in fragile X syndrome: Results of two randomized, double-blind, placebo-controlled trials. Sci Transl Med. 2016; 8:321ra5.
 24. Quiroz J, Wasef E, Wong CY, Kurian A, Deptula D, Banken L, Rabbia M, Fontoura P, Santarelli L. Effect of Two Doses of Basimglurant on Behavioral Symptoms in Adolescent & Adult Patients with Fragile X Syndrome: Results from FRAGXIS, a Double-Blind, Placebo Controlled Study. In: International Meeting for Autism Research (Salt Lake City, UT, 2015). <https://imfar.confex.com/imfar/2015/webprogram/Paper19054.html> (accessed July 28, 2016).
 25. Quiroz J, Wong CY, Wasef E, Deptula D, Banken L, Rabbia M, Fontoura P, Santarelli L. Safety and Exploratory Efficacy of Basimglurant in Pediatric Patients with Fragile X Syndrome: A Randomized, Double-Blind, Placebo-Controlled Study. In: International Meeting for Autism Research (Salt Lake City, UT, 2015). <https://imfar.confex.com/imfar/2015/webprogram/Paper18427.html> (accessed July 28, 2016).
 26. Olmos-Serrano JL, Paluszkiwicz SM, Martin BS, Kaufmann WE, Corbin JG, Huntsman MM. Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. J Neurosci. 2010; 30:9929-9938.
 27. D'Hulst C, Heulens I, Brouwer JR, Willemsen R, De Geest N, Reeve SP, De Deyn PP, Hassan BA, Kooy RF. Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS). Brain Res. 2009; 1253:176-183.
 28. D'Hulst C, Kooy RF. The GABAA receptor: A novel target for treatment of fragile X? Trends Neurosci. 2007; 30:425-431.
 29. D'Hulst C, De Geest N, Reeve SP, Van Dam D, De Deyn PP, Hassan BA, Kooy RF. Decreased expression of the GABAA receptor in fragile X syndrome. Brain Res. 2006; 1121:238-245.
 30. Heulens I, D'Hulst C, Van Dam D, De Deyn PP, Kooy RF. Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model. Behav Brain Res. 2012; 229:244-249.
 31. Chang S, Bray SM, Li Z, Zarnescu DC, He C, Jin P, Warren ST. Identification of small molecules rescuing fragile X syndrome phenotypes in Drosophila. Nat Chem Biol. 2008; 4:256-263.
 32. Gatto CL, Broadie K. Genetic controls balancing excitatory and inhibitory synaptogenesis in neurodevelopmental disorder models. Front Synaptic Neurosci. 2010; 2:4.
 33. Olmos-Serrano JL, Corbin JG, Burns MP. The GABA(A) receptor agonist THIP ameliorates specific behavioral deficits in the mouse model of fragile X syndrome. Dev Neurosci. 2011; 33:395-403.
 34. Braat S, D'Hulst C, Heulens I, De Rubeis S, Mientjes E, Nelson DL, Willemsen R, Bagni C, Van Dam D, De Deyn PP, Kooy RF. The GABAA receptor is an FMRP target with therapeutic potential in fragile X syndrome. Cell Cycle. 2015; 14:2985-2995.
 35. Gantois I, Vandesompele J, Speleman F, Reyniers E, D'Hooge R, Severijnen LA, Willemsen R, Tassone F, Kooy RF. Expression profiling suggests underexpression of the GABA(A) receptor subunit delta in the fragile X knockout mouse model. Neurobiol Dis. 2006; 21:346-357.
 36. D'Hulst C, Heulens I, Van der Aa N, Goffin K, Koole M, Porke K, Van De Velde M, Rooms L, Van Paesschen W, Van Esch H, Van Laere K, Kooy RF. Positron Emission Tomography (PET) Quantification of GABAA Receptors in the Brain of Fragile X Patients. PloS One. 2015; 10:e0131486.
 37. Reddy DS, Rogawski MA. Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. J Pharmacol Exp Ther. 2000; 295:1241-1248.
 38. Isaacson JS, Hille B. GABA(B)-mediated presynaptic inhibition of excitatory transmission and synaptic vesicle dynamics in cultured hippocampal neurons. Neuron. 1997; 18:143-152.
 39. Pacey LK, Heximer SP, Hampson DR. Increased GABA(B) receptor-mediated signaling reduces the susceptibility of fragile X knockout mice to audiogenic seizures. Mol Pharmacol. 2009; 76:18-24.
 40. Berry-Kravis EM, Hessel D, Rathmell B, Zarevics P, Cherubini M, Walton-Bowen K, Mu Y, Nguyen DV, Gonzalez-Heydrich J, Wang PP, Carpenter RL, Bear MF, Hagerman RJ. Effects of STX209 (arbaclofen) on neurobehavioral function in children and adults with fragile X syndrome: A randomized, controlled, phase 2 trial. Sci Transl Med. 2012; 4:152ra127.
 41. Sansone SM, Widaman KF, Hall SS, Reiss AL, Lightbody A, Kaufmann WE, Berry-Kravis E, Lachiewicz A, Brown EC, Hessel D. Psychometric study of the Aberrant Behavior Checklist in Fragile X Syndrome and implications for targeted treatment. J Autism Dev Disord. 2012; 42:1377-1392.
 42. Berry-Kravis E, Visootsak J, Hagerman R, Budimirovic D, Kaufmann W, Bear M, Walton-Bowen K, Carpenter R, Wang P. Arbaclofen in fragile X syndrome: Results of phase 3 trials. Ann Neurol. 2014; 76.
 43. Erickson CA, Mullett JE, McDougale CJ. Brief report: Acamprosate in fragile X syndrome. J Autism Dev Disord. 2010; 40:1412-1416.
 44. Erickson CA, Wink LK, Ray B, Early MC, Stieglmeier E, Mathieu-Frasier L, Patrick V, Lahiri DK, McDougale

- CJ. Impact of acamprosate on behavior and brain-derived neurotrophic factor: An open-label study in youth with fragile X syndrome. *Psychopharmacology (Berl)*. 2013; 228:75-84.
45. Erickson CA, Ray B, Maloney B, Wink LK, Bowers K, Schaefer TL, McDougle CJ, Sokol DK, Lahiri DK. Impact of acamprosate on plasma amyloid-beta precursor protein in youth: A pilot analysis in fragile X syndrome-associated and idiopathic autism spectrum disorder suggests a pharmacodynamic protein marker. *J Psychiatr Res*. 2014; 59:220-228.
 46. Bailey AR, Giunta BN, Obregon D, Nikolic WV, Tian J, Sanberg CD, Sutton DT, Tan J. Peripheral biomarkers in Autism: Secreted amyloid precursor protein-alpha as a probable key player in early diagnosis. *Int J Clin Exp Med*. 2008; 1:338-344.
 47. Sokol DK, Chen D, Farlow MR, Dunn DW, Maloney B, Zimmer JA, Lahiri DK. High levels of Alzheimer beta-amyloid precursor protein (APP) in children with severely autistic behavior and aggression. *J Child Neurol*. 2006; 21:444-449.
 48. Ray B, Long JM, Sokol DK, Lahiri DK. Increased secreted amyloid precursor protein-alpha (sAPPalpha) in severe autism: Proposal of a specific, anabolic pathway and putative biomarker. *PloS One*. 2011; 6:e20405.
 49. Dziembowska M, Wlodarczyk J. MMP9: A novel function in synaptic plasticity. *Int J Biochem Cell Biol*. 2012; 44:709-713.
 50. Dziembowska M, Pretto DI, Janusz A, Kaczmarek L, Leigh MJ, Gabriel N, Durbin-Johnson B, Hagerman RJ, Tassone F. High MMP-9 activity levels in fragile X syndrome are lowered by minocycline. *Am J Med Genet A*. 2013; 161A:1897-1903.
 51. Bilousova TV, Dansie L, Ngo M, Aye J, Charles JR, Ethell DW, Ethell IM. Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. *J Med Genet*. 2009; 46:94-102.
 52. Sidhu H, Dansie LE, Hickmott PW, Ethell DW, Ethell IM. Genetic removal of matrix metalloproteinase 9 rescues the symptoms of fragile X syndrome in a mouse model. *J Neurosci*. 2014; 34:9867-9879.
 53. Dansie LE, Phommahaxay K, Okusanya AG, Uwadia J, Huang M, Rotschafer SE, Razak KA, Ethell DW, Ethell IM. Long-lasting effects of minocycline on behavior in young but not adult Fragile X mice. *Neuroscience*. 2013; 246:186-198.
 54. Utari A, Chonchaiya W, Rivera SM, Schneider A, Hagerman RJ, Faradz SM, Ethell IM, Nguyen DV. Side effects of minocycline treatment in patients with fragile X syndrome and exploration of outcome measures. *Am J Intellect Dev Disabil*. 2010; 115:433-443.
 55. Paribello C, Tao L, Folino A, Berry-Kravis E, Tranfaglia M, Ethell IM, Ethell DW. Open-label add-on treatment trial of minocycline in fragile X syndrome. *BMC Neurol*. 2010; 10:91.
 56. Leigh MJ, Nguyen DV, Mu Y, Winarni TI, Schneider A, Chechi T, Polussa J, Doucet P, Tassone F, Rivera SM, Hessel D, Hagerman RJ. A randomized double-blind, placebo-controlled trial of minocycline in children and adolescents with fragile x syndrome. *J Dev Behav Pediatr*. 2013; 34:147-155.
 57. Schneider A, Leigh MJ, Adams P, Nanakul R, Chechi T, Olichney J, Hagerman R, Hessel D. Electrocardiogram changes associated with minocycline treatment in fragile X syndrome. *J Psychopharmacol*. 2013; 27:956-963.
 58. Castren M, Paakkonen A, Tarkka IM, Ryyanen M, Partanen J. Augmentation of auditory N1 in children with fragile X syndrome. *Brain Topogr*. 2003; 15:165-171.
 59. Hessel D, Berry-Kravis E, Cordeiro L, Yuhas J, Ornitz EM, Campbell A, Chruscinski E, Hervey C, Long JM, Hagerman RJ. Prepulse inhibition in fragile X syndrome: Feasibility, reliability, and implications for treatment. *Am J Med Genet B Neuropsychiatr Genet*. 2009; 150B:545-553.
 60. Van der Molen MJ, Van der Molen MW, Ridderinkhof KR, Hamel BC, Curfs LM, Ramakers GJ. Auditory change detection in fragile X syndrome males: A brain potential study. *Clin Neurophysiol*. 2012; 123:1309-1318.
 61. Van der Molen MJ, Van der Molen MW, Ridderinkhof KR, Hamel BC, Curfs LM, Ramakers GJ. Attentional set-shifting in fragile X syndrome. *Brain Cogn*. 2012; 78:206-217.
 62. Hessel D, Tassone F, Cordeiro L, Koldewyn K, McCormick C, Green C, Wegelin J, Yuhas J, Hagerman RJ. Brief report: Aggression and stereotypic behavior in males with fragile X syndrome – moderating secondary genes in a "single gene" disorder. *J Autism Dev Disord*. 2008; 38:184-189.
 63. Chugani DC, Muzik O, Chakraborty P, Mangner T, Chugani HT. Human brain serotonin synthesis capacity measured *in vivo* with α -[C-11]methyl-L-tryptophan. *Synapse*. 1998; 28:33-43.
 64. Boccutto L, Chen CF, Pittman AR, Skinner CD, McCartney HJ, Jones K, Bochner BR, Stevenson RE, Schwartz CE. Decreased tryptophan metabolism in patients with autism spectrum disorders. *Mol Autism*. 2013; 4:16.
 65. Chandana SR, Behen ME, Juhasz C, Muzik O, Rothermel RD, Mangner TJ, Chakraborty PK, Chugani HT, Chugani DC. Significance of abnormalities in developmental trajectory and asymmetry of cortical serotonin synthesis in autism. *Int J Dev Neurosci*. 2005; 23:171-182.
 66. West PJ, Marcy VR, Marino MJ, Schaffhauser H. Activation of the 5-HT(6) receptor attenuates long-term potentiation and facilitates GABAergic neurotransmission in rat hippocampus. *Neuroscience*. 2009; 164:692-701.
 67. Mori K, Togashi H, Kojima T, Matsumoto M, Ohashi S, Ueno K, Yoshioka M. Different effects of anxiolytic agents, diazepam and 5-HT(1A) agonist tandospirone, on hippocampal long-term potentiation *in vivo*. *Pharmacol Biochem Behav*. 2001; 69:367-372.
 68. Bianchi P, Ciani E, Guidi S, Trazzi S, Felice D, Grossi G, Fernandez M, Giuliani A, Calza L, Bartesaghi R. Early pharmacotherapy restores neurogenesis and cognitive performance in the Ts65Dn mouse model for Down syndrome. *J Neurosci*. 2010; 30:8769-8779.
 69. Descamps OS, Tenoutasse S, Stephenne X, *et al*. Management of familial hypercholesterolemia in children and young adults: Consensus paper developed by a panel of lipidologists, cardiologists, paediatricians, nutritionists, gastroenterologists, general practitioners and a patient organization. *Atherosclerosis*. 2011; 218:272-280.
 70. Xu XQ, McGuire TF, Blaskovich MA, Sebt SM, Romero G. Lovastatin inhibits the stimulation of

- mitogen-activated protein kinase by insulin in HIRcB fibroblasts. *Arch Biochem Biophys.* 1996; 326:233-237.
71. Li W, Cui Y, Kushner SA, Brown RA, Jentsch JD, Frankland PW, Cannon TD, Silva AJ. The HMG-CoA reductase inhibitor lovastatin reverses the learning and attention deficits in a mouse model of neurofibromatosis type 1. *Curr Biol.* 2005; 15:1961-1967.
 72. Bhakar AL, Dolen G, Bear MF. The pathophysiology of fragile X (and what it teaches us about synapses). *Annu Rev Neurosci.* 2012; 35:417-443.
 73. Osterweil EK, Chuang SC, Chubykin AA, Sidorov M, Bianchi R, Wong RK, Bear MF. Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron.* 2013; 77:243-250.
 74. Osterweil EK, Krueger DD, Reinhold K, Bear MF. Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. *J Neurosci.* 2010; 30:15616-15627.
 75. Caku A, Pellerin D, Bouvier P, Riou E, Corbin F. Effect of lovastatin on behavior in children and adults with fragile X syndrome: An open-label study. *Am J Med Genet A.* 2014; 164A:2834-2842.
 76. Berry-Kravis E, Sumis A, Hervey C, Nelson M, Porges SW, Weng N, Weiler IJ, Greenough WT. Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr.* 2008; 29:293-302.
 77. Hall SS, Lightbody AA, McCarthy BE, Parker KJ, Reiss AL. Effects of intranasal oxytocin on social anxiety in males with fragile X syndrome. *Psychoneuroendocrinology.* 2012; 37:509-518.
 78. Deacon RM, Glass L, Snape M, Hurley MJ, Altimiras FJ, Biekofsky RR, Cogram P. NNZ-2566, a novel analog of (1-3) IGF-1, as a potential therapeutic agent for fragile X syndrome. *Neuromolecular Med.* 2015; 17:71-82.
 79. Treagus R. Neuren's trofinetide successful in proof of concept Phase 2 clinical trial in Fragile X syndrome. <http://www.neurenpharma.com/IRM/PDF/1557/TrofinetidesuccessfulinPhase2trialinFragileX> (accessed July 28, 2016).
 80. Berry-Kravis E, Rubin J, Harary E, Daniely Y. A 6-week, randomized, multicenter, double-blind, parallel, flexed- and fixed-dose study of MDX (Metadoxine extended-release; MG01CI) compared with placebo in adolescents and adults with fragile X syndrome. http://files.shareholder.com/downloads/AMDA-1SVKDP/0x0x858015/E85D78F1-33D2-46F4-B4C7-96472D1F9A22/AACAP_AL014_poster_final.pdf (accessed July 28, 2016).
 81. De Sonia A, Visootsak J, Smith M, *et al.* FXCRC analysis of arbaclofen responses in Fragile X Syndrome. In: 14th International Fragile X Conference (Orange County, CA, 2014). http://onramp.meetingexpectations.com/Presentation/FragileX_PresentationSearch.aspx?eventID=6H1m3bLeW1kyNTaZzmVDaw%3D%3D&ClientID=50&gwwSearchResultsChangePage=22_20 (accessed July 28, 2016).
 82. Berry-Kravis E, Krause SE, Block SS, *et al.* Effect of CX516, an AMPA-modulating compound, on cognition and behavior in fragile X syndrome: A controlled trial. *J Child Adolesc Psychopharmacol.* 2006; 16:525-540.
 83. de Diego-Otero Y, Calvo-Medina R, Quintero-Navarro C, Sanchez-Salido L, Garcia-Guirado F, del Arco-Herrera I, Fernandez-Carvajal I, Ferrando-Lucas T, Caballero-Andaluz R, Perez-Costillas L. A combination of ascorbic acid and alpha-tocopherol to test the effectiveness and safety in the fragile X syndrome: Study protocol for a phase II, randomized, placebo-controlled trial. *Trials.* 2014; 15:345.
 84. Sahu JK, Gulati S, Sapra S, Arya R, Chauhan S, Chowdhury MR, Gupta N, Kabra M, Gupta YK, Dwivedi SN, Kalra V. Effectiveness and safety of donepezil in boys with fragile x syndrome: A double-blind, randomized, controlled pilot study. *J Child Neurol.* 2013; 28:570-575.
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Targeting mRNA for the treatment of facioscapulohumeral muscular dystrophy

Bo Bao¹, Rika Maruyama¹, Toshifumi Yokota^{1,2,*}

¹ Department of Medical Genetics, School of Human Development, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB, Canada;

² Muscular Dystrophy Canada Research Chair, University of Alberta, Edmonton AB, Canada.

Summary

Facioscapulohumeral muscular dystrophy (FSHD) is an inherited autosomal dominant disorder characterized clinically by progressive muscle degeneration. Currently, no curative treatment for this disorder exists. FSHD patients are managed through physiotherapy to improve function and quality of life. Over the last two decades, FSHD has been better understood as a disease genetically characterized by a pathogenic contraction of a subset of macrosatellite repeats on chromosome 4. Specifically, several studies support an FSHD pathogenesis model involving the aberrant expression of the *double homeobox protein 4 (DUX4)* gene. Hence, potential therapies revolving around inhibition of *DUX4* have been explored. One of the potential treatment options is the use of effective antisense oligonucleotides (AOs) to knockdown expression of the myopathic *DUX4* gene and its downstream molecules including *paired-like homeodomain transcription factor 1 (PITX1)*. Success in the suppression of *PITX1* expression has already been demonstrated systemically *in vivo* in recent studies. In this article, we will review the pathogenesis of FSHD and the latest research involving the use of antisense knockdown therapy.

Keywords: Antisense oligonucleotide therapy, *DUX4*, morpholino, gene therapy, *PITX1*, skeletal muscle

1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant gain-of-function genetic disorder involving asymmetric muscle weakness and atrophy particularly observed in the face, shoulder, upper arms, further extending into the trunk and legs (1). While there are a dozen forms of muscular dystrophy, FSHD is the third most common muscular dystrophy after Duchenne muscular dystrophy (DMD) and myotonic dystrophy, affecting approximately 1 in 8,000 - 20,000 individuals (2,3). However, since an individual can remain asymptomatic or exhibit mild symptoms, the

frequency of FSHD occurrence could be underestimated. While several candidate genes for FSHD have been identified and explored thus far, the role of *DUX4* as the causative gene in the pathogenesis of FSHD has predominated in the literature (4,5). Hence, the inhibition of *DUX4* expression and the suppression of its downstream molecules can potentially offer therapeutic benefit. The potential of antisense oligonucleotide (AO) therapy as a therapeutic treatment for neuromuscular diseases has recently been highlighted by several clinical trials involving DMD and spinal muscular atrophy (e.g. ClinicalTrials.gov identifier: NCT02193074). Recently, *in vitro* studies have demonstrated success in the suppression of *DUX4* mRNA expression by administering AOs into primary skeletal muscle cells of FSHD patients (5). Nonetheless, desired progress has been impeded by the lack of FSHD animal models and inefficient uptake of AOs into FSHD skeletal muscle fibers. This article will cover the pathogenesis of FSHD, the applicability of antisense oligonucleotide therapy in FSHD, as well as the limitations of antisense therapy in neuromuscular disorders.

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*Address correspondence to:

Dr. Toshifumi Yokota; Department of Medical Genetics, School of Human Development, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB, Canada T6G 2H7.

E-mail: toshifum@ualberta.ca

2. Pathogenesis of FSHD

FSHD is a genetic and epigenetic disorder primarily involving skeletal muscles. Unique to FSHD are asymmetric muscle weaknesses particularly seen in the face, shoulder and extremities (1). Typically, the onset of symptoms observed in FSHD patients occurs from 15-30 years of age. Depending on the genetic and epigenetic factors, disease manifestations will differ. Initially, FSHD phenotype involves facial muscle weakness resulting in difficulty with labial consonants, whistling and drinking through a straw (6). Upon progression of the disease, atrophy involving the upper arms, pelvic girdle, and lower limbs will occur. Hence, 10-20% of FSHD patients will progressively lose independent ambulation and become wheelchair bound by the age of 50 years (7). The most common presenting symptom is shoulder abduction weakness, seen in 82% of symptomatic patients. Shoulder weakness is the result of a more lateral positioning of the scapula than normal leading to a winged scapula appearance. Additionally, a clinical finding specific to FSHD is the Beevor's sign, which describes the ascending movement of the umbilicus when flexing the neck due to early truncal weakness. Extra-muscular manifestations of FSHD involve high-frequency hearing loss, and retinal telangiectasias in 75% and 60% of patients, respectively (8). Factors that contribute to the severity of phenotype include the age of symptom onset and also the extent of genetic changes.

2.1. Genetics of FSHD

Several candidate genes have reportedly been involved in the FSHD phenotype described previously: *DUX4*, *FSHD region gene 1 (FRG1)*, *FSHD region gene 2 (FRG2)*, and *adenine nucleotide translocase 1 (ANT1)* (9). Recent studies have primarily attributed pathogenesis of FSHD to the aberrant expression of a normally dormant gene, *DUX4* (10). *DUX4* is a double homeodomain transcription factor encoded within the D4Z4 tandem repeat. In a healthy individual, the subtelomeric region of chromosome 4q contains 11-100 copies of the 3.3 kb D4Z4 macrosatellite repeat, each with a copy of *DUX4* (11). However, *DUX4* is not expressed in normal functioning somatic tissues such as well-differentiated muscles fibers. While *DUX4* is expressed in early development, it is transcriptionally silenced during cellular differentiation of somatic tissues by CpG methylation of D4Z4 repeats (12). In early development, *DUX4* may play a role in activating a stem-cell-like transcriptional pathway (10). Expression of *DUX4* is maintained in the spermatogonia of the male testis (13,14). While the role of *DUX4* in the seminiferous tubule is not clearly defined, it may be involved in germ cell maintenance and development of stem cells (15). Snider *et al.* illustrated the expression

of full-length *DUX4* mRNA in induced pluripotent stem cells (iPSCs). However, the aberrant expression of *DUX4* is severely toxic to muscle tissues, resulting in oxidative stress and apoptosis (16-19). A recent study indicates that expression of *DUX4* in B cells was even capable of generating leukemia in mice *in vivo* (20). Additionally, *DUX4*-induced expression of antigenic proteins such as ERV may be involved in the inflammatory response seen in FSHD muscle histopathology, contributing to muscle atrophy (13).

2.2. Epigenetics of FSHD

In FSHD patients, several epigenetic changes take place to result in the pathogenic expression of *DUX4* in skeletal muscle cells. The first is the contraction of the D4Z4 array. Specifically, the deletion of D4Z4 repeat array in the subtelomeric region of chromosome 4 called 4q35 to less than 10 units results in reduced methylation and subsequently chromatin remodeling (21). This defect was first described as a reduction seen in EcoRI fragment of genomic DNA as compared to healthy individuals. While healthy individuals possess 11 to 150 D4Z4 repeats with EcoRI fragments being 40-300 kb in size, FSHD patients have between 1 to 10 repeats and EcoRI fragments at 10-38 kb in size (22). Following reduced DNA methylation due to contracted D4Z4 repeat, a more relaxed chromatin structure allows greater expression of genes located on that locus. The smaller the D4Z4 repeat size, the greater severity of the disease. Secondly, the presence of pLAM1 polyadenylated mRNA site at the distal D4Z4 unit is another condition for disease manifestation (23,24). Interestingly, the polyadenylation site is only intact on chromosome 4qA and not 4qB (25). As such, possible therapeutic strategy for FSHD may include inhibition of polyadenylation in chromosome 4qA leading to *DUX4* gene silencing. Ultimately, the deletion of D4Z4 array leads to a combination of DNA hypomethylation and polyadenylation allowing the aberrant expression of *DUX4*. Hence, *DUX4* are occasionally expressed in skeletal muscle nuclei (14). Detectable levels of *DUX4* up-regulation in myoblast was illustrated by Snider *et al.*, where 1 in 1,000 nuclei was positive for *DUX4* in proliferating primary FSHD myoblasts. Tassin *et al.* also confirmed low expression of *DUX4* in proliferating FSHD myoblasts *via* Western blot analysis. The study demonstrated increased *DUX4* protein expression within 1 in 200 nuclei after allowing FSHD primary myoblasts to differentiate for 4 days. Hence, *DUX4* transcription can be influenced by physiological stage of the cells and its surrounding environment (26).

2.3. Types of FSHD

Two types of FSHD exist: FSHD1 and FSHD2. The most common form, FSHD1, occurs in over 95% of

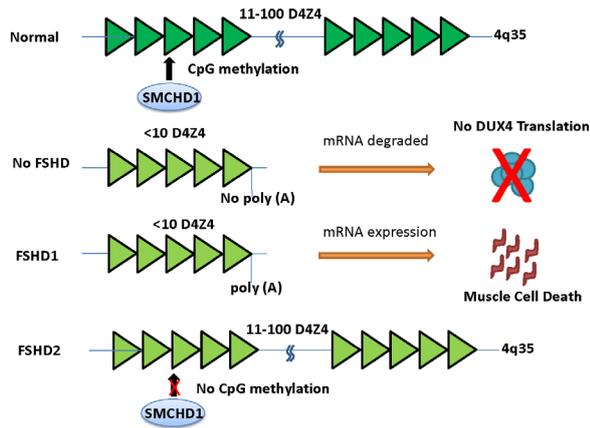


Figure 1. Role of chromosome 4q35 D4Z4 repeat array in the pathogenesis of facioscapulohumeral muscular dystrophy (FSHD). While contraction of D4Z4 repeats in FSHD1 results in the expression of *DUX4* and subsequent myopathy, expression of *DUX4* in FSHD2 is due to a mutation in the *SMCHD1* gene.

FSHD patients (21). Genetic analysis links FSHD 1 to the genetic contraction of macrosatellite D4Z4 repeat array on chromosome 4. FSHD2, however, has a normal number of D4Z4 repeats and instead involves a heterozygous mutation in the *SMCHD1* gene on chromosome 18p, a chromatin modifier (27) (Figure 1). Nonetheless, patients with FSHD1 and FSHD2 share similar clinical presentations. Over the last two decades, progress has been made in the better understanding of the pathogenesis of FSHD, potentially leading to therapeutic strategies for the treatment of FSHD.

3. Therapeutic Approaches to FSHD

No definitive curative treatment for FSHD has been established despite the recent progress made in understanding the genetic and pathophysiologic mechanism of the disease. Current standard clinical management options include physical therapy, aerobic exercise, respiratory function therapy, and orthopedic intervention (28,29). The current guideline for the management of individuals with FSHD is based on a principle of improving function and quality of life. Current drug therapy trials for the management of FSHD include myostatin inhibitor luspatercept and anti-inflammatory biologics (ATYR1940). The basis for anti-inflammatory biologics is to suppress inflammation commonly seen in muscle pathology of FSHD patients in order to slow phenotype progression. All the while, gene therapy has been explored to reduce pathogenic *DUX4* protein production in FSHD by controlling D4Z4 methylation, suppressing *DUX4* mRNA, and inhibiting *DUX4* pathway (5,17,30-32). Several inhibitory tools are available for use including small interfering RNA (siRNA), small hairpin RNA (shRNA), microRNA (miRNA) and antisense oligonucleotides (33,34).

3.1. RNA interference

RNA interference-based approach has been explored by several studies as a prospective treatment for FSHD. siRNA are small double-stranded RNA molecules that act in the cytoplasm of cells to silence mRNA of targeted gene *via* a process of transcript degradation or translational inhibition (35). siRNA has been used to target the 3' untranslated region transcribed from pLAM (5). While shRNA (or artificial miRNA) shares a similar process of silencing target genes as siRNA, it acts on the nucleus of the cell instead. Hence, the advantage of shRNA lies in its ability to have long lasting effects at low doses. Wallace *et al.* have demonstrated *in vivo* success with an artificial miRNA by delivering miDUX4 through adeno-associated viral (AAV) vector into an AAV-based *DUX4* mouse model (32). The study illustrates a 90% reduction in *DUX4* protein and 64% reduction in *DUX4* mRNA. One of the limitations of RNA interference approach is its high dose cytotoxicity derived from its off-target effects (36,37). Additionally, the negative charge, size, and rigid structure of siRNA can complicate its passive diffusion across the target cell. Therefore, they require viral vectors for *in vivo* systemic delivery, which can cause significant side effects such as immune response.

3.2. Antisense oligonucleotides

Antisense oligonucleotides (AOs) on the other hand are small single-stranded DNA-like molecules of 8 to 30 base pairs in length which can be chemically modified specifically to interfere with mRNA processing and stability (38). AOs can either act *via* exon skipping, splice modulation, or inhibition of gene expression. Importantly they do not require viral vectors for *in vivo* systemic delivery. The potential of AOs was initially demonstrated following discovery that transfection of short DNA sequence can inhibit gene expression (39). Currently, antisense therapy is used in preclinical studies and clinical trials of a variety of neuromuscular disorders including DMD, spinal muscular atrophy (SMA), and Fukuyama congenital muscular dystrophy (FCMD) (40-46). Currently, Sarepta therapeutics, Nippon-Shinyaku, and Prosensa are conducting clinical trials involving phosphorodiamidate morpholino oligomer (PMO) and 2'O methyl phosphorothioate oligonucleotide (2'OMePS). Beyond neuromuscular disease, antisense-mediated gene suppression therapy has taken ground in a spectrum of disease including cancer, thrombosis, and Ebola (47-50).

4. Antisense oligonucleotide therapy for FSHD

In light of recent success antisense therapy has in the study of neuromuscular disorders, its application to FSHD has been investigated in multiple studies (51).

Antisense therapy uses antisense oligonucleotides (AOs) which are short single-stranded DNA-like molecules to selectively hybridize pre-mRNA *via* base pairing (38). Since oligonucleotides have difficulty penetrating the lipid bilayer of cells and are also degradable by nucleases, several AO chemistries have been designed to continuously improve efficacy including PMO, octa-guanidine dendrimer conjugated PMOs (vPMO), and peptide-phosphorodiamidate morpholino oligomer (PPMO). Challengingly, the *DUX4* open reading frame (ORF) is located in the first exon and hence makes disruption of its reading frame by antisense-mediated exon skipping difficult (52). However, effective interference of *DUX4* mRNA using antisense oligonucleotide has been illustrated by Vanderplanck *et al. in vitro* (5). The study utilizes 2'OMePs to target splice sites of exon 2 and 3 and thereby disrupts the polyadenylation signal at the 3'UTR. Upon Western blot analysis, no *DUX4* protein can be detected following treatment with 600 nM of AOs. As well, the 2'OMePs administered was able to achieve 50% reduction in the intensity of *DUX4* gene upon RT-PCR analysis of *DUX4* gene fragments. However, the 2'OMePs chemistry developed by Prosenza targeting DMD has recently failed phase III clinical trial due to its toxicity and ineffectiveness (53,54).

4.1. Phosphorodiamidate morpholino oligomer (PMO)

PMO is one of the most commonly used modified AO chemistry to offer sequence-specific inhibition of gene expression (55). PMO consists of short chains of 20-30 nucleic acid bases, a morpholino ring and a non-ionic phosphorodiamidate intersubunit linkage (56). Its structural chemistry provides high nuclease resistance, high affinity to target RNA, resistance to metabolic degradation, and reduced activation of toll-like receptors (57,58). The phosphorodiamidate backbone, in particular, helps the morpholino evade targeting by nucleases. The modified backbone also provides additional stability by helping the molecule evade immune responses. As well, compared to equivalent DNA-based antisense oligonucleotides, morpholino also has a higher binding affinity (56,57,59). Hence, morpholinos lead to less off-target effects. In addition, morpholinos have longer effective half-life due to its substitution for a six-membered morpholine ring. *In vivo* DMD studies have shown the efficacy of PMO by illustrating its ability to penetrate dysfunctional muscle fibers, increase dystrophin expression and ultimately improve muscle function (60-63). Marsollier *et al.* have shown the efficacy of transfecting PMO in immortalized FSHD cells to target *DUX4* mRNA polyadenylation signal in order to suppress the expression of *DUX4*. One of the challenges in building a therapeutic strategy around PMOs is its difficulty in crossing the lipid bilayer of cells and thereby resulting in reduced

delivery to skeletal muscles (38). While the leaky muscle membrane of DMD assists in the uptake of AOs into a target cell, FSHD myofibers lack this leakiness (64-66). Hence, to achieve and maintain therapeutic efficacy, PMOs may need to be administered in large and repeated doses. However, a higher dose could result in harmful effects.

4.2. Octa-guanidine dendrimer-conjugated vivo-morpholinos (vPMO)

In order to improve the cell-penetrating ability of antisense oligonucleotides, second-generation oligonucleotide such as octa-guanidine dendrimer-conjugated vivo-morpholinos (vPMO) have been used. Vivo-morpholinos essentially conjugates with a triazine core scaffold of eight guanidinium head groups to help penetrate the cell membrane and improve delivery of the morpholino (66-68) (Figure 2). The positive charge that accompanies vPMO assist in uptake and competes with splicing factors. *In vivo* studies with vPMO carried out by Yokota *et al.* have demonstrated greater efficacy in inducing exon 6-9 multiple exon skipping in dystrophic dogs compared to unconjugated PMO. In addition, no vPMO toxicity has been recorded upon systemic injection into mice up to 12 mg/kg (69). However, the positive charge does increase the risk of blood clot formation (70).

4.3. Peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO)

Another candidate antisense oligonucleotide that has improved delivery efficacy while also minimizing toxicity is peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO). Multiple peptide-conjugated oligonucleotide derivatives have been explored in recent studies, including arginine-rich peptide B-peptide, Pip-5c, Pip-6 and Pip6a (71-74). In particular, the newest modification, Pip-6a demonstrates improved stability and cardiac muscle penetration (71). Recent studies involving administration of PPMO into the *mdx* mouse model of DMD have shown promising results characterized by restored dystrophin at low doses, increased uptake and prolonged functionality (65,74-77). Specifically, an intramuscular injection of 2 µg of PPMO resulted in 85% dystrophin-positive fiber expression compared to only 14% observed in PMO treatment (65). Similarly, > 95% of exon-skipped RNA transcript was observed after IV injection of 20 mg/kg PPMO. Additionally, functional improvements were observed in various skeletal muscles after administration of PPMO (75). The improved effectiveness of PPMO compared to PMO is attributable to its active uptake process involving caveolae-mediated endocytosis (71). While access to the target cell has improved over the years, challenges

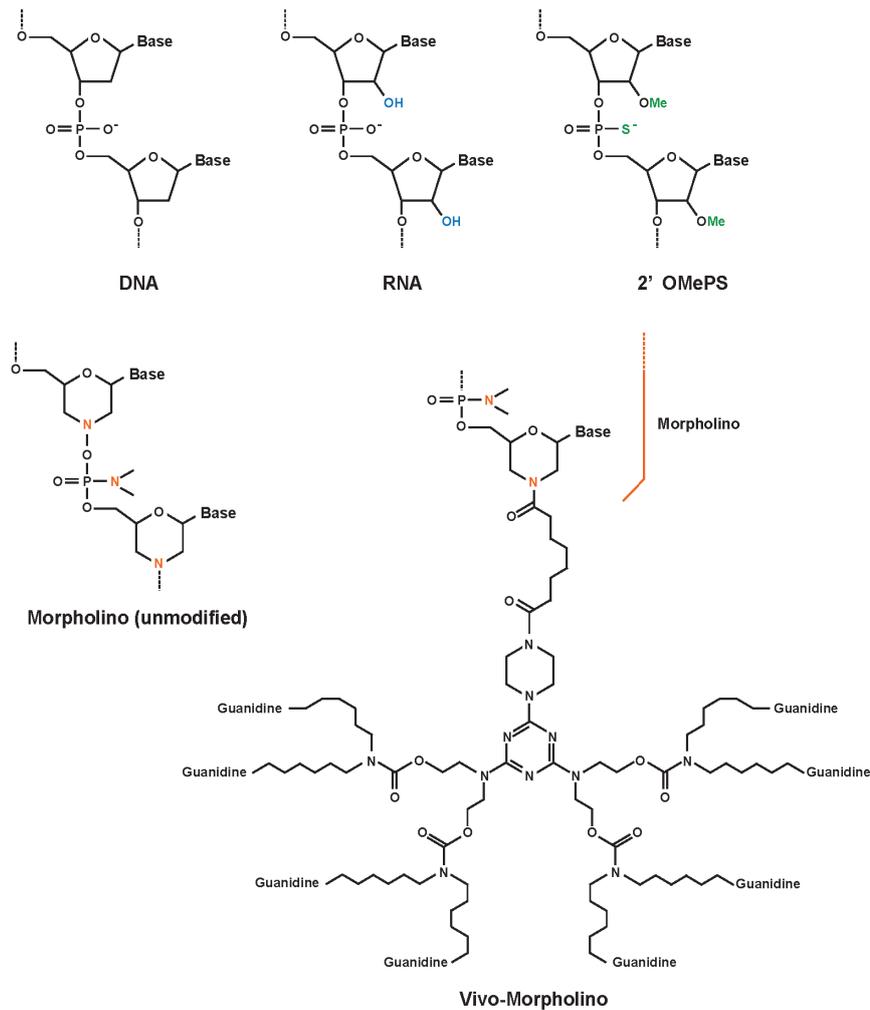


Figure 2. Molecular structure of various antisense oligonucleotide chemistries derived over the years. New generations of antisense chemistries such as 2'-O-methylated antisense oligonucleotides (2'OMePS) and octa-guanidine dendrimer conjugated PMOs (vPMO) are artificially modified to improve delivery into target cells.

remain in promoting the internal distribution of PMO to the nucleus for it to be active (65). Most of the PPMO are nonetheless distributed in the cytoplasm away from its site of action. As well, the toxicity of PPMO remains a challenge. PPMO however, benefits from its low therapeutic dose and sustained effects on the target cell. An ideal oligonucleotide therapy will be one that demonstrates long-term effects, sufficient efficacy at a low dose, and low toxicity.

5. AO-based therapy targeting *PITX1*

Since the aberrant expression of *DUX4*, a transcription factor, can lead to pathogenic deregulation of multiple genes in muscle, targeting of a downstream gene regulated by *DUX4* has also been explored recently. *PITX1*, a homeobox transcription factor, is a direct transcriptional target of *DUX4* (24). *PITX1* has previously been illustrated to be elevated in muscle fibers of FSHD patients and is understood to be

involved in the myopathy characterized in FSHD. Studies have found that the *PITX1* gene is 10-20 times up-regulated in the muscle fibers of FSHD patients. The role of *PITX1* in myopathy was shown *in vivo* via a tet-repressible muscle-specific *PITX1* transgenic mouse model (78,79). The *PITX1* transgenic mouse model with overexpression of *PITX1* in skeletal muscles demonstrates a similar disease phenotype to the muscular dystrophy seen in FSHD patients. Specifically, mice with over-expressed *PITX1* display reduced muscle fiber size and muscle strength. Hence, up-regulation of *PITX1* via *DUX4* overexpression contributes to the atrophy and wasting of skeletal muscles in FSHD patients. All in all, the downstream molecular changes due to ectopic *DUX4* expression are cytotoxic. Padley *et al.* have also illustrated the feasibility of suppressing *PITX1* using morpholinos *in vivo* (78). The study involves administration of 10 mg/kg of vPMOs into a tet-repressible muscle-specific *PITX1* overexpressing transgenic mouse model for 6

weeks. The vPMOs is used to inhibit the translation of *PITX1* by targeting the 25 base sequence at the translation start site of the *PITX1* mRNA transcript (78). Immunohistochemistry results illustrated 70% reduction in *PITX1* expression in triceps and 60% expression reduction in quadriceps. Muscle pathology results also illustrated a reduction in *PITX1* positive nuclei in muscle fibers as evidenced by 44% reduction in the number of angular shaped atrophic myofibers seen. Antisense targeting of the *PITX1* gene involved in myopathy is, therefore, an efficient therapeutic strategy for FSHD.

6. FSHD animal model

Despite advances in the design of oligonucleotide chemistry to promote increased uptake and efficacy, we still lack an adequate FSHD animal model to evaluate functional benefit and toxicity of antisense therapy. Namely, *DUX4* transgenic mouse model has not been able to capture the full disease phenotype of FSHD. For instance, the D4Z4-2.5 mice have normal histology of the limb, grip strength and creatine kinase (12,17). The challenge in generating a proper animal model for FSHD stems from the fact that D4Z4 macrosatellite encoding *DUX4* is unique to primates (80). Hence, introducing *DUX4* expression into natural laboratory models will be challenging. Currently, the best available system for *in vivo* study is the AAV-model developed by Wallace *et al.*, which demonstrates myopathy consistent with FSHD. The model is established by using adeno-associated viral vectors to deliver *DUX4* into mouse muscle fibers (34). Successful establishment of an FSHD animal model based on *DUX4* expression will assist in the understanding of the pathogenesis of disease and development of therapeutic approaches for FSHD.

7. Conclusion

Over the last two decades, progress has been made in our understanding of FSHD pathogenesis. As a gain-of-function disease characterized by the aberrant expression of *DUX4*, a knock-down approach involving antisense oligonucleotide has been explored. In particular, AOs have been especially useful by selectively inhibiting translation of target mRNA. Application of antisense oligonucleotide in the treatment of neuromuscular disorder has progressed in recent years, and its potential benefit has been observed from *in vitro* studies demonstrating successful suppression of *DUX4* expression. Additionally, promising benefits have been observed in the treatment of transgenic mouse model expressing *PITX1* with AOs. With the advancement of modified oligonucleotides providing enhanced delivery and increased efficacy, the movement towards gene therapy seems plausible.

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References

- Mul K, Lassche S, Voermans NC, Padberg GW, Horlings CG, van Engelen BG. What's in a name? The clinical features of facioscapulohumeral muscular dystrophy. *Pract Neurol*. 2016; 16:201-207.
- Deenen JC, Arnts H, van der Maarel SM, Padberg GW, Verschuuren JJ, Bakker E, Weinreich SS, Verbeek AL, van Engelen BG. Population-based incidence and prevalence of facioscapulohumeral dystrophy. *Neurology*. 2014; 83:1056-1059.
- Mostacciuolo ML, Pastorello E, Vazza G, Miorin M, Angelini C, Tomelleri G, Galluzzi G, Trevisan CP. Facioscapulohumeral muscular dystrophy: Epidemiological and molecular study in a north-east Italian population sample. *Clin Genet*. 2009; 75:550-555.
- Gabriels J, Beckers MC, Ding H, De Vriese A, Plaisance S, van der Maarel SM, Padberg GW, Frants RR, Hewitt JE, Collen D, Belayew A. Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element. *Gene*. 1999; 236:25-32.
- Vanderplanck C, Ansseau E, Charron S, Stricwant N, Tassin A, Laoudj-Chenivresse D, Wilton SD, Coppee F, Belayew A. The FSHD atrophic myotube phenotype is caused by *DUX4* expression. *PLoS One*. 2011; 6:e26820.
- Wang LH, Tawil R. Facioscapulohumeral Dystrophy. *Curr Neurol Neurosci Rep*. 2016; 16:66.
- Statland JM, Tawil R. Risk of functional impairment in Facioscapulohumeral muscular dystrophy. *Muscle Nerve*. 2014; 49:520-527.
- Kan HE, Janssen BH, Voet NBM. Facioscapulohumeral Dystrophy. In: *Neuromuscular Imaging* (Mike P, Wattjes DF, eds.). Springer, New York, USA, 2013; pp. 295-304.
- Gabellini D, D'Antona G, Moggio M, Prella A, Zecca C, Adami R, Angeletti B, Ciscato P, Pellegrino MA, Bottinelli R, Green MR, Tupler R. Facioscapulohumeral muscular dystrophy in mice overexpressing *FRG1*. *Nature*. 2006; 439:973-977.
- Tawil R, van der Maarel SM, Tapscott SJ. Facioscapulohumeral dystrophy: The path to consensus on pathophysiology. *Skelet Muscle*. 2014; 4:12.
- van der Maarel SM, Frants RR. The D4Z4 repeat-

- mediated pathogenesis of facioscapulohumeral muscular dystrophy. *Am J Hum Genet.* 2005; 76:375-386.
12. Krom YD, Thijssen PE, Young JM, *et al.* Intrinsic epigenetic regulation of the D4Z4 macrosatellite repeat in a transgenic mouse model for FSHD. *PLoS genet.* 2013; 9:e1003415.
 13. Young JM, Whiddon JL, Yao Z, Kasinathan B, Snider L, Geng LN, Balog J, Tawil R, van der Maarel SM, Tapscott SJ. *DUX4* binding to retroelements creates promoters that are active in FSHD muscle and testis. *PLoS genet.* 2013; 9:e1003947.
 14. Snider L, Geng LN, Lemmers RJ, Kyba M, Ware CB, Nelson AM, Tawil R, Filippova GN, van der Maarel SM, Tapscott SJ, Miller DG. Facioscapulohumeral dystrophy: incomplete suppression of a retrotransposed gene. *PLoS genet.* 2010; 6:e1001181.
 15. Geng LN, Yao Z, Snider L, Fong AP, Cech JN, Young JM, van der Maarel SM, Ruzzo WL, Gentleman RC, Tawil R, Tapscott SJ. *DUX4* activates germline genes, retroelements, and immune mediators: Implications for facioscapulohumeral dystrophy. *Dev Cell.* 2012; 22:38-51.
 16. Bosnakovski D, Lamb S, Simsek T, Xu Z, Belayew A, Perlingeiro R, Kyba M. *DUX4c*, an FSHD candidate gene, interferes with myogenic regulators and abolishes myoblast differentiation. *Exp Neurol.* 2008; 214:87-96.
 17. Bosnakovski D, Xu Z, Gang EJ, Galindo CL, Liu M, Simsek T, Garner HR, Agha-Mohammadi S, Tassin A, Coppee F, Belayew A, Perlingeiro RR, Kyba M. An isogenetic myoblast expression screen identifies *DUX4*-mediated FSHD-associated molecular pathologies. *The EMBO J.* 2008; 27:2766-2779.
 18. Kowaljow V, Marcowycz A, Anseau E, *et al.* The *DUX4* gene at the FSHD1A locus encodes a pro-apoptotic protein. *Neuromuscul Disord.* 2007; 17:611-623.
 19. Snider L, Asawaicharn A, Tyler AE, Geng LN, Petek LM, Maves L, Miller DG, Lemmers RJ, Winokur ST, Tawil R, van der Maarel SM, Filippova GN, Tapscott SJ. RNA transcripts, miRNA-sized fragments and proteins produced from D4Z4 units: New candidates for the pathophysiology of facioscapulohumeral dystrophy. *Hum Mol Genet.* 2009; 18:2414-2430.
 20. Yasuda T, Tsuzuki S, Kawazu M, *et al.* Recurrent *DUX4* fusions in B cell acute lymphoblastic leukemia of adolescents and young adults. *Nature genet.* 2016; 48:569-574.
 21. Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, Gruter AM, Hofker MH, Moerer P, Williamson R. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. *Nature genet.* 1992; 2:26-30.
 22. Upadhyaya M, Maynard J, Rogers MT, Lunt PW, Jardine P, Ravine D, Harper PS. Improved molecular diagnosis of facioscapulohumeral muscular dystrophy (FSHD): Validation of the differential double digestion for FSHD. *J Med Genet.* 1997; 34:476-479.
 23. Lemmers RJ, van der Vliet PJ, Klooster R, *et al.* A unifying genetic model for facioscapulohumeral muscular dystrophy. *Science.* 2010; 329:1650-1653.
 24. Dixit M, Anseau E, Tassin A, *et al.* *DUX4*, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of *PITX1*. *Proc Natl Acad Sci U S A.* 2007; 104:18157-18162.
 25. Lemmers RJ, Wohlgemuth M, van der Gaag KJ, van der Vliet PJ, van Teijlingen CM, de Knijff P, Padberg GW, Frants RR, van der Maarel SM. Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy. *Am J Hum Genet.* 2007; 81:884-894.
 26. Pandey SN, Khawaja H, Chen YW. Culture conditions affect expression of *DUX4* in FSHD Myoblasts. *Molecules.* 2015; 20:8304-8315.
 27. Lemmers RJ, Tawil R, Petek LM, *et al.* Digenic inheritance of an SMCHD1 mutation and an FSHD-permissive *D4Z4* allele causes facioscapulohumeral muscular dystrophy type 2. *Nature genet.* 2012; 44:1370-1374.
 28. Attarian S, Salort-Campana E, Nguyen K, Behin A, Andoni Urtizbera J. Recommendations for the management of facioscapulohumeral muscular dystrophy in 2011. *Rev Neurol (Paris).* 2012; 168:910-918.
 29. Tawil R, Kissel JT, Heatwole C, Pandya S, Gronseth G, Benatar M, Guideline Development D, Implementation Subcommittee of the American Academy of N, Practice Issues Review Panel of the American Association of N, Electrodiagnostic M. Evidence-based guideline summary: Evaluation, diagnosis, and management of facioscapulohumeral muscular dystrophy: Report of the Guideline Development, Dissemination, and Implementation Subcommittee of the American Academy of Neurology and the Practice Issues Review Panel of the American Association of Neuromuscular & Electrodiagnostic Medicine. *Neurology.* 2015; 85:357-364.
 30. Bosnakovski D, Choi SH, Strasser JM, Toso EA, Walters MA, Kyba M. High-throughput screening identifies inhibitors of *DUX4*-induced myoblast toxicity. *Skelet Muscle.* 2014; 4:4.
 31. Rival-Gervier S, Lo MY, Khattak S, Pasceri P, Lorincz MC, Ellis J. Kinetics and epigenetics of retroviral silencing in mouse embryonic stem cells defined by deletion of the D4Z4 element. *Mol Ther.* 2013; 21:1536-1550.
 32. Wallace LM, Liu J, Domire JS, Garwick-Coppens SE, Guckes SM, Mendell JR, Flanigan KM, Harper SQ. RNA interference inhibits *DUX4*-induced muscle toxicity *in vivo*: implications for a targeted FSHD therapy. *Mol Ther.* 2012; 20:1417-1423.
 33. Bortolanza S, Nonis A, Sanvito F, Maciotta S, Sitia G, Wei J, Torrente Y, Di Serio C, Chamberlain JR, Gabellini D. AAV6-mediated systemic shRNA delivery reverses disease in a mouse model of facioscapulohumeral muscular dystrophy. *Mol Ther.* 2011; 19:2055-2064.
 34. Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA interference improves myopathic phenotypes in mice over-expressing FSHD region gene 1 (*FRG1*). *Mol Ther.* 2011; 19:2048-2054.
 35. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. *Nature.* 2004; 431:343-349.
 36. McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I, Gilmore BL, Burstein H, Peluso RW, Polisky B, Carter BJ, Davidson BL. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: Implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A.* 2008; 105:5868-5873.
 37. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature.* 2006; 441:537-541.
 38. Touznic A, Lee JJ, Yokota T. New developments in

- exon skipping and splice modulation therapies for neuromuscular diseases. *Expert Opin Biol Ther.* 2014; 14:809-819.
39. Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A.* 1978; 75:285-288.
 40. Taniguchi-Ikeda M, Kobayashi K, Kanagawa M, Yu CC, Mori K, Oda T, Kuga A, Kurahashi H, Akman HO, DiMauro S, Kaji R, Yokota T, Takeda S, Toda T. Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy. *Nature.* 2011; 478:127-131.
 41. Wirth B, Barkats M, Martinat C, Sendtner M, Gillingwater TH. Moving towards treatments for spinal muscular atrophy: hopes and limits. *Expert Opin Emerg Drugs.* 2015; 20:353-356.
 42. Kaczmarek A, Schneider S, Wirth B, Riessland M. Investigational therapies for the treatment of spinal muscular atrophy. *Expert Opin Investig Drugs.* 2015; 24:867-881.
 43. Mitropant C, Porensky P, Zhou H, Price L, Muntoni F, Fletcher S, Wilton SD, Burghes AH. Improved antisense oligonucleotide design to suppress aberrant SMN2 gene transcript processing: towards a treatment for spinal muscular atrophy. *PLoS One.* 2013; 8:e62114.
 44. Zhou H, Janghra N, Mitropant C, Dickinson RL, Anthony K, Price L, Eperon IC, Wilton SD, Morgan J, Muntoni F. A novel morpholino oligomer targeting ISS-N1 improves rescue of severe spinal muscular atrophy transgenic mice. *Hum Gene Ther.* 2013; 24:331-342.
 45. Rigo F, Hua Y, Krainer AR, Bennett CF. Antisense-based therapy for the treatment of spinal muscular atrophy. *J Cell Biol.* 2012; 199:21-25.
 46. Hua Y, Sahashi K, Rigo F, Hung G, Horev G, Bennett CF, Krainer AR. Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature.* 2011; 478:123-126.
 47. Heald AE, Iversen PL, Saoud JB, Sazani P, Charleston JS, Axtelle T, Wong M, Smith WB, Vutikullird A, Kaye E. Safety and pharmacokinetic profiles of phosphorodiamidate morpholino oligomers with activity against ebola virus and marburg virus: results of two single-ascending-dose studies. *Antimicrob Agents Chemother.* 2014; 58:6639-6647.
 48. Swenson DL, Warfield KL, Warren TK, Lovejoy C, Hassinger JN, Ruthel G, Blouch RE, Moulton HM, Weller DD, Iversen PL, Bavari S. Chemical modifications of antisense morpholino oligomers enhance their efficacy against Ebola virus infection. *Antimicrob Agents Chemother.* 2009; 53:2089-2099.
 49. Ma W, Lin Y, Xuan W, Iversen PL, Smith LJ, Benchimol S. Inhibition of p53 expression by peptide-conjugated phosphorodiamidate morpholino oligomers sensitizes human cancer cells to chemotherapeutic drugs. *Oncogene.* 2012; 31:1024-1033.
 50. Buller HR, Gailani D, Weitz JI. Factor XI antisense oligonucleotide for venous thrombosis. *N Engl J Med.* 2015; 372:1672.
 51. Bao B, Yokota T. Potential of antisense therapy for facioscapulohumeral muscular dystrophy. *Expert Opin Orphan Drugs.* 2015; 3:1365-1374.
 52. Anseau E, Domire JS, Wallace LM, Eidahl JO, Guckes SM, Giesige CR, Pyne NK, Belayew A, Harper SQ. Aberrant splicing in transgenes containing introns, exons, and V5 epitopes: lessons from developing an FSHD mouse model expressing a D4Z4 repeat with flanking genomic sequences. *PLoS One.* 2015; 10:e0118813.
 53. Hoffman E. A rebirth for drisapersen in Duchenne muscular dystrophy? *Lancet Neurol.* 2014; 13:963-965.
 54. Lu QL, Cirak S, Partridge T. What can we learn from clinical trials of exon skipping for DMD? *Mol Ther Nucleic Acids.* 2014; 3:e152.
 55. Taylor MF, Paulauskis JD, Weller DD, Kobzik L. *In vitro* efficacy of morpholino-modified antisense oligomers directed against tumor necrosis factor-alpha mRNA. *J Biol Chem.* 1996; 271:17445-17452.
 56. Summerton J, Stein D, Huang SB, Matthews P, Weller D, Partridge M. Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. *Antisense Nucleic Acid Drug Dev.* 1997; 7:63-70.
 57. Summerton J, Weller D. Morpholino antisense oligomers: Design, preparation, and properties. *Antisense Nucleic Acid Drug Dev.* 1997; 7:187-195.
 58. Hudziak RM, Barofsky E, Barofsky DF, Weller DL, Huang SB, Weller DD. Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. *Antisense Nucleic Acid Drug Dev.* 1996; 6:267-272.
 59. Eisen JS, Smith JC. Controlling morpholino experiments: don't stop making antisense. *Development.* 2008; 135:1735-1743.
 60. Yokota T, Duddy W, Partridge T. Optimizing exon skipping therapies for DMD. *Acta Myol.* 2007; 26:179-184.
 61. Vitiello L, Bassi N, Campagnolo P, Zaccariotto E, Occhi G, Malerba A, Pigozzo S, Reggiani C, Ausoni S, Zaglia T, Gamba P, Baroni MD, Ditadi AP. *In vivo* delivery of naked antisense oligos in aged mdx mice: Analysis of dystrophin restoration in skeletal and cardiac muscle. *Neuromuscul Disord.* 2008; 18:597-605.
 62. Yokota T, Lu QL, Partridge T, Kobayashi M, Nakamura A, Takeda S, Hoffman E. Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol.* 2009; 65:667-676.
 63. Malerba A, Thorogood FC, Dickson G, Graham IR. Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice. *Hum Gene Ther.* 2009; 20:955-965.
 64. Aoki Y, Nagata T, Yokota T, Nakamura A, Wood MJ, Partridge T, Takeda S. Highly efficient *in vivo* delivery of PMO into regenerating myotubes and rescue in laminin-alpha2 chain-null congenital muscular dystrophy mice. *Hum Mol Genet.* 2013; 22:4914-4928.
 65. Moulton HM, Moulton JD. Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy. *Biochim Biophys Acta.* 2010; 1798:2296-2303.
 66. Hoffman EP, Bronson A, Levin AA, Takeda S, Yokota T, Baudy AR, Connor EM. Restoring dystrophin expression in duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through. *Am J Pathol.* 2011; 179:12-22.
 67. Reissner KJ, Sartor GC, Vazey EM, Dunn TE, Aston-Jones G, Kalivas PW. Use of vivo-morpholinos for control of protein expression in the adult rat brain. *J Neurosci Methods.* 2012; 203:354-360.
 68. Yokota T, Nakamura A, Nagata T, Saito T, Kobayashi M, Aoki Y, Echigoya Y, Partridge T, Hoffman EP, Takeda S. Extensive and prolonged restoration of dystrophin expression with vivo-morpholino-mediated multiple exon

- skipping in dystrophic dogs. *Nucleic Acid Ther.* 2012; 22:306-315.
69. Wu B, Li Y, Morcos PA, Doran TJ, Lu P, Lu QL. Octa-guanidine morpholino restores dystrophin expression in cardiac and skeletal muscles and ameliorates pathology in dystrophic mdx mice. *Mol Ther.* 2009; 17:864-871.
70. Ferguson DP, Dangott LJ, Lightfoot JT. Lessons learned from vivo-morpholinos: How to avoid vivo-morpholino toxicity. *Biotechniques.* 2014; 56:251-256.
71. Lehto T, Castillo Alvarez A, Gauck S, Gait MJ, Coursindel T, Wood MJ, Lebleu B, Boisguerin P. Cellular trafficking determines the exon skipping activity of Pip6a-PMO in mdx skeletal and cardiac muscle cells. *Nucleic Acids Res.* 2014; 42:3207-3217.
72. Betts C, Saleh AF, Arzumanov AA, Hammond SM, Godfrey C, Coursindel T, Gait MJ, Wood MJ. Pip6-PMO, A new generation of peptide-oligonucleotide conjugates with improved cardiac exon skipping activity for DMD treatment. *Mol Ther Nucleic Acids.* 2012; 1:e38.
73. Yin H, Saleh AF, Betts C, Camelliti P, Seow Y, Ashraf S, Arzumanov A, Hammond S, Merritt T, Gait MJ, Wood MJ. Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice. *Mol Ther.* 2011; 19:1295-1303.
74. Yin H, Moulton HM, Seow Y, Boyd C, Boutilier J, Iverson P, Wood MJ. Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. *Hum Mol Genet.* 2008; 17:3909-3918.
75. Moulton HM, Wu B, Jearawiriyapaisarn N, Sazani P, Lu QL, Kole R. Peptide-morpholino conjugate: a promising therapeutic for Duchenne muscular dystrophy. *Ann N Y Acad Sci.* 2009; 1175:55-60.
76. Goyenvalle A, Babbs A, Powell D, Kole R, Fletcher S, Wilton SD, Davies KE. Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. *Mol Ther.* 2010; 18:198-205.
77. Crisp A, Yin H, Goyenvalle A, Betts C, Moulton HM, Seow Y, Babbs A, Merritt T, Saleh AF, Gait MJ, Stuckey DJ, Clarke K, Davies KE, Wood MJ. Diaphragm rescue alone prevents heart dysfunction in dystrophic mice. *Hum Mol Genet.* 2011; 20:413-421.
78. Pandey SN, Lee YC, Yokota T, Chen YW. Morpholino treatment improves muscle function and pathology of *Pitx1* transgenic mice. *Mol Ther.* 2014; 22:390-396.
79. Pandey SN, Cabotage J, Shi R, Dixit M, Sutherland M, Liu J, Muger S, Harper SQ, Nagaraju K, Chen YW. Conditional over-expression of *PITX1* causes skeletal muscle dystrophy in mice. *Biol Open.* 2012; 1:629-639.
80. Leidenroth A, Hewitt JE. A family history of DUX4: phylogenetic analysis of DUXA, B, C and Duxbl reveals the ancestral *DUX* gene. *BMC Evol Biol.* 2010; 10:364-2148-2110-2364.

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Endocarditis in left ventricular assist device

Braghadheeswar Thyagarajan^{1,*}, Monisha Priyadarshini Kumar², Rutuja R Sikachi³, Abhinav Agrawal¹

¹ Department of Medicine, Monmouth Medical Center, Long Branch, New Jersey, USA;

² Madras Medical College, Chennai, India;

³ Department of Anesthesiology, Lilavati Hospital and Research Center, Mumbai, India.

Summary

Heart failure is one of the leading causes of death in developed nations. End stage heart failure often requires cardiac transplantation for survival. The left ventricular assist device (LVAD) has been one of the biggest evolvments in heart failure management often serving as bridge to transplant or destination therapy in advanced heart failure. Like any other medical device, LVAD is associated with complications with infections being reported in many patients. Endocarditis developing secondary to the placement of LVAD is not a frequent, serious and difficult to treat condition with high morbidity and mortality. Currently, there are few retrospective studies and case reports reporting the same. In our review, we found the most common cause of endocarditis in LVAD was due to bacteria. Both bacterial and fungal endocarditis were associated with high morbidity and mortality. In this review we will be discussing the risk factors, organisms involved, diagnostic tests, management strategies, complications, and outcomes in patients who developed endocarditis secondary to LVAD placement.

Keywords: Endocarditis, left ventricular assist device (LVAD)

1. Introduction

Heart failure is one of the leading causes of death in developed nations (1). As per Center for Disease Control, in 2013 around 5.1 million people were reportedly diagnosed with heart failure in the United States. Management of heart failure costs approximately 32 billion dollars each year. In 2009, 1 in 9 deaths were reported with congestive heart failure as the underlying cause of death. Cardiac transplantation is a widely known management for end-stage heart failure patients. But the patients who demand a transplant exceeds the donor pool and thus the time spent on the waiting list is too long. The introduction of a left ventricular assist device (LVAD) made a drastic evolvment in management of heart failure. It can be used as a

bridging therapy while waiting for the recovery of the donor (2) and also can be used as destination therapy (3). Thus, it serves as an excellent solution to overcome the constraints of a limited donor pool and improves the overall survival of the patient. LVAD is reported to influence and improve myocardial contractility (4). It also reduces the ongoing hypertrophy and fibrosis, thus resulting in the reversal of remodeling (5).

As any other device-oriented medical therapy, LVAD has its own limitations and complications, with infections being reported in 60% of the patients (6). Patients who develop endocarditis secondary to LVAD placement have a very high mortality rate (7). Early diagnosis and management will help in reducing this mortality. The primary objective of this review is to outline and discuss the different types of endocarditis associated with LVAD, risk factors, diagnostic methods, management, complications, and outcomes.

2. Methodology

A systematic review of the MEDLINE database was conducted using the PubMed search engine. We included all articles published between January 1, 1990, and May

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*Address correspondence to:

Dr. Braghadheeswar Thyagarajan, Department of Medicine, Monmouth Medical Center, 300 Second Avenue, Long Branch, New Jersey 07740, USA.

E-mail: bragmd@gmail.com

1, 2016. In PubMed, the combination of medical text terms used included "endocarditis", "left ventricular assist device" and "LVAD". We included all prospective or retrospective studies, review articles, case series and case reports. We found in our search that there was a total of 9 studies reporting endocarditis in patients with LVAD. 3 of the studies were retrospective reviews and 6 of them were case reports. We also searched the reference lists of the manuscripts by this strategy and selected those found to be relevant. All pertinent reports and reference lists were searched to identify any additional studies that could be included. All data were accessed between February and May 2016.

3. Left ventricular assist device

The approach towards end stage heart failure has been revolutionized with the introduction of LVAD therapy, which acts as a mechanical pump to improve the patient's circulatory status. The Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure trial (REMATCH), randomized 129 end-stage heart failure patient's ineligible for cardiac transplant to medical therapy versus pulsatile LVAD therapy and demonstrated a significant improvement in one-year survival of patients with LVAD (53% in LVAD arm versus 25% in medical therapy arm) with a hazard ratio for LVAD being 0.5 compared to medical treatment. It was thus concluded that LVAD can serve as a long-term treatment therapy in patients with end-stage heart failure and an effective bridge or alternative therapy to cardiac transplantation (8). Over time, the device has now evolved into an efficient flow pump with smaller size and lighter weight specifications in comparison with the older heavy, large and fill to empty devices (9). The two types of devices available currently are continuous flow (Heartmate II LVAD and Heartware HVAD) and pulsatile flow (Heartmate XVE) LVAD. Continuous flow LVAD weighs 390 gm while pulsatile flow weighs 1250 gm. Both devices significantly enhance the functional capacity as well as the quality of life. Commonly, the pumps are implanted through a median sternotomy incision. The pump is textured to prevent thrombus formation (10). It consists of inflow duct, unidirectional valve and outflow duct. The implanted LVAD pumps draw blood from the left ventricle and delivers it to the ascending aorta. The pumps are connected to an external power source and controller which delivers electricity *via* percutaneous leads (1,11). By the year 2013, the number of mechanical circulatory support device implants in the United States was more than the number of heart transplants (12).

4. Infections in LVAD

Infection is a commonly associated complication of any implanted cardiac device. As mentioned previously,

it has been reported that 60% of patients undergoing LVAD develop some sort of infection (6). These include bloodstream infection, sepsis, and endocarditis (1). The major cause of readmission in LVAD patients is an infection (13). In the REMATCH trial, infection and device failure in ventricular assist device patients were reported to be the dominant factors contributing to the drop in 2-year survival rates from 53% to 23%. The trial also listed sepsis, pump infection and peri-operative bleeding as the main predictors of cost-effectiveness (8). The pump pockets, drive line, and cannula/intravascular pump are susceptible to biofilm formation and acts as a nidus for chronic infection and bacteremia. Surgical site infection can also occur. Less common infections include peritonitis, mediastinitis, and pseudoaneurysms. Poor prognosis is associated with bloodstream infection, which can be complicated, by cerebral emboli and multiple organ failures (1). It has also been reported that few patients who underwent cardiac transplantation followed by removal of LVAD device developed late onset driveline infection leading to complications (14).

5. Endocarditis in LVAD

Endocarditis in patients with LVAD has a 50% mortality rate (7). LVAD-associated endocarditis is defined as clinical evidence of pump and/or cannula infection along with the presence of vegetation on echocardiography or a vascular phenomenon as defined by modified Duke's criteria (15).

5.1. Risk factors

LVAD devices usually get infected during or after implantation (1). Commonly the pathogens colonize the internal surface of LVAD *via* bloodstream infiltration and the external surface *via* local infiltration (10). The colonization of organisms on the device depends on multiple factors such as turbulence of flow, the device surface and the adherent nature of the pathogen. The surface of the device is commonly a textured polyurethane membrane, which is coated with a pseudo-neointimal layer. Platelets and fibrinogen adhere here and form a fibrin matrix, which acts as a trap for other types of cells. Connective tissue cells such as myofibroblasts attach here and form a collagenous matrix. This serves as a potential site for the adherence of pathogens, thus leading to infection (16-19).

5.2. Bacterial Endocarditis

In our review, bacterial endocarditis has been reported by 2 retrospective studies and 4 case reports (Table 1). The microbiological profile of LVAD endocarditis is very diverse. The common pathogen includes *Staphylococcus*, *Pseudomonas* and *Streptococcus*

Table 1. Studies reporting Bacterial Endocarditis in patients with LVAD

Reference	Study Date	Type of study	NP ^a (n)	NPE ^b (n)	Diagnostic method	Organism	Management	Outcome
Riaz <i>et al.</i> (22)	1/2005 to 12/2011	Retrospective review	247	3	Blood cultures, TEE	<i>Pseudomonas aeruginosa</i> , MRSA, Coagulase negative Staphylococci	Removal of LVAD, antibiotics	1 patient survived and 2 patients expired
Mendes <i>et al.</i> (24)	4/2011	Case report	1	1	Blood cultures, TEE	MR Staphylococcus epidermis, linezolid-resistant Streptococcus sanguinis, <i>Pseudomonas Aeruginosa</i>	Linezolid, Vancomycin, daptomycin.	Patient expired from other complications
De Jong <i>et al.</i> (25)	5/1998	Case report	1	1	Immunoscintigraphy with Tc-99m labeled anti-NCA 95 anti granulocyte antibodies	Staphylococcus aureus	Exchange of valves in the inflow and outflow tracts, oxacillin	Patient survived
Hill <i>et al.</i> (2)	9/2014	Case report	1	1	Positive blood cultures, Ultrasonography showing small fluid collections in the driveline	<i>Pseudomonas aeruginosa</i>	Ceftazidime and oral ciprofloxacin	Patient developed intraparenchymal hemorrhage due to a mycotic aneurysm of the brain and expired eventually
Motomura <i>et al.</i> (26)	6/2011	Case report	1	1	Positive blood cultures, negative TEE, positive CT scan for SMA and hemorrhagic lesions in the brain	Coagulase-negative gram-positive cocci	Vancomycin, micafungin, piperacillin and tazobactam	Patient expired due to multiple brain lesions and cerebral edema
Lahpor <i>et al.</i> (23)	3/1993 to 12/2001	Retrospective review	38	1 (suspected)	Positive blood cultures, negative TEE	NA	Long term antibiotics, explantation of device	Patient survived, explantation of device revealed no vegetations

^aNP, Number of patients; ^bNPE, Number of patient with endocarditis.

species (20). *Staphylococcus aureus* is the most common pathogen in LVAD endocarditis, which has the propensity to adhere itself due to possession of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) (21). In a retrospective review done at the Mayo Clinic by Riaz *et al.*, which included 247 patients who underwent LVAD implantation, three patients developed endocarditis. All cases had either concurrent or prior LVAD infection apart from endocarditis. The microbiology revealed the agents to be *pseudomonas aeruginosa* in one case, methicillin-resistant *staphylococcus aureus* (MRSA) in another and coagulase negative *staphylococcus* in the third. The diagnosis was confirmed by means of positive blood cultures and positive transesophageal echocardiography (TEE). All patients underwent removal of the LVAD and were treated with a prolonged course of antibiotics. Only one patient survived (22). In another retrospective review done in the Netherlands, which included 38 patients who received LVAD between 1993 to 2001,

12 patients had complications due to infections. Endocarditis was suspected in one patient who required prolonged antibiotics. However, explantation of the device revealed no vegetation and the patient survived (23). Mendes *et al.* reported a case of a patient who had an LVAD placed for ischemic cardiomyopathy and eventually developed endocarditis. The culture revealed methicillin-resistant *staphylococcus epidermis* (MRSE) and the patient was treated with linezolid with no significant improvement. A repeated microbiological study with PCR and sequencing revealed linezolid-resistant *streptococcus sanguinis* with a 23S rRNA mutation leading to the development of cross-resistance to rRNA-targeting drug agents including linezolid made the treatment even more challenging. The patient was treated with different antibiotics and later blood cultures also revealed he developed *pseudomonas aeruginosa* bacteremia. Eventually, his blood cultures came back negative after a prolonged course of antibiotics but the patient died due to other complications (24).

Table 2. Studies reporting Fungal Endocarditis in patients with LVAD

Reference	Study Date	Type of study	NP ^a (n)	NPE ^b (n)	Diagnostic method	Organism	Management	Outcome
Nurozler <i>et al.</i> (29)	7/1991 to 12/1999	Retrospective review	165	5	1 patient had positive fungemia,	<i>Candida parapsilosis</i> , <i>Candida albicans</i> , <i>Syncephalastrum racemosum</i>	1 patient had LVAD changed and transplant, 3 patients had transplant and 1 patient had LVAD explanted	4 patient survived and 1 patient expired
Barbone <i>et al.</i> (31)	11/2004	Case report	1	1	Postmortem revealed friable material with fungal hyphae in the inflow and outflow valves, negative blood cultures	<i>Aspergillus</i> species	NA	Patient Expired in 21 days after implant of LVAD
Maly <i>et al.</i> (20)	3/2011	Case report	1	1	Explanted LVAD revealed thrombotic like obstruction of the outflow cannula, negative TEE and blood cultures	<i>Aspergillus</i> species, <i>Candida albicans</i>	LVAD was explanted due to worsening function and patient underwent urgent heart transplant	Patient survived after heart transplant

^aNP, Number of patients; ^bNPE, Number of patient with endocarditis.

De Jonge *et al.* presented a case of a patient who developed high-grade temperatures after three years of LVAD implantation with blood cultures growing staphylococcus aureus. The routine investigation did not reveal any source of infection. T99m labeled anti-NCA 95 anti-granulocyte antibodies found a suspected focus of infection at the outflow tract. The patient underwent a successful exchange of the inflow and outflow tract and experienced accelerated recovery (25). Hill *et al.* reported a patient on LVAD who initially developed an abscess in the driveline with blood cultures growing pseudomonas aeruginosa requiring prolonged antibiotic therapy. This patient eventually developed a small mycotic aneurysm in the brain which was inoperable and eventually died (2). Motomura *et al.* reported a case of superior mesenteric artery mycotic aneurysm secondary to LVAD endocarditis. The patient was a 31-year-old male who underwent LVAD placement for non-ischemic cardiomyopathy and had a previous history of intravenous drug abuse. Seven months' post implant he was admitted to the hospital for sepsis and blood cultures grew coagulase-negative gram-positive cocci. During his hospital course, he developed a superior mesenteric artery mycotic aneurysm and eventually he developed multiple hemorrhagic lesions in his brain leading to death (26).

5.3. Fungal Endocarditis

Fungal endocarditis is a rare but fatal complication of LVAD placement (27). We came across 1 retrospective study and 2 case reports discussing LVAD fungal endocarditis (Table 2). Opportunistic fungal infections commonly occur in these patients due to diverse

factors, which include poor nutritional status and reduced immunity. Long-term antibiotic use makes these patients susceptible to fungal infection flourishing (28). *Candida* is reported to be the most common fungal agent involved in LVAD endocarditis (29). 50-70% of fungal endocarditis present with a positive blood culture (30). In a retrospective review by Nurozler *et al.* involving 165 patients with LVAD, he reported that 22% of the patients developed some sort of fungal infection out of which 5 patients (3%) had fungal endocarditis. One of the five patients had a positive blood culture while the other patients had negative blood cultures. The organisms in the other four patients were identified as fungal growth during explantation of the LVAD due to persistent fever and leukocytosis. The organism's reports were *Candida parapsilosis*, *Candida albicans*, and *Syncephalastrum racemosum*. All the patients had their LVAD explanted and four of them had cardiac transplants. The microbiology of the material found in the LVAD revealed the above-mentioned organisms. 4 out of the 5 patients survived (29). Barbone *et al.* reported a patient who died on postoperative day 21 following the implant of a LVAD due to LVAD dysfunction and intractable high temperature. The patient had normal white blood cells and negative blood cultures. The patient was treated with empiric antibiotics with no response. The postmortem study revealed friable fungal (*aspergillus*) vegetation in inflow and outflow valves (31). Multiple authors recommend the use of empiric antifungal therapy in culture negative sepsis unresponsive to broad-spectrum antibiotics in patients with LVAD (31). Maly *et al.* reported a patient on LVAD who developed outflow tract obstruction secondary to fungal infection

thrombus formation. Months after the LVAD implant procedure, the patient presented with a dry cough and fatigue. He was afebrile. Lab abnormalities included hemoglobinuria and elevated inflammatory markers. Initial blood cultures were negative and TEE did not reveal any vegetation. During this readmission, a donor's heart became available and Cardiac transplantation was successfully done. The explanted LVAD revealed the fungal thrombus obstructing the outflow track with histopathology showing aspergillus. This emphasizes the fact that a normal TEE does not always rule out endocarditis (20).

6. Diagnosis

When LVAD driveline or pump pocket infection is suspected, blood cultures with gram stain should be obtained before the initiation of broad-spectrum antibiotic therapy (32). LVAD endocarditis is similar to prosthetic valve endocarditis, which can lead to a series of complications such as LVAD dysfunction, LVAD thrombosis and septic embolization (1,6). The patient can present with persistently elevated temperature, positive blood culture, skin signs of endocarditis such as Osler's nodes, Janeway lesions and mycotic emboli to systemic organs such as brain or kidneys. Certain patients also present with mild symptoms such as cachexia, low-grade temperature or anorexia (33). Also, there have been reports of asymptomatic patients who had an incidental diagnosis of LVAD endocarditis made through the histopathological study of the explanted device (1). Modified Duke criteria for diagnosis of Infective endocarditis is found to be more sensitive than Duke criteria or Von Reyn criteria (34). Implementing echocardiography to the modified Duke criteria has increased its sensitivity to 100% (35). Emphasis on signs, symptoms, and identification of causative pathogen using serological markers, additional cultures, recent molecular techniques and histological studies increased the therapeutic specificity and sensitivity of Modified Duke's criteria. Thus finding it to be more effective in diagnosing endocarditis even in patients with negative blood cultures (36). In the case of bloodstream infections, transesophageal echocardiography (TEE) is done to look for any vegetation on the LVAD surface. But TEE need not necessarily rule out the possibility of seeding at the reflective internal blood contacting metal surface of the device. TEE should be also considered in patients with negative blood cultures possibly due to recent antibiotic use (15) There have been reports of using Immunoscintigraphy with Tc-99m labeled anti-NCA 96 anti-granulocyte antibodies for the diagnosis of the infective focus (25) and also the use of ultrasonography to detect abscesses along the surfaces of the LVAD (2). Despite absent vegetation on TEE and the other tests, inability to clear bloodstream infection with appropriate antibiotics should raise concern for

LVAD endocarditis (15).

7. Management

Initial management of LVAD driveline or pump pocket infection involves the use of broad-spectrum antibiotics after blood cultures have been obtained. In addition to systemic antibiotics, driveline infection also requires surgical drainage and incision of the driveline site with driveline revision, which allows for removal of dead tissue for faster recovery. Vacuum-assisted closure devices can also be used in driveline infection (32,37). In the case of pump pocket infection, if there is fluid collection around the device, exploration of the site with surgical incision and drainage is required. Antibiotic beads can also be used in these types of infections (38). Severe cases of pump pocket infection must be aggressively managed as LVAD endocarditis. The driveline or pump pocket infection in patients with LVAD can be managed with device removal and a limited course of antibiotic therapy but it's insufficient in case of LVAD endocarditis. The endovascular surface of LVAD must be presumed seeded in cases of implant device infection complicated by endocarditis. These cases should be managed with chronic suppressive antibiotic therapy until the infected LVAD is removed and replaced with a new device or until the patient undergoes cardiac transplantation (22). Conservative management of endocarditis without lead removal is reported as an ineffective treatment approach. Failure of treatment is strongly associated with failure to remove the infected LVAD (1). Currently, there is no data regarding specific approaches in the management of LVAD endocarditis, device exchange or explantation is generally based on the patient's overall clinical status. In our review, out of the 8 patients reported with bacterial endocarditis among all the studies, all of them received a prolonged course of antibiotics, 2 patients had explantation of the device and one patient had an exchange of the inflow and outflow valves (2,22-26). Aggressive management of infection, with prompt device removal and prolonged antibiotic therapy targeting the specific organism, is crucial to prevent catastrophic events (1).

The same approach applies to fungal endocarditis as well. Early detection of non-specific signs and symptoms as well as appropriate antifungal treatment in a timely manner is highly demanded to treat this deadly complication (29). The risk of opportunistic fungal infection is extremely high in patients who are immunosuppressed and it is recommended to administer prophylactic antifungal therapy to these patients (27). All high-risk patients on LVAD should be treated with fluconazole prophylaxis. Patients diagnosed with candida endocarditis should be treated with an echinocandin (20). Prophylaxis for aspergillosis is not routinely administered. Voriconazole is the first drug choice to treat the suspected invasive aspergillosis in

these patients (31). Out of the 7 patients reported with fungal endocarditis, 6 of them had anti-fungal treatment and LVAD explantation. Heart transplantation was done in 5 of the patient due to the availability of donor's heart (20,29,31). But it's strongly emphasized that eradication of fungemia with drugs alone without LVAD removal is an impossible task (15). In summary, the effective treatment methodologies for positive outcomes in patients with LVAD endocarditis were documented to be treating a patient with systemic antibiotic suppression therapy alone, LVAD replacement, LVAD transplantation and LVAD explantation without transplantation (7). More clinical data is required for a specific treatment approach for LVAD endocarditis regarding the use of just antibiotics versus device exchange and explantation.

8. Similarities and differences in prosthetic valve endocarditis and LVAD endocarditis

In both prosthetic valve endocarditis and LVAD endocarditis, there are signs of bloodstream infection causing symptoms such as fever, cachexia, low-grade temperature or anorexia, positive blood cultures, skin signs of endocarditis such as Osler's nodes, Janeway lesions and mycotic emboli to systemic organs such as brain or kidneys. However, in prosthetic valve endocarditis, TEE has a higher sensitivity in diagnosing the condition compared to that of LVAD endocarditis. Similar to LVAD endocarditis, immunoscintigraphy with indium-111 is useful in detecting myocardial abscesses or diffuse tissue infiltrations in prosthetic valve endocarditis (39). Treatment of both prosthetic valve endocarditis and LVAD endocarditis requires the use of a prolonged course of antibiotics. The primary difference in treatment of the two endocarditis situations is that in LVAD endocarditis, explantation of the device is always indicated along with antibiotic treatment. However, in prosthetic valve endocarditis, surgical intervention is required only if it meets one of the following criteria which includes large vegetation (> 10 mm), mobile vegetation, thromboembolic events with the presence of vegetation, persistent sepsis despite 48 hours of antibiotic treatment, congestion not relieved with medical treatment, and acute renal failure (40). Another important difference is the need for prophylaxis. Currently, there is no literature indicating the need for prophylaxis antibiotics in patients with LVAD to prevent endocarditis for procedures, however, antibiotic prophylaxis has been indicated for patients with a prosthetic valve for procedures involving the oropharynx, gastrointestinal tract, and urogenital tract (39).

9. Complications

Complications associated with the device implant include infection, bleeding, right ventricular failure, septic emboli, thromboembolism, and stroke (1). In

bacterial endocarditis, the reported complications include mycotic embolism causing intraparenchymal bleeding and systemic mycotic emboli (2,22-26). In fungal endocarditis, the reported complications include vegetation obstructing the inflow and outflow valves and also obstruction of the outflow cannula (20,29,31).

10. Outcomes

The extensive review of the literature revealed only limited results on the outcomes of LVAD endocarditis. In our review, out of the 8 patients reported with bacterial endocarditis 3 patients survived (37.5%) and 5 patients died (62.5%). Two patients (25%) were reported to have peripheral emboli from the endocarditis. Among the 7 patients reported with fungal endocarditis 5 patients survived (71.4%) and 2 patients died (28.5%) (2,22-26). There is no significant difference in survival of transplanted patients with or without perioperative infection whereas patients with LVAD endocarditis are reported to have increased risk of morbidity and mortality (41). Overall mortality from sepsis in patients with LVAD is 4%. Other causes of death in patients with a continuous-flow left ventricular assist device are hemorrhagic stroke (9%), right heart failure (5%), external power interruption (4%), bleeding (3%), respiratory failure (3%), and cardiac arrest (3%). Among patients with a pulsatile flow LVAD, the leading causes of death are hemorrhagic stroke (10%), right heart failure (8%), multisystem organ failure (7%), and ischemic stroke (5%) (11). The overall estimated survival at the end of the 1st and 2nd year in the case of continuous flow LVAD is found to be 68% and 58% respectively while with pulsatile flow LVAD is found to be 55% and 24% (11).

11. Conclusion

In conclusion, endocarditis secondary to LVAD placement is a serious and difficult to treat condition with high morbidity and mortality. Both bacterial and fungal endocarditis have been reported in patients with LVAD. A negative TEE does not always rule out endocarditis associated with LVAD and persistent bacteremia should raise suspicion of endocarditis in these patients. Complications include systemic mycotic embolization and vegetation causing obstruction of the inflow or outflow tract leading to LVAD dysfunction. Explantation of the LVAD along with prompt antibiotic or antifungal therapy is needed for the treatment of endocarditis associated with LVAD.

References

1. Gordon RJ, Quagliarello B, Lowy FD. Ventricular assist device-related infections. *Lancet Infect Dis*. 2006; 6:426-437.

2. Hill JA, Mokadam NA, Rakita RM. Intracranial mycotic aneurysm associated with left ventricular assist device infection. *Ann Thorac Surg.* 2014; 98:1088-1089.
3. Rose EA, Gelijns AC, Moskowitz AJ, *et al.* Long-term mechanical left ventricular assistance for end-stage heart failure. *N Engl J Med.* 2001; 345:1435-1443.
4. Heerdt PM, Holmes JW, Cai B, Barbone A, Madigan JD, Reiken S, Lee DL, Oz MC, Marks AR, Burkhoff D. Chronic unloading by left ventricular assist device reverses contractile dysfunction and alters gene expression in end-stage heart failure. *Circulation.* 2000; 102:2713-2719.
5. Bruckner BA, Stetson SJ, Perez-Verdia A, Youker KA, Radovancevic B, Connelly JH, Koerner MM, Entman ME, Frazier OH, Noon GP, Torre-Amione G. Regression of fibrosis and hypertrophy in failing myocardium following mechanical circulatory support. *J Heart Lung Transplant.* 2001; 20:457-464.
6. Koval CE, Rakita R; AST Infectious Diseases Community of Practice. Ventricular assist device related infections and solid organ transplantation. *Am J Transplant.* 2013; 13:348-354.
7. Oz MC, Argenziano M, Catanese KA, Gardocki MT, Goldstein DJ, Ashton RC, Gelijns AC, Rose EA, Levin HR. Bridge experience with long-term implantable left ventricular assist devices. Are they an alternative to transplantation? *Circulation.* 1997; 95:1844-1852.
8. Rose EA, Moskowitz AJ, Packer M, *et al.* The REMATCH trial: Rationale, design, and end points. Randomized evaluation of mechanical assistance for the treatment of congestive heart failure. *Ann Thorac Surg.* 1999; 67:723-730.
9. Nowotny BH, Boner DH, Maltais S. Ventricular assist device implantation: Perioperative nursing considerations. *AORN J.* 2016; 103:388-403; quiz 404-406.
10. Fischer SA, Trenholme GM, Costanzo MR, Piccione W. Infectious complications in left ventricular assist device recipients. *Clin Infect Dis.* 1997; 24:18-23.
11. Slaughter MS, Rogers JG, Milano CA, Russell SD, Conte JV, Feldman D, Sun B, Tatooles AJ, Delgado RM 3rd, Long JW, Wozniak TC, Ghumman W, Farrar DJ, Frazier OH; HeartMate II Investigators. Advanced heart failure treated with continuous-flow left ventricular assist device. *N Engl J Med.* 2009; 361:2241-2251.
12. Shah SP, Mehra MR. Durable left ventricular assist device therapy in advanced heart failure: Patient selection and clinical outcomes. *Indian Heart J.* 2016; 68(Suppl 1):S45-51.
13. DaSilva M, MacIver J, Rodger M, Jaffer M, Raju S, Billia F, Rao V. Readmissions following implantation of a continuous-flow left ventricular assist device. *J Card Surg.* 2016; 31:361-364.
14. Dulanto Chang A, Narsana N, Ruiz ME. Late sequelae of left ventricular assist device infection presenting after heart transplant. *Transpl Infect Dis.* 2016; 18:453-456.
15. Nienaber JJ, Kusne S, Riaz T, Walker RC, Baddour LM, Wright AJ, Park SJ, Vikram HR, Keating MR, Arabia FA, Lahr BD, Sohail MR; Mayo Cardiovascular Infections Study Group. Clinical manifestations and management of left ventricular assist device-associated infections. *Clinical Infect Dis.* 2013; 57:1438-1448.
16. Graham TR, Dasse K, Coumbe A, Salih V, Marrinan MT, Frazier OH, Lewis CT. Neo-intimal development on textured biomaterial surfaces during clinical use of an implantable left ventricular assist device. *Eur J Cardiothorac Surg.* 1990; 4:182-190.
17. Salih V, Graham TR, Berry CL, Coumbe A, Smith SC, Dasse K, Frazier OH. The lining of textured surfaces in implantable left ventricular assist devices. An immunocytochemical and electronmicroscopic study. *Am J Cardiovasc Pathol.* 1993; 4:317-325.
18. Scott-Burden T, Frazier OH. Cellular linings of ventricular assist devices. *Ann Thorac Surg.* 1995; 60:1561-1562.
19. Rafi S, Oz MC, Seldomridge JA, Ferris B, Asch AS, Nachman RL, Shapiro F, Rose EA, Levin HR. Characterization of hematopoietic cells arising on the textured surface of left ventricular assist devices. *Ann Thorac Surg.* 1995; 60:1627-1632.
20. Maly J, Szarszoi O, Dorazilova Z, Besik J, Pokorny M, Kotulak T, Netuka I. Case report: Atypical fungal obstruction of the left ventricular assist device outflow cannula. *J Cardiothorac Surg.* 2014; 9:40.
21. Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* 1998; 6:484-488.
22. Riaz T, Nienaber JJ, Baddour LM, Walker RC, Park SJ, Sohail MR. Cardiovascular implantable electronic device infections in left ventricular assist device recipients. *Pacing Clin Electrophysiol.* 2014; 37:225-230.
23. Lahpor JR, de Jonge N, van Swieten HA, Wesenhagen H, Klöpping C, Geertman JH, Oosterom A, Rodermans B, Kirkels JH. Left ventricular assist device as bridge to transplantation in patients with end-stage heart failure: Eight-year experience with the implantable HeartMate LVAS. *Neth Heart J.* 2002; 10:267-271.
24. Mendes RE, Deshpande LM, Kim J, Myers DS, Ross JE, Jones RN. *Streptococcus sanguinis* isolate displaying a phenotype with cross-resistance to several rRNA-targeting agents. *J Clin Microbiol.* 2013; 51:2728-2731.
25. deJonge KC, Laube HR, Dohmen PM, Ivancevic V, Konertz WF. Diagnosis and management of left ventricular assist device valve-endocarditis: LVAD valve replacement. *Ann Thorac Surg.* 2000; 70:1404-1405.
26. Motomura T, Bruckner B, Leon-Becerril J, Anaya-Ayala JE, de Rienzo-Madero B, Bismuth J, Bunge R, Irwin S, Loebe M. Superior mesenteric artery mycotic aneurysm in patients with left ventricular assist device support and intravenous drug abuse. *Artif Organs.* 2011; 35:E164-E167.
27. Goldstein DJ, el-Amir NG, Ashton RC Jr, Catanese K, Rose RA, Levin HR, Oz MC. Fungal infections in left ventricular assist device recipients. Incidence, prophylaxis, and treatment. *ASAIO J.* 1995; 41:873-875.
28. Ankersmit HJ, Tugulea S, Spanier T, Weinberg AD, Artrip JH, Burke EM, Flannery M, Mancini D, Rose EA, Edwards NM, Oz MC, Itescu S. Activation-induced T-cell death and immune dysfunction after implantation of left-ventricular assist device. *Lancet.* 1999; 354:550-555.
29. Nurozler F, Argenziano M, Oz MC, Naka Y. Fungal left ventricular assist device endocarditis. *Ann Thorac Surg.* 2001; 71:614-618.
30. Seelig MS, Speth CP, Kozinn PJ, Toni EF, Taschdjian CL. *Candida* endocarditis after cardiac surgery. Clues to earlier detection. *J Thorac Cardiovasc Surg.* 1973; 65:583-601.
31. Barbone A, Pini D, Grossi P, Bandera A, Manasse E, Citterio E, Eusebio A, Silvaggio G, Settepani F, Muncinò A, Colombo P, Casari E, Ornaghi D, Gronda E, Gallotti R. *Aspergillus* left ventricular assist device endocarditis. *Ital Heart J.* 2004; 5:876-880.
32. Maniar S, Kondareddy S, Topkara VK. Left ventricular

- assist device-related infections: Past, present and future. *Expert Rev Med Devices*. 2011; 8:627-634.
33. Sivaratnam K, Duggan JM. Left ventricular assist device infections: Three case reports and a review of the literature. *ASAIO J*. 2002; 48:2-7.
 34. Habib G, Derumeaux G, Avierinos JF, *et al*. Value and limitations of the Duke criteria for the diagnosis of infective endocarditis. *J Am Coll Cardiol*. 1999; 33:2023-2029.
 35. Naber CK, Bartel T, Eggebrecht H, Erbel R. Diagnosis of endocarditis today: Duke criteria or clinical judgment? *Herz*. 2001; 26:379-390. (in German)
 36. Prendergast BD. Diagnostic criteria and problems in infective endocarditis. *Heart*. 2004; 90:611-613.
 37. Garatti A, Giuseppe B, Russo CF, Marco O, Ettore V. Drive-line exit-site infection in a patient with axial-flow pump support: Successful management using vacuum-assisted therapy. *J Heart Lung Transplant*. 2007; 26:956-959.
 38. McKellar SH, Allred BD, Marks JD, Cowley CG, Classen DC, Gardner SC, Long JW. Treatment of infected left ventricular assist device using antibiotic-impregnated beads. *Ann Thorac Surg*. 1999; 67:554-555.
 39. Piper C, Körfer R, Horstkotte D. Prosthetic valve endocarditis. *Heart*. 2001; 85:590-593.
 40. Horstkotte D, Piper C, Wiemer M, Arendt G, Steinmetz H, Bergemann R, Schulte HD, Schultheiss HP. Emergency heart valve replacement after acute cerebral embolism during florid endocarditis. *Med Klin (Munich)*. 1998; 93:284-293. (in German)
 41. Argenziano M, Catanese KA, Moazami N, Gardocki MT, Weinberg AD, Clavenna MW, Rose EA, Scully BE, Levin HR, Oz MC. The influence of infection on survival and successful transplantation in patients with left ventricular assist devices. *J Heart Lung Transplant*. 1997; 16:822-831.
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Development of chidamide for peripheral T-cell lymphoma, the first orphan drug approved in China

Xianping Lu*, Zhiqiang Ning*, Zhibin Li, Haixiang Cao, Xinhao Wang

Chipscreen Biosciences Ltd, Shenzhen, China.

Summary

Peripheral T-cell lymphoma (PTCL) is a set of rare and highly heterogeneous group of mature T- and NK-cell neoplasms associated with poor outcomes and lack of standard and effective therapies. The total number of newly diagnosed cases of PTCL yearly in China is estimated about 50,000. Chidamide (CS055) is a novel and orally active benzamide class of histone deacetylase (HDAC) inhibitor that selectively inhibits activity of HDAC1, 2, 3 and 10, the enzymes that are involved and play an important role in tumor initiation and development in both tumor cells and their surrounding micro-environment. Functioning as a genuine epigenetic modulator, chidamide induces growth arrest and apoptosis in tumor cells and enhances cellular antitumor immunity. Based on the overall results from preclinical and phase I clinical studies, exploratory and pivotal phase II trials of chidamide for relapsed or refractory PTCL were conducted from March 2009 to May 2012, and the results led to CFDA approval of chidamide for the indication in December 2014, being the first approved orphan drug according to the research & development approach of orphan drugs in China, as well as the first orally active drug for PTCL in China and worldwide.

Keywords: Chidamide, HDAC inhibitor, epigenetic, T-cell lymphoma, orphan drug

1. Introduction

Peripheral T-cell lymphoma (PTCL) is a set of rare and heterogeneous groups of mature T- and natural killer (NK)-cell neoplasms associated with poor outcomes. The median overall survival (OS) is about 1 to 3 years for various types of PTCL (1-3). PTCL makes up 25-30% of all NHL cases in China, with an estimated 50,000 new patients diagnosed annually. Subtype distribution of PTCL is significantly different between China and North American or European countries (4). According to the WHO classification, the most common subtype of PTCL in China is extranodal NK/T-cell lymphoma, nasal type (ENKL), followed by PTCL not otherwise specified (PTCL NOS), anaplastic large-cell lymphoma (ALCL), and angioimmunoblastic

T-cell lymphoma (AITL) (4,5).

Worldwide, there is still no consensus on first-line therapy for patients with PTCL due to the rarity of the disease and the lack of randomized clinical trials. For relapsed or refractory chemotherapy sensitive patients, autologous or allogeneic stem cell transplant (SCT) following high-dose therapies is the treatment goal. To obtain this goal, clinical trials or second-line chemotherapies are suggested. The treatment options for patients who are in two or more relapses are clinical trials, best supportive care, alternative chemotherapy and palliative radiotherapy (6).

In Western countries, the development of new agents for the treatment of chemorefractory PTCL as second-line therapy has made great progress in recent years. Pralatrexate (an antifolate agent), romidepsin (a cyclic peptide HDAC inhibitor), and belinostat (a hydroxamate pan HDAC inhibitor), were approved by the US Food and Drug Administration (FDA) for patients with relapsed or refractory PTCL in September 2009, June 2011, and July 2014, respectively. The overall response rates (ORR) by independent central review for those three approved drugs were 29%, 25% and 26% for pralatrexate, romidepsin and belinostat, respectively (7-9).

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*Address correspondence to:

Drs. Xianping Lu and Zhiqiang Ning, 2-601, BIO-Incubator, Gaoxin C, 1st Ave., Shenzhen Hi-Tech Industrial Park, Nanshan District, Shenzhen, Guangdong 518057, China.
E-mail: xplu@chipscreen.com (Lu XP); zqning@chipscreen.com (Ning ZQ)

Epigenetic modifications, including DNA methylation, histone modification and nucleosome remodeling, function cooperatively to determine chromatin configuration and unique transcriptional profiles in cells. Disruption of epigenetic processes may cause altered gene function and play an essential role in malignant cellular transformation and progression (10). These findings have provided the rationale for epigenetic agents targeting DNA methyltransferases (DNMT) and histone deacetylases (HDAC) for cancer treatment (11).

Chidamide (CS055), discovered and developed by Chipscreen Biosciences, is a novel orally active HDAC inhibitor with subtype selective activity against HDAC1, 2, 3 and 10. Functioning as a genuine epigenetic modulator, chidamide induces growth arrest and apoptosis in tumor cells and enhances cellular antitumor immunity. In the current article, we present the main results from preclinical and clinical studies, with emphasis on the single agent chidamide for PTCL and its development and regulatory path as the first approved orphan drug according to the research & development approach of orphan drugs in China, as well as the first orally active drug for PTCL in China and worldwide.

2. Discovery and preclinical development

2.1. Discovery and mechanisms of action

HDACs are involved in the remodeling of chromatin and play a key role in the epigenetic regulation of gene expression. At least 18 human HDACs have been identified and are grouped into four classes, including class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 7, and 9 as IIa, and HDAC6 and 10 as IIb), and class IV (HDAC11) (12). Elevated expression or activity of HDACs is implicated in the development and progression of cancer (13). Inhibition of HDAC enzymes results in increased histone acetylation, thereby inducing an open chromatin conformation and transcription of previously dormant genes. Although the precise biological functions of individual HDACs are still largely unknown, the importance of HDAC enzymes in the malignant phenotype has been most closely associated with Class I HDACs 1-3 (12-14).

Back in 2001, when Chipscreen was set up, we initiated an exploratory program in discovery of novel HDAC inhibitors with high subtype selectivity and oral bioavailability. Based on the large members of HDAC enzyme families and variety of enzyme structures of individual subtypes, we hypothesized that the existing HDAC inhibitors at the time with different chemical structures should have had different selectivity in HDAC subtypes, and thus elicit different biological responses. We carried out chemical genomic analysis to differentiate whether these chemically divergent inhibitors were biologically different. As a result, we

found that among the HDAC inhibitors evaluated, only the benzamide class compounds, but not hydroxamic acid-based ones, exhibited induction of expression of epithelial differentiation related genes (e.g., EMP1, EPLIN), T-cell receptor (TCR) and MHC I cluster genes, and death receptor 6 (DR6)-related apoptosis genes. Preferential repression of genes related to drug resistant and protein modification/degradation pathways was also observed in benzamide class compounds (15). These findings led us to focus on the chemical scaffold of benzamide class of HDAC inhibitors, and CS055 (later named chidamide) was discovered from a variety of benzamide-prototype compounds based on computational and medicinal chemistry, and further evaluated by chemical genomic-based analysis and other molecular biological means both *in vitro* and *in vivo*. In summary, chidamide has demonstrated to selectively inhibit activity of HDAC1, 2, 3 and 10, and to perform its anti-cancer functions as a genuine epigenetic modulator by the following mechanisms: induction of growth arrest and apoptosis in blood and lymphoid-derived tumor cells, reversal of epithelial-mesenchymal transitions and drug resistance of tumor cells, and importantly, enhancement of NK-cell and antigen-specific CD8+ cytotoxic T-lymphocyte-mediated cellular antitumor immunity (15-19).

2.2. Preclinical studies

Chidamide was initially assessed in preclinical animal studies that employed a daily dose regimen. Chidamide exhibits a broad-spectrum of anti-tumor activity *in vivo*, including activities against lung, colon, breast and liver carcinoma, evaluated by using athymic nude mice subcutaneously inoculated with different human tumor cell lines (16). Using a daily dose regimen, the ED₅₀ in average for those animal models was 11.5 mg/kg.

Nonclinical pharmacokinetic studies were conducted in rodent and non-rodent animals after single and multiple oral dosing with a daily dose regimen. Plasma concentrations in animals were observed to be slightly less than dose-proportional across the species. Oral dosing was characterized by variable plasma elimination half-lives in different animal species, ranging from 21 to 38 hours, that was apparently independent of dose levels/exposure. In rat studies, chidamide was shown to mainly distribute to the gastrointestinal tract, pancreas, lungs and immune organs.

IND-enabling safety studies of chidamide were conducted in rats and dogs with repeat dosing for 28 days with a daily dose regimen. In rats, an every-three-day dosing regimen was also employed. All the studies incorporated toxicokinetic analyses. Overall target organ toxicities were similar in rats and dogs, regardless of dosing regimens employed. Typical findings included dose-dependent reductions in body weight and food consumption, hematologic

abnormalities, and changes in clinical chemistry parameters. In repeat toxicity studies, rats tolerated 10 times more dose exposure in animals with an every-three-day dosing regimen compared with those under a daily dose regimen, although significant accumulation of plasma drug substance was seen for both regimens from single-dose to the last dose administration in 28 days. Interestingly to note, there was no significant loss of efficacy in mice when chidamide was administered with regimens of either every-other-day or every-three-day dosing compared with a daily dose regimen when the total dose-exposure was similar with different dosing regimens in a total 28-day period of evaluations. Taken together, the efficacy and tolerance window was dramatically increased by interval dosing regimens, which formed a good foundation rationale for a phase I trial in humans.

3. Phase I study

A phase I clinical study was conducted in patients with advanced solid tumors or lymphomas (20). A total of 31 patients were enrolled who received oral doses of 5, 10, 17.5, 25, 32.5, or 50 mg chidamide twice weekly (BIW), or 32.5 or 50 mg chidamide three-times weekly (TIW) for four consecutive weeks, followed by a two-week drug-free holiday. A complete treatment cycle was 6 weeks. Treatment-related adverse events (AE) were mostly grade 1 (72%), with 17% grade 2 and 11% grade 3. The most common AEs were fatigue (35%, limited to grade 1), thrombocytopenia (26%), anorexia (26%), leucopenia or neutropenia (23%), reduced hemoglobin (19%), nausea (16%), and diarrhea (16%). In general, the number and severity of AEs increased with drug exposure, particularly with respect to myelosuppression and GI events. No dose-limiting toxicities (DLT) were identified in the BIW cohorts up to 50 mg. DLTs were grade 3 diarrhea and vomiting in two patients in the TIW cohort at 50 mg, respectively.

Out of the 25 patients who had measurable lesions for efficacy evaluation, there were 5 patients with partial response (PR). Four of the 5 patients (80%) with PRs were T-cell non-Hodgkin's lymphoma (T-NHL) patients assigned to the 5, 32.5, and 50 mg BIW cohorts and the 32.5 TIW cohort. The other PR patient was enrolled with adenoid cystic carcinoma of the submandibular gland and was treated in the 32.5 mg BIW cohort.

Single-dose PK studies were performed in patients who received 25, 32.5, and 50 mg chidamide, regardless of dosing schedules. Peak plasma concentrations for the majority of patients were observed within 0.5-2 h of drug administration and returned to close to baseline level within 48 h, but remained quantifiable at 72 h after a single dose. Systemic exposures (C_{max} and AUC) were generally dose dependent across the 25-50 mg dose range. The elimination half-life ($t_{1/2}$) was

similar among the different dose groups, with mean values ranging from 16.8 to 18.3 h. Preliminary multi-dose PK analysis suggested an increased systemic exposure on the TIW dosing schedule.

Inhibition of HDAC enzymes results in increased histone acetylation, which is usually considered as an important parameter for a pharmacodynamics (PD) study on HDAC inhibitors (21). PD analysis was carried out by examining histone H3 acetylation in peripheral white blood cells (WBCs) from 19 patients. In general, peak induction of H3 acetylation in WBCs was observed between 24 and 48 h after treatment, with increased acetylation persisting for up to > 72 h after a single dose of chidamide at 25, 32.5, and 50 mg.

Microarray gene expression studies were performed on peripheral WBCs from patients with T-cell lymphoma before and after administration of the first dose of chidamide. The expression of genes involved in immune cell-mediated antitumor functions was significantly up-regulated by chidamide dosing. Further laboratory studies have demonstrated that *ex vivo* treatment with nanomolar concentrations of chidamide enhances immune cell-mediated cytotoxicity by human peripheral mononuclear cells, accompanied by increased expression of proteins involved in NK-cell activities (16).

In conclusion, the phase I study showed that chidamide was generally well tolerated in patients with advanced solid tumors or lymphomas in tested regimens. Favorable PK and PD profiles were also demonstrated. Encouraging preliminary anti-tumor activity was observed, particularly from patients with T-cell lymphomas.

4. Clinical development for PTCL as an orphan drug

4.1. Exploratory phase II trial

The exploratory phase II trial was a multi-centered, open label, non-randomized study. Eligible patients with PTCL NOS subtype were assigned randomly to receive either 30 mg or 50 mg twice per week for 2 consecutive weeks in a 3-week-cycle. The total drug exposure for the two dosing cohorts in a 3-week period was 120 and 200 mg, respectively. Response assessment was performed once every 6 weeks, using the International Workshop to Standardize Response Criteria for Non-Hodgkin's Lymphoma (IWC). The primary endpoint was ORR, and secondary endpoints included duration of response (DOR) and progression free survival (PFS). Safety was evaluated once every 3 weeks, and AEs were graded by the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0 (CTCAE).

A total of 19 patients were enrolled in the exploratory phase II trial, including 9 patients in the 30 mg cohort and 10 patients in the 50 mg cohort. Baseline

Table 1. Baseline characteristics of the exploratory phase II trial

Characteristics	30 mg cohort (n = 9)	50 mg cohort (n = 10)	All (n = 19)
Gender			
Male	6 (66.7%)	8 (80.0%)	14 (73.7%)
Female	3 (33.3%)	2 (20.0%)	5 (26.3%)
Age (years)			
Median	51.0	52.0	51.0
Mean	53.2	51.9	52.5
Disease stage			
II	2 (22.2%)	1 (10.0%)	3 (15.8%)
III	3 (33.3%)	2 (20.0%)	5 (26.3%)
IV	4 (44.4%)	7 (70.0%)	11 (57.9%)
Years since first diagnosis			
Median	1.5	1.1	1.4
Range	0.2-8.1	0.2-2.5	0.2-8.1

Table 2. Adverse events in ≥ 2 patients in the exploratory phase II trial

Event	30 mg cohort (n = 9)		50 mg cohort (n = 10)		All (n = 19)	
	Total (%)	\geq Grade 3 (%)	Total (%)	\geq Grade 3 (%)	Total (%)	\geq Grade 3 (%)
Thrombocytopenia	3 (33.3)	2 (22.2)	6 (60.0)	4 (40.0)	9 (47.4)	6 (31.6)
Leucopenia	1 (11.1)	1 (11.1)	4 (40.0)	1 (10.0)	5 (26.3)	2 (10.5)
Fever	1 (11.1)	0	3 (30.0)	0	4 (21.1)	0
Fatigue	0	0	3 (30.0)	0	3 (15.8)	0
Anemia	1 (11.1)	1 (11.1)	1 (10.0)	0	2 (10.5)	1 (5.3)
Nausea	0	0	2 (20.0)	0	2 (10.5)	0

characteristics of patients are presented in Table 1, which did not show a significantly different distribution in these two cohorts.

One patient obtained complete response (CR) in the 30 mg cohort. Four patients responded to the treatment in the 50 mg cohort, including 1 patient with CR, 1 patient with complete response unconfirmed (CRu) and 2 patients with PR. The ORR was 11.1% (95% CI: 0.3%-48.2%) and 40.0% (95% CI: 12.2%-73.8%) for the 30 mg and 50 mg cohort, respectively. The DOR for the patient with CR in the 30 mg cohort was 1,091 days at the data cutoff date, and continued with the treatment after that. The median PFS for the 30 mg cohort was 84 days. As for the 50 mg cohort, the DORs for the 4 responding patients were 59, 259, 440 and 1,010 days. The patient with the longest DOR (1,010 days) continued the treatment after the data cutoff date. The median PFS for the cohort was 44 days.

Fourteen patients (73.7%) out of the 19 patients had at least one AE. The most common AEs (≥ 2 patients in the two cohorts in total), as listed in Table 2, were thrombocytopenia (9, 47.4%), leucopenia (5, 26.3%), fever (4, 21.1%), fatigue (3, 15.8%), anemia (2, 10.5%) and nausea (2, 10.5%). Most AEs were grade 1-2. Grade 3 AEs included thrombocytopenia (2 patients in each cohort), leucopenia (1 patient in each cohort), anemia (1 patient in the 30 mg cohort), and edema (1 patient in the 30 mg cohort). The only two Grade 4 AEs of thrombocytopenia were reported from the 50 mg cohort.

4.2. Pivotal phase II trial

Based on the overall results from the phase I study on patients with solid tumors/lymphomas and exploratory phase II trial in patients with PTCL NOS, together with the progress that pralatrexate was approved by FDA for relapsed or refractory PTCL under the orphan drug designation in December 2010, we initiated the process to communicate with and had positive feedback from the Center for Drug Evaluation (CDE) of the China Food and Drug Administration (CFDA) to explore the possibility to design and conduct a pivotal phase II trial for chidamide in relapsed or refractory PTCL.

The pivotal phase II trial was an open-label, single-arm, multicenter study of chidamide monotherapy. Patients with relapsed or refractory PTCL of any subtype that investigators considered as suitable were eligible for this study, but underwent a central pathology review to evaluate final eligibility. By overall evaluation of efficacy and safety profiles from the two dosing cohorts in the exploratory phase II trial, patients in the pivotal trial were administered 30 mg chidamide twice weekly without 1-week-drug-free breaks, which accounted for a total dosage of 180 mg in a 3-week period. Patients continued to receive chidamide treatment until progression of the disease, unacceptable toxicity, or patient/investigator discretion. The primary endpoint was ORR as assessed by an independent review committee. Of the 83 patients enrolled, 79 with eligible PTCL histology were for efficacy assessments.

Table 3. Baseline characteristics of the pivotal trials from the four approved drugs

Characteristics	Pralatrexate (n = 111)	Romidepsin (n = 130)	Belinostat (n = 120)	Chidamide (n = 79)
Age (years)				
Median	58.0	61	64.0	53.0
Mean	57.7	/	/	49.6
Gender				
Male	76 (68%)	88 (38%)	62 (52%)	53 (67%)
Female	35 (32%)	42 (32%)	58 (48%)	26 (33%)
Years since first diagnosis	1.3	1.3	1.0	1.1
Prior systemic therapy				
Median	3	2	2	3
Range	1~13	1~8	1~8	1~9
Subtype n (%)				
PTCL NOS	59 (54)	69 (53)	77 (64)	27 (34)
ALCL	17 (15)	22 (17)	15 (13)	17 (22)
ALK negative	11 (10)	21 (16)	13 (11)	11 (14)
ALK positive/unknown	6 (5)	1 (1%)	2 (2)	6 (8)
AITL	13 (12)	27 (21)	22 (18)	10 (13)
ENKL	2 (2)	1 (1)	2 (2)	16 (20)
Others	18 (17)	11 (8)	4 (3)	9 (11)

PTCL NOS, PTCL not otherwise specified; ALCL, anaplastic large cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; ENKL, extranodal NK/T-cell lymphoma, nasal type.

Table 4. Efficacy parameters of the pivotal trials from the four approved drugs

Parameter	Pralatrexate (n = 109)	Romidepsin (n = 130)	Belinostat (n = 120)	Chidamide (n = 79)
PR, n (%)	20 (18)	14 (11)	18 (15)	11 (14)
CR + CRu, n (%)	12 (11)	19 (15)	13 (11)	11 (14)
ORR, n (%)	32 (29)	33 (25)	31 (26)	22 (28)
DOR > 3m, n (%)	13 (12)	/	/	19 (24)
PFS (month)				
Median	3.5	4.0	1.6	2.1
Range	1 day-23.9	/	/	1 day-44.9
OS (month)				
Median	14.5	11.3	7.9	21.4
Range	1-24.1	/	/	0.3-50.1
Response rates for individual subtypes, %				
PTCL NOS	32 (19/59)	29 (20/69)	23 (18/77)	22 (6/27)
ALCL	35 (6/17)	23 (5/22)	13 (2/15)	41 (7/17)
ALK negative	NA	24 (5/21)	15 (2/13)	45 (5/11)
ALK positive/unknown	NA	0 (0/1)	0 (0/2)	33 (2/6)
AITL	8 (1/13)	30 (8/27)	45 (10/22)	50 (5/10)
ENKL	0 (0/2)	0 (0/1)	50 (1/2)	19 (3/16)
Others	33 (6/18)	0 (0/11)	0 (0/4)	11 (1/9)

PR, partial response; CR, complete response; CRu, complete response unconfirmed; DOR, duration of response; PFS, progression free survival; OS, overall survival; PTCL NOS, PTCL not otherwise specified; ALCL, anaplastic large cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; ENKL, extranodal NK/T-cell lymphoma, nasal type.

The ORR was 28% (22 of 79) including 14% (11 of 79) with CR/CRu. Median PFS and OS were 2.1 and 21.4 months, respectively. Patients with AITL tended to have a higher response rate and more durable responses to chidamide treatment. The most common AEs \geq grade 3 were thrombocytopenia (22%), leucopenia (13%), and neutropenia (11%), respectively. Results led to CFDA approval of chidamide in relapsed or refractory PTCL in December 2014. The full report of the pivotal phase II study can be found in a recent publication (22).

It would be interesting to compare the results of chidamide with the other three FDA approved PTCL drugs, all derived from their pivotal phase II trials

(7-9,22). While there were similar patient baseline characteristics from these trials in terms of age, gender, years since first diagnosis and prior systemic therapy numbers, a significant difference presented in the pathological subtypes of patients enrolled between the chidamide trial and the others (Table 3). Four major subtypes of PTCL (PTCL NOS, ALCL, AITL and ENKL) accounted for 83-97% of patients for all four trials. However, the chidamide trial enrolled a lower percentage of PTCL NOS patients, but significantly higher numbers of NKTL patients. Patients enrolled with ENKL in this study were 20% in total, whereas only 1-2% of patients with this subtype were included

in other trials, reflecting a significant difference in geographic or racial population of this PTCL subtype in China compared with the studies carried out in Western countries for romidepsin, pralatrexate or belinostat.

Although the ORRs were very similar in general among these drugs at a range of 25-29%, there were remarkable differences in response rates in some individual PTCL subtypes (Table 4). For instance, patients with chemorefractory AITL tended to have higher response rates in response to treatment with chidamide, as well as romidepsin and belinostat, the three HDAC inhibitors. In contrast, only 8% of patients (1 of 13 patients) responded to the pralatrexate treatment. Chidamide showed an apparently longer OS in relapsed or refractory PTCL including 20% of patients with ENKL known usually to have very poor prognosis. However, we should be cautious to look at this potential benefit for chidamide and make a direct comparison with other drugs, because of limited patient numbers, different geographic or racial populations, as well as different subtype distributions, all account for the efficacy profiles in these different trials.

We believe that the successful development of chidamide in PTCL in China, may have significance in the field: first, chidamide is the first HDAC inhibitor with subtype-selectivity and oral availability approved for PTCL worldwide. The results summarized in the current article not only form the foundation for providing a much needed treatment option for PTCL patients in China, but also have made a significant scientific contribution to the field. Second, subtype distribution of PTCL patients enrolled in our pivotal phase II trial, which was the largest clinical trial in a Chinese PTCL population to date, was significantly different compared with studies in Western countries, particularly in the ENKL subtype. The subtype distribution profiles in different trials not only reflect the significant differences in geographic or racial population, but also may have impacts on interpretation of overall results of efficacy from different trials for the indication. Our study on chidamide would be very important for and an essential addition to new drug clinical trials for PTCL patients worldwide. And third, PTCL patients treated with chidamide showed a potential long-term survival benefit even compared with recently approved drugs, particularly to patients of certain subtypes, such as AITL, which may be associated with unique epigenetic modulating mechanisms of chidamide (23,24).

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References

1. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. *Blood*. 1997; 89:3909-3918.
2. Armitage JO, Vose JM, Weisenburger DD. Towards understanding the peripheral T-cell lymphomas. *Ann Oncol*. 2004; 15:1447-1449.
3. Savage KJ. Peripheral T-cell lymphomas. *Blood Rev*. 2007; 21:201-216.
4. Sun J, Yang Q, Lu Z, He M, Gao L, Zhu M, Sun L, Wei L, Li M, Liu C, Zheng J, Liu W, Li G, Chen J. Distribution of lymphoid neoplasms in China: Analysis of 4,638 cases according to the World Health Organization classification. *Am J Clin Pathol*. 2012; 138:429-434.
5. Vose J, Armitage J, Weisenburger D. International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: Pathology findings and clinical outcomes. *J Clin Oncol*. 2008; 26:4124-4130.
6. National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology™: Non-Hodgkin's Lymphoma, v2.2016. http://www.nccn.org/professionals/physician_gls/pdf/nhl.pdf (accessed March 15, 2016)
7. O'Connor OA, Pro B, Pinter-Brown L, *et al*. Pralatrexate in patients with relapsed or refractory peripheral T-Cell lymphoma: Results from the pivotal PROPEL study. *J Clin Oncol*. 2011; 29:1182-1189.
8. Coiffier B, Pro B, Prince HM, *et al*. Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol*. 2012; 30:631-636.
9. O'Connor OA, Horwitz S, Masszi T, *et al*. Belinostat in patients with relapsed or refractory peripheral T-Cell lymphoma: Results of the pivotal phase II BELIEF (CLN-19) study. *J Clin Oncol*. 2015; 33:2492-2499.
10. Shen H, Laird PW. Interplay between the cancer genome and epigenome. *Cell*. 2013; 153:38-55.
11. Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: Causes and therapies. *Nat Rev Cancer*. 2001; 1:194-202.
12. deRuijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): Characterization of the classical HDAC family. *Biochem J*. 2003; 370:737-749.
13. Witt O, Deubzer HE, Milde T, Oehme I. HDAC family:

- What are the cancer relevant targets? *Cancer Lett.* 2009; 277:8-21.
14. Delcuve GP, Khan DH, Davie JR. Targeting class I histone deacetylases in cancer therapy. *Expert Opin Ther Targets.* 2013; 17:29-41.
 15. Pan DS, Yang QJ, Fu X, Shan S, Zhu JZ, Zhang K, Li ZB, Ning ZQ, Lu XP. Discovery of an orally active subtype-selective HDAC inhibitor, chidamide, as an epigenetic modulator for cancer treatment. *MedChemComm.* 2014; 5:1789-1796.
 16. Ning ZQ, Li ZB, Newman MJ, Shan S, Wang XH, Pan DS, Zhang J, Dong M, Du X, Lu XP. Chidamide (CS055/HBI-8000): A new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity. *Cancer ChemotherPharmacol.* 2012; 69:901-909.
 17. Gong K, Xie J, Yi H, Li W. CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G1 arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells. *Biochem J.* 2012; 443:735-746.
 18. Yao Y, Zhou J, Wang L, Gao X, Ning Q, Jiang M, Wang J, Wang L, Yu L. Increased PRAME-Specific CTL killing of acute myeloid leukemia cells by either a novel histone deacetylase inhibitor chidamide alone or combined treatment with decitabine. *PLoS One.* 2013; 8:e70522.
 19. Zhou Y, Pan DS, Shan S, Zhu JZ, Zhang K, Yue XP, Nie LP, Wan J, Lu XP, Zhang W, Ning ZQ. Non-toxic dose chidamide synergistically enhances platinum-induced DNA damage responses and apoptosis in Non-Small-Cell lung cancer cells. *Biomed Pharmacother.* 2014; 68:483-491.
 20. Dong M, Ning ZQ, Xing PY, Xu JL, Cao HX, Dou GF, Meng ZY, Shi YK, Lu XP, Feng FY. Phase I study of chidamide (CS055/HBI-8000), a new histone deacetylase inhibitor, in patients with advanced solid tumors and lymphomas. *Cancer Chemother Pharmacol.* 2012; 69:1413-1422.
 21. Fournel M, Bonfils C, Hou Y, *et al.* MGCD0103, a novel isotype-selective histone deacetylase inhibitor, has broad spectrum antitumor activity *in vitro* and *in vivo*. *Mol Cancer Ther.* 2008; 7:759-768.
 22. Shi Y, Dong M, Hong X, *et al.* Results from a multicenter, open-label, pivotal phase II study of chidamide in relapsed or refractory peripheral T-cell lymphoma. *Ann Oncol.* 2015; 26:1766-1771.
 23. Odejide O, Weigert O, Lane AA, *et al.* A targeted mutational landscape of angioimmunoblastic T-cell lymphoma. *Blood.* 2014; 123:1293-1296.
 24. Lu XP, Ning ZQ, Li ZB, Pan DS, Shan S, Guo X, Cao HX, Yu JD, Yang QJ. Discovery and development of HDAC subtype selective inhibitor chidamide: Potential immunomodulatory activity against cancers. In: *Successful drug discovery: Vol.II* (Fischer J, Childers W, eds.). Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2016; pp. 89-115.

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Urine-derived induced pluripotent stem cells as a modeling tool to study rare human diseases

Liang Shi^{1,2}, Yazhou Cui¹, Jing Luan¹, Xiaoyan Zhou¹, Jinxiang Han^{1,*}

¹School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China;

²Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China.

Summary

Rare diseases with a low prevalence are a key public health issue because the causes of those diseases are difficult to determine and those diseases lack a clearly established or curative treatment. Thus, investigating the molecular mechanisms that underlie the pathology of rare diseases and facilitating the development of novel therapies using disease models is crucial. Human induced pluripotent stem cells (iPSCs) are well suited to modeling rare diseases since they have the capacity for self-renewal and pluripotency. In addition, iPSC technology provides a valuable tool to generate patient-specific iPSCs. These cells can be differentiated into cell types that have been affected by a disease. These cells would circumvent ethical concerns and avoid immunological rejection, so they could be used in cell replacement therapy or regenerative medicine. To date, human iPSCs could have been generated from multiple donor sources, such as skin, adipose tissue, and peripheral blood. However, these cells are obtained *via* invasive procedures. In contrast, several groups of researchers have found that urine may be a better source for producing iPSCs from normal individuals or patients. This review discusses urinary iPSC (UiPSC) as a candidate for modeling rare diseases. Cells obtained from urine have overwhelming advantages compared to other donor sources since they are safely, affordably, and frequently obtained and they are readily obtained from patients. The use of iPSC-based models is also discussed. UiPSCs may prove to be a key means of modeling rare diseases and they may facilitate the treatment of those diseases in the future.

Keywords: iPSCs, "urine cells", rare disease models

1. Introduction

A rare disease is defined by the World Health Organization (WHO) as a disease with a prevalence of less than 0.65%-1%, but it is seriously debilitating even life-threatening (1). Because of its large population, China has the largest rare disease population in terms of prevalence. However, the causes of rare diseases have

yet to be identified and they are difficult to manage since there are currently no curative or disease-modified treatments available (2). More than 80% of intractable and rare diseases involve a genetic cause (3). Thus, disease models are indispensable tools for studying molecular mechanisms of rare diseases and developing therapeutic approaches. Such models would need to recreate the phenotypic and pathological variants of the disease *in vitro*.

Human embryonic stem cells (ESCs) can differentiate into all somatic cells and they can be grown indefinitely in culture. This is why human ESC technology has garnered worldwide attention for its therapeutic applications *in vivo* (4). However, human ESC treatment is plagued by immune rejection and ethical concerns. Prior to 2006, Japanese researchers induced four transcription factors (*OCT3/4*, *SOX2*, *c-MYC*, and *KLF4*) in murine fibroblasts with

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*Address correspondence to:

Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong 250062, China.

E-mail: jxhan9888@aliyun.com

Table 1. Human iPSCs can be generated from different donor sources

Donor sources	Cell types	Obtained process	Ref.
Fibroblasts	Facial dermal fibroblasts, periodontal ligament fibroblasts, and gingival fibroblasts	Invasive (biopsy)	(11,12)
Keratinocytes	Keratin-dense epithelial cells	Not strictly noninvasive	(13)
Melanoma cells	Skin melanocytes	Invasive (skin punch biopsies)	(14)
Adipose stem cells	White preadipocytes and adipose-derived mesenchymal stem cells	Invasive (liposuction)	(15-17)
Cord blood	Cord blood-derived stem cells and endothelial cells	Noninvasive	(18,19)
Peripheral blood	Mononuclear, T-, and myeloid cells	Minimal invasion (venipuncture)	(20-22)
Neural cells	Neural stem cells	Invasive	(23)
Astrocytes	Human astrocytes	Invasive	(24)
Hepatocytes	Primary human hepatocytes	Invasive	(25)
Amniocytes	Human amniotic fluid-derived cells	Invasive (amniocentesis)	(26,27)

Table 2. Human iPSCs can be induced without viral integration

Integration-free methods	Cell types	Reprogramming factors*	Efficiency (%)	Ref.
Sendai viruses	Fibroblasts	OSKM	~ 1	(42)
Adenoviruses	Fibroblasts and liver cells	OSKM	~ 0.001	(43)
Plasmids	Fibroblasts	OSNL	~ 0.001	(44,45)
Episomes	Fibroblasts	OSNL	~ 0.1	(46)
Transposons	Fibroblasts	OSKM	~ 0.1	(47)
Proteins	Fibroblasts	OS	~ 0.001	(48,49)
Chemicals or small Molecules	Umbilical vein endothelial cells	OCT4 + small molecules	~ 0.01	(50-53)
mRNA	Fibroblasts	OSKM or OSKML + VPA	~ 1-4.4	(54)
MicroRNA	Adipose stromal cells and dermal fibroblasts	miR-200c, miR-302s or miR-369s	~ 0.1	(55)

*Abbreviations represent a combination of reprogramming factors: K, KLF4; L, LIN28; M, c-MYC; N, NANOG; O, OCT4; S, SOX2; VPA, valproic acid.

retroviruses and they generated the first induced pluripotent stem cells (iPSCs) (5). Human iPSCs are highly similar to ESCs: they display ESC-like morphology, they express pluripotency markers, they share a similar level of gene expression and epigenetic states (6,7), and they possess the capacity to develop into three germ layers *in vitro* and *in vivo* (8). Given these facts, iPSCs could provide a valuable model to study germ cell determination *in vitro*, but they could also serve as a promising way to differentiate stem cells into diseased cells in order to model disease, screen drugs, and examine the effects of cell therapy. Several groups of researchers have indicated that iPSCs might be derived from urine samples (9,10). Compared to the procedures used to obtain other donor cells, urine collection is safe, affordable, ubiquitous, and readily accepted by patients. This article aims to present the benefits of iPSCs derived from urine for the study of rare diseases, and this article also highlights the applications of rare disease models.

2. Urine as an efficient source of cells for generation of iPSCs

Human iPSCs can be generated from a large variety of donor sources (Table1). However, the ideal cell source would be obtained non-invasively, simple and cost-effective to obtain, and obtained universally (from patients of any age, sex, ethnic group, or body type) (28). The first successful culture of urinary tract cells

occurred in 1972 (29), and that feat was repeated by many researchers (30-37). Zhou *et al.* generated iPSCs from urine-derived cells (9). Here, urine-derived cells will be referred to simply as urine cells. UiPSCs are advantageous in many ways. First, UiPSCs can be generated at a low cost. Urine is merely a body waste, so the only cost is the culture medium. Second, obtaining cell samples from patients, and especially young children with rare diseases, is difficult, but this can be avoided by repeated urine collections without medical assistance. Third, UiPSCs may have fewer genetic alterations than iPSCs from the skin due to less direct exposure to radiation (38,39). Moreover, UiPSCs have a high level of reprogramming efficiency, between 0.1% and 4% in general (40). This is because most urinary cells of an epithelial origin do not require mesenchymal-to-epithelial transition (MET) during reprogramming, unlike fibroblasts.

Different types of somatic cells are used to generate iPSCs, and different reprogramming techniques with varying levels of efficiency are used to reprogram cells with different origins (41). Avoiding viral integration and accomplishing safe and efficient reprogramming is the main goal. iPSCs can be generated without viral integration for basic applications (Table 2). Taking safety and workload into account, Xue *et al.* described a useful method of generating 93 iPS cell lines from 20 individuals with various backgrounds; their method reprogrammed human urine-derived cells without using a virus, serum, feeder, or oncogene *c-MYC* (56). This

non-viral iPSC bank with genetic information from different individuals revealed that UiPSCs could serve as a powerful *in vitro* model for the study of the disease. As their research continued, they found that different batches of cells derived from the same person's urine had dramatically different levels of reprogramming efficiency. In addition, half of the cells isolated from urine proliferated only to a limited extent, posing a major hurdle to the generation of iPSCs. Thus, Xue *et al.* developed three methods of reprogramming urine cells with diverse properties through the use of small molecules and autologous urine cells as feeders. iPSCs can be generated from almost any batch of cells isolated from urine, resulting in further advances in the banking of patient-specific iPSCs (57).

3. Modeling rare diseases using UiPSCs

Patient-specific iPSCs can differentiate into large numbers of affected cell types with the same genetic background as the patient since they are immortal and pluripotent (58). Using these cells to construct rare disease models will prove highly useful in the discovery of effective and safe drugs and in the study of cell replacement therapies. Many groups of researchers have successfully generated patient-specific UiPSCs. These cells are derived from patients with conditions such as renal disease (59), pediatric disease (60), a bleeding disorder (61), neurological disease (62), and a bone disorder (63,64). Obtaining these cells is the critical first step to elucidating the mechanisms that underlie the pathology of those diseases.

As UiPSC generation has become more efficient, many patient-specific UiPSCs have been successfully generated. Cryptorchidism is the most frequent congenital anomaly in human males. Cryptorchidism involves multiple causes, such as genetic mutations and environmental factors (65,66). Researchers have established cryptorchid-specific iPSC lines with genetic variations and they have found that urine cells may represent a better source for generation of iPSCs, especially in the study of pediatric diseases (60). Most patients with complex disorders are treated with various medications over a prolonged period, which may have an impact on donor cell propagation and nuclear reprogramming. Expansion of fibroblasts from patients with end-stage renal disease was problematic since cell division stopped after several passages (67). In contrast, use of immunosuppressive drugs has little effect on urine cell propagation and generation of iPSCs. Thus, urine-derived cells appeared to be a valuable donor source for patients with systemic lupus erythematosus (SLE) (59). Whether these drugs substantially impact the generation of iPSCs needs to be explored further under different conditions.

Although cells of multiple origins may be involved in the pathogenesis of rare diseases, specific cell types

could be used to recreate a phenocopy of the disease *in vitro* so that the cell type could be identified as where the disease originates. Investigating a rare disorder presents many challenges, but UiPSCs represent a world of potential applications – for recreating the phenotypic and pathological variants and also for identifying drug candidates and transplanting autologous cells into patients. Two studies have posited that new treatments for rare diseases can be developed in the future using UiPSCs-based models with integration-free episomal vectors (61,64).

The first study derived iPSCs from patients with hemophilia A (HA), which is a rare bleeding disorder with a prevalence of 1‰-2‰ caused by clotting factor VIII (*FVIII*) mutations resulting in deficient production of FVIII protein (68,69). HA-iPSCs are generated from patients' urine cells using an integration-free transfection technique (46,70). Differentiated hepatocyte-like cells derived from HA-iPSCs (HA-iPSC-Hep) display the phenotype of the defective FVIII found in selected patients. At the genetic level, HA-iPSC-Hep carry the mRNA of the defective *FVIII* gene. FVIII protein is absent on a protein level as well. Furthermore, FVIII activity in the culture supernatant is much lower than that in the reference group. Therefore, this new model is remarkable for two main reasons. Previous researchers investigated the mechanisms underlying HA in animal models. However, different species have differing physiologies, which may partly explain why many novel drugs are not effective in patients when tested in clinical trials (71). Models like HA-iPSC-Hep will help to explain the pathogenesis of disease and facilitate the development of new therapeutics. In patients with a bleeding disorder, an invasive procedure can be life-threatening, so an easily accessible source of donor cells must be obtained in a completely non-invasive manner. Urine cells can be obtained non-invasively and safely, so those cells are a useful source of iPSCs. A novel model using integration-free episomal vectors could be used in cell therapy along with gene editing.

The second study generated iPSCs from patients with fibrodysplasia ossificans progressiva (FOP). FOP is caused by a recurrent heterozygous missense mutation in activin receptor-like kinase 2 (*ACVR1/ALK2*) (72). FOP is a rare genetic disease characterized by congenital malformation of the halluces and by progressive heterotopic ossification (HO), and FOP is the most catastrophic disorder of HO in humans (73). Endothelial cells (ECs) and pericytes derived from FOP iPSCs can recreate some aspects of the disease phenotype *in vitro*. Researchers found that ECs were not readily generated from FOP iPSCs and had limited proliferation, which may be related to the crosstalk between bone morphogenetic protein (BMP) signaling and vascular endothelial growth factor (VEGF) signaling. This issue needs to be further explored.

Researchers have also found that FOP iPSC-pericytes are prone to mineralization. Moreover, use of ALK2 inhibitor led to a reduction in alkaline phosphatase (ALP) activity. Models such as FOP iPSCs provide a useful tool to determine the underlying mechanisms of endothelial-mesenchymal transition (EndMT), to explore the bioactivity of ALK2 inhibitors, and to screen drugs (64).

The variability between iPSC clones and the intrinsic epigenetic features of donor cells may have an impact on the properties of iPSCs. Reliable control iPSC clones could be generated *via* gene editing to eliminate any potential confounding effects of variations in genetic background. In other words, isogenic iPSCs may differ only at specific loci while all other locations remain identical (74,75).

4. Applications of iPSC-based models

These two models described indicate that UiPSCs have opened new avenues for modeling rare disorders and they offer proof of principle for basic biological research in the short term. In the long term, the clinical use of UiPSCs in drug discovery and cell replacement therapies will also receive a great deal of attention. With reliable disease models, researchers will be able to dissect the pathophysiological basis of rare diseases and screen drugs in affected cell types. Furthermore, these models will advance the field a step closer to patient-matched cells or tissues for clinical transplantation, which may represent the ultimate goal of stem cell therapy.

4.1. Disease pathogenesis and drug discovery

Many rare human diseases are still poorly understood, with a complicated pathogenesis and multiple causes. Patient-specific iPSC models can recreate the phenotypic and pathological variants of the disease *in vitro* and can then be used to identify drugs to rescue these phenotypes. Thus, disease models offer an unprecedented opportunity to understand underlying mechanisms of disease and to develop therapeutic candidates.

Some genetic mutations underlying human diseases may affect the generation of patient-specific iPSCs or the maintenance of their pluripotent state (76,77). Nonetheless, iPSCs provide a unique human system for understanding molecular mechanisms of reprogramming and pathogenesis and can thus be used to develop effective drugs. Researchers have recently identified two molecular mechanisms responsible for inhibition of the generation of FOP-iPSCs from patient fibroblasts: incomplete reprogramming of pluripotent and fibroblastic genes and the forced differentiation of cells during and after reprogramming (78). This inhibition was due to the constitutive activation of *ALK2*

and was mostly overcome by specific suppression of *ALK2* expression. While screening a library of chemical substances, the researchers identified a new *ALK2* inhibitor candidate to restore generation of FOP-iPSCs. As mentioned earlier, ALP activity was reduced by use of an *ALK* inhibitor, and this may represent a potential treatment for FOP. Two mechanisms were involved in the pathology of FOP: ligand-independent BMP signaling and ligand-dependent hyper-responsiveness to BMP stimulation. A landmark study by Toguchida's research group generated induced mesenchymal stromal cells (iMSCs) from FOP-iPSCs that, when implanted into immunodeficient mice with Activin-A-expressing cells, induced bone and cartilage formation *in vivo* (79). *In vitro* treatment with TGF- β and BMP inhibitors eliminated increased chondrogenesis in FOP-iMSCs. In addition to the two molecular mechanisms that were involved in the pathology of FOP, Toguchida's research group identified a novel third mechanism: Activin-A activates TGF- β and aberrant BMP signaling that results in increased chondrogenesis in FOP-iMSCs. Based on the role and mechanism of action of Activin-A in HO, Activin-A triggers increased chondrogenesis in FOP-iMSCs, but this action can be inhibited by several Activin-A inhibitors. Therefore, Activin-A inhibitors could be a novel therapy for patients with FOP.

Taken together, these findings provide proof of the principle that new effective treatments for FOP can be discovered. In addition, iPSC-based models can be useful in identifying novel drug compounds and in testing drug toxicity and responsiveness (80).

4.2. Cell therapy

With the recent development of gene editing tools, the idea of patient-personalized therapies and replacement of diseased or damaged organ tissues has come closer to becoming a clinical reality. In the two models mentioned thus far, iPSCs were generated from urine cells using integration-free episomal vectors, so the two models could be used in cell therapy in combination with gene editing. A mutation causing a disease could be corrected in patient-specific iPSCs.

Approximately half of the severe cases of HA are caused by chromosomal inversions in the portion of the *FVIII* gene. Given this fact, Park *et al.* used a transcription activator-like effector nuclease (TALEN) to revert the inverted 140-kbp segment in an iPSC-based model of hemophilia (81). Endothelial cells derived from *FVIII*-corrected iPSCs produced FVIII protein *in vitro*. Interestingly, the iPSC-based model of hemophilia was created using the same TALEN pair that induced chromosomal inversions affecting the *FVIII* gene in wild-type iPSCs. This approach could be used in autologous stem cell therapy and to provide a method of disease modeling. However, the challenge is to invert a much larger region that is eight times more prevalent

than aforementioned 140-kbp inversion. Park *et al.* successfully used the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system to correct these regions in patient-specific iPSCs (82). Combining these gene editing tools with iPSC technology would provide a source of endothelial progenitor cells for transplantation therapies.

A recent study suggested that a one-step procedure can generate gene-corrected *ALK2*-iPSCs, and the advantages of this approach are that it saves time, labor, and money (83). Activated BMP signaling by an FOP R206H mutation adversely affects the generation of FOP iPSCs since BMPs can induce differentiation of human ESCs (84). Previous studies indicated that iPSCs from FOP-derived skin fibroblasts were generated inefficiently and were also unable to maintain their pluripotent state (85). Therefore, a one-step strategy to concurrently perform reprogramming and gene correction in the CRISPR/Cas9 system would circumvent the iPSC stage. Matsumoto *et al.* successfully established gene-corrected FOP-iPSCs through the use of bacterial artificial chromosome (BAC)-based homologous recombination (86).

Chronic granulomatous disease (CGD) is a rare genetic disease with a single point mutation (T > G) at the end of intron 1 of *CYBB* in most patients (87). iPSCs were generated from a patient with mutant *CYBB*, and the mutations were corrected using a CRISPR-Cas9 site-specific nuclease system (88). Phagocytes derived from these *CYBB*-corrected iPSCs restored the oxidative burst.

Thus, a combination of iPSC technology and gene editing could provide an autologous source of corrected iPSC-derived cells for transplantation therapies. Natronobacterium gregoryi Argonaute (NgAgo) is the newest tool with which to edit the genes of human cells (89). No studies have used this tool to model disease in iPSCs thus far, but it has potential advantages over Cas9 such as lower off-targeting levels. Further research is needed to prove that NgAgo can be used effectively with iPSC technology in cell replacement therapy and to expand the clinical use of UiPSCs to model rare diseases.

5. Advantages of and barriers to the study of rare diseases using UiPSCs

Urine is merely a body waste, but it can reproducibly be used as an efficient source of cells with which to generate human iPSCs. A point worth noting is that the isolation of urine cells is completely non-invasive and is readily accepted by patients, and especially young children. In addition, researchers have used a non-viral episome system to compare the reprogramming efficiency of fibroblasts and epithelial cells from foreskin and urine. iPSCs derived from urine were generated with an efficiency of approximately 1.5%,

which is two orders of magnitude higher than the efficiency with which fibroblasts were generated (0.01%) (90). This significant difference may be due to the fact that epithelial cells do not have to undergo the MET that fibroblasts must undergo. Nonetheless, researchers have contended that human iPSCs may have remnants of epigenetic memory of donor origin, resulting in biased differentiation towards lineages related to the donor cell type (91,92). iPSCs derived from fibroblasts favor differentiation towards the mesodermal lineage, but an interesting fact is that such a tendency has not been found in UiPSCs.

Rare diseases can profoundly affect humans, so the study of those diseases has garnered worldwide attention. Nonetheless, the causes of those diseases are difficult to determine and those diseases are difficult to diagnose and treatment. The presence of additional complications also increases the difficulty of their treatment. Therefore, a human model of these complex and varying conditions may serve as a useful platform for understanding disease pathogenesis and for developing new therapeutics. Because iPSCs resemble ESCs in terms of pluripotency and the potential for self-renewal, patient-specific iPSCs could represent a promising strategy for investigating the molecular mechanisms of disease and discovery of drugs to treat them (93,94). Recent studies have reported that generating UiPSCs from patients with rare diseases would provide insight into the mechanisms of those diseases (59-64). The availability of limitless amounts of diseased cells from patients with a rare disease could, along with gene editing, prove useful in clinical practice (Figure 1). These cells represent a potential boon to regenerative medicine: cells from a patient's urine sample can be reprogrammed into iPSCs and then differentiated into diseased cells or tissues needed to treat the disease. This customized therapy would overcome the hurdles – immune rejection and ethical concerns – faced by cells derived from embryos. Researchers have indicated that UiPSCs can be used for further regenerative therapies. Urine cells have been used to generate iPSCs in a non-viral episome system and those iPSCs have been differentiated into epithelial sheets. Those sheets developed into ameloblasts in a tooth-like structure with a success rate of up to 30% (95). Moreover, this approach demonstrated that human UiPSCs could be an ideal source for the regeneration of patient-specific teeth.

Clinical studies of rare diseases face severe challenges since patients are often geographically dispersed or are not readily available. One solution to this problem is to use gene editing to introduce mutations associated with a disease into iPSCs from healthy donors. A recent study efficiently introduced specific point mutations with CRISPR/Cas9 and it edited just one copy of a gene, rather than both copies (96). Another crucial point in disease modeling is what

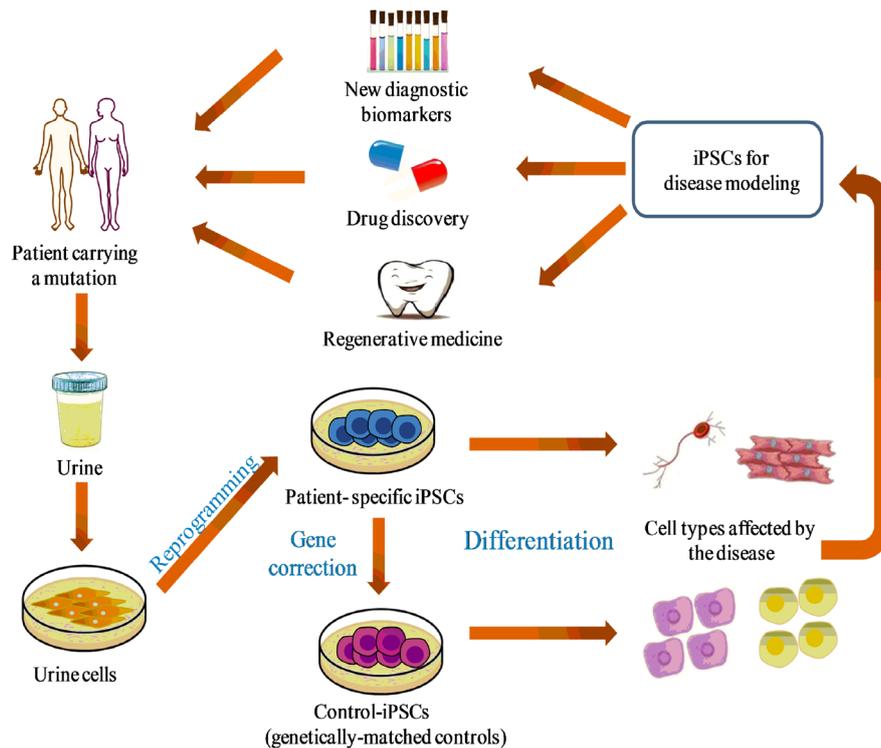


Figure 1. Creation of models and applications of UiPSCs in the study of rare diseases. A diagram showing the creation of models of specific rare diseases using UiPSCs and their applications. Cells are isolated from patient urine samples and reprogrammed into iPSCs. Isogenic controls are created with gene-editing tools and then differentiated into diseased cells that can recreate crucial aspects of the disease *in vitro*. Patient-specific disease models can be used to identify new diagnostic biomarkers and to screen effective and novel drugs as well as to replace cells or tissues in regenerative medicine.

to use as a control. In many cases, researchers compare a patient's iPSCs with those from a healthy individual. However, the cells behave too differently in culture because of differences in the genomic background or gene expression. Researchers have turned to a refined method of gene editing – gene targeting – to produce genetically-matched controls.

There are several issues that need to be addressed in more detail before UiPSCs can be successfully used in clinical practice, but the creation of faithful disease models will aid in further clinical use of UiPSCs. The generation of UiPSCs is universal except in some rare cases, such as renal failure or cancer requiring a cystectomy (40). Some genetic mutations underlying human diseases could affect the generation of patient-specific iPSCs or the maintenance their pluripotent state. Further study is needed to determine what effect these mutations have. In addition, several iPSC clones generated from the same patient's cells should be assessed for consistent functional readout in order to minimize the risk of acquired genetic (or epigenetic) abnormalities. Moreover, differentiation protocols are inefficient and produce only impaired and/or heterogeneous cell populations in many cases. The challenge is to obtain mature cell types. Furthermore, a wide range of growth factors and specific culture conditions need to be used to differentiate iPSCs. Clinical trials must first thoroughly assess whether

the use of different molecules is safe (97). Given that undifferentiated iPSCs can cause teratomas *in vivo*, the crucial step is to remove residual undifferentiated cells before transplantation. Therefore, researchers are now working to ensure the identity and safety of cell lines in terms of genomic variability, patterns of gene expression, and the aspects mentioned earlier. One way to resolve these issues is to devise a set of clinical-grade practices of quality control for the banking of allogeneic iPSCs. These practices would ensure that donor cells in large-scale collections are immunologically compatible and they would provide a foundation for the treatment of rare diseases. Banking of iPSCs would have numerous advantages for patients, no matter when or where those cells are needed.

6. Conclusion

In summary, the use of patient-specific UiPSCs offers a novel strategy for modeling disease, and this approach has already demonstrated its potential to provide a better understanding of the molecular mechanisms of disease. Some exemplary applications mentioned here can be expanded to study countless other rare diseases using UiPSCs. As generation of patient-specific UiPSCs becomes more routine and more scalable, the models they allow will serve as a useful tool to determine the mechanisms of disease, to identify drug candidates,

and to develop personalized regenerative medicine. Further study is needed to develop therapeutic applications of UiPSC technology for treatment of rare diseases. Further technical improvements, particularly in generation and differentiation of patient-specific UiPSCs and increased banking of allogeneic iPSCs, will further advance the field. That said, extreme caution is required when considering use of UiPSC technology in clinical practice. Many challenges still need to be overcome and a great deal more knowledge is needed before UiPSC technology can become a routine clinical approach to the treatment of rare diseases.

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References

- Song P, Gao J, Inagaki Y, Kokudo N, Tang W. Intractable and rare diseases research in Asia. *Biosci Trends*. 2012; 6:48-51.
- Cui Y, Han J. A proposed definition of rare diseases for China: From the perspective of return on investment in new orphan drugs. *Orphanet J Rare Dis*. 2015; 10:28.
- Wuhl E, van Stralen KJ, Wanner C, Ariceta G, Heaf JG, Bjerre AK, Palsson R, Duneau G, Hoitsma AJ, Ravani P, Schaefer F, Jager KJ. Renal replacement therapy for rare diseases affecting the kidney: An analysis of the ERA-EDTA Registry. *Nephrol Dial Transplant*. 2014; 29:iv1-iv8.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998; 282:1145-1147.
- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006; 126:663-676.
- Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell*. 2007; 1:55-70.
- Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A. Dissecting direct reprogramming through integrative genomic analysis. *Nature*. 2008; 454:49-55.
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917-1920.
- Zhou T, Benda C, Dunzinger S, *et al*. Generation of human induced pluripotent stem cells from urine samples. *Nat Protoc*. 2012; 7:2080-2089.
- Song B, Niclis JC, Alikhan MA, Sakkal S, Sylvain A, Kerr PG, Laslett AL, Bernard CA, Ricardo SD. Generation of induced pluripotent stem cells from human kidney mesangial cells. *J Am Soc Nephrol*. 2011; 22:1213-1220.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131:861-872.
- Wada N, Wang B, Lin NH, Laslett AL, Gronthos S, Bartold PM. Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts. *J Periodontol Res*. 2011; 46:438-447.
- Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilic J, Pekarik V, Tiscornia G, Edel M, Boue S, Izpisua Belmonte JC. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol*. 2008; 26:1276-1284.
- Utikal J, Maherali N, Kulalert W, Hochedlinger K. *SOX2* is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J Cell Sci*. 2009; 122:3502-3510.
- Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F, Hu S, Cherry AM, Robbins RC, Longaker MT, Wu JC. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci U S A*. 2009; 106:15720-15725.
- Sugii S, Kida Y, Kawamura T, Suzuki J, Vassena R, Yin YQ, Lutz MK, Berggren WT, Izpisua Belmonte JC, Evans RM. Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2010; 107:3558-3563.
- Aoki T, Ohnishi H, Oda Y, Tadokoro M, Sasao M, Kato H, Hattori K, Ohgushi H. Generation of induced pluripotent stem cells from human adipose-derived stem cells without *c-MYC*. *Tissue Eng Part A*. 2010; 16:2197-2206.
- Giorgetti A, Montserrat N, Aasen T, Gonzalez F, Rodriguez-Piza I, Vassena R, Raya A, Boue S, Barrero MJ, Corbella BA, Torrabadella M, Veiga A, Izpisua Belmonte JC. Generation of induced pluripotent stem cells from human cord blood using *OCT4* and *SOX2*. *Cell Stem Cell*. 2009; 5:353-357.
- Haase A, Olmer R, Schwanke K, *et al*. Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell*. 2009; 5:434-441.
- Staerk J, Dawlaty MM, Gao Q, Maetzel D, Hanna J, Sommer CA, Mostoslavsky G, Jaenisch R. Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell*. 2010; 7:20-24.
- Loh YH, Hartung O, Li H, *et al*. Reprogramming of T cells from human peripheral blood. *Cell Stem Cell*. 2010; 7:15-19.
- Seki T, Yuasa S, Fukuda K. Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus. *Nat Protoc*. 2012; 7:718-728.
- Kim JB, Greber B, Arauzo-Bravo MJ, Meyer J, Park KI, Zachres H, Scholer HR. Direct reprogramming of human neural stem cells by *OCT4*. *Nature*. 2009; 461:649-643.
- Ruiz S, Brennand K, Panopoulos AD, Herrerias A, Gage FH, Izpisua-Belmonte JC. High-efficient generation of induced pluripotent stem cells from human astrocytes. *PLoS One*. 2010; 5:e15526.
- Liu H, Ye Z, Kim Y, Sharkis S, Jang YY. Generation of

- endoderm-derived human induced pluripotent stem cells from primary hepatocytes. *Hepatology*. 2010; 51:1810-1819.
26. Cai J, Li W, Su H, *et al*. Generation of human induced pluripotent stem cells from umbilical cord matrix and amniotic membrane mesenchymal cells. *J Biol Chem*. 2010; 285:11227-11234.
 27. Li C, Zhou J, Shi G, Ma Y, Yang Y, Gu J, Yu H, Jin S, Wei Z, Chen F, Jin Y. Pluripotency can be rapidly and efficiently induced in human amniotic fluid-derived cells. *Hum Mol Genet*. 2009; 18:4340-4349.
 28. Zhou T, Benda C, Duzinger S, *et al*. Generation of induced pluripotent stem cells from urine. *J Am Soc Nephrol*. 2011; 22:1221-1228.
 29. Sutherland GR, Bain AD. Culture of cells from the urine of newborn children. *Nature*. 1972; 239:231.
 30. Linder D. Culture of cells from the urine and bladder washings of adults. *Somatic Cell Genet*. 1976; 2:281-283.
 31. Herz F, Schermer A, Koss LG. Short-term culture of epithelial cells from urine of adults. *Proc Soc Exp Biol Med*. 1979; 161:153-157.
 32. Felix JS, Littlefield JW. Urinary tract epithelial cells cultured from human urine. *Int Rev Cytol Suppl*. 1979;11-23.
 33. Herz F. Culture of urinary cells. *Birth Defects Orig Artic Ser*. 1980; 16:85-93.
 34. Felix JS, Littlefield JW. Human newborn urine as a source of epithelial cells. *Birth Defects Orig Artic Ser*. 1980; 16:231-237.
 35. Felix JS, Sun TT, Littlefield JW. Human epithelial cells cultured from urine: Growth properties and keratin staining. *In Vitro*. 1980; 16:866-874.
 36. Dorrenhaus A, Muller JI, Golka K, Jedrusik P, Schulze H, Follmann W. Cultures of exfoliated epithelial cells from different locations of the human urinary tract and the renal tubular system. *Arch Toxicol*. 2000; 74:618-626.
 37. Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J. Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. *Diabetes*. 2005; 54:3427-3434.
 38. Gore A, Li Z, Fung HL, *et al*. Somatic coding mutations in human induced pluripotent stem cells. *Nature*. 2011; 471:63-67.
 39. Hussein SM, Batada NN, Vuoristo S, *et al*. Copy number variation and selection during reprogramming to pluripotency. *Nature*. 2011; 471:58-62.
 40. Benda C, Zhou T, Wang X, Tian W, Grillari J, Tse H-F, Grillari-Voglauer R, Pei D, Esteban MA. Urine as a Source of Stem Cells. *Adv Biochem Eng Biotechnol*. 2013; 129:19-32.
 41. Gonzalez F, Boue S, Izpisua Belmonte JC. Methods for making induced pluripotent stem cells: Reprogramming a la carte. *Nat Rev Genet*. 2011; 12:231-242.
 42. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci*. 2009; 85:348-362.
 43. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science*. 2008; 322:945-949.
 44. Si-Tayeb K, Noto FK, Sepac A, Sedlic F, Bosnjak ZJ, Lough JW, Duncan SA. Generation of human induced pluripotent stem cells by simple transient transfection of plasmid DNA encoding reprogramming factors. *BMC Dev Biol*. 2010; 10:81.
 45. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science*. 2008; 322:949-953.
 46. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA. Human induced pluripotent stem cells free of vector and transgene sequences. *Science*. 2009; 324:797-801.
 47. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*. 2009; 458:766-770.
 48. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Scholer HR, Duan L, Ding S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell*. 2009; 4:381-384.
 49. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell*. 2009; 4:472-476.
 50. Ichida JK, Blanchard J, Lam K, *et al*. A small-molecule inhibitor of tgf-Beta signaling replaces *SOX2* in reprogramming by inducing *NANOG*. *Cell Stem Cell*. 2009; 5:491-503.
 51. Zhu S, Li W, Zhou H, Wei W, Ambasadhan R, Lin T, Kim J, Zhang K, Ding S. Reprogramming of human primary somatic cells by *OCT4* and chemical compounds. *Cell Stem Cell*. 2010; 7:651-655.
 52. Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, Melton DA. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol*. 2008; 26:795-797.
 53. Lyssiotis CA, Foreman RK, Staerk J, *et al*. Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of *KLF4*. *Proc Natl Acad Sci U S A*. 2009; 106:8912-8917.
 54. Warren L, Manos PD, Ahfeldt T, *et al*. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*. 2010; 7:618-630.
 55. Miyoshi N, Ishii H, Nagano H, *et al*. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell Stem Cell*. 2011; 8:633-638.
 56. Xue Y, Cai X, Wang L, Liao B, Zhang H, Shan Y, Chen Q, Zhou T, Li X, Hou J, Chen S, Luo R, Qin D, Pei D, Pan G. Generating a non-integrating human induced pluripotent stem cell bank from urine-derived cells. *PLoS One*. 2013; 8:e70573.
 57. Li D, Wang L, Hou J, *et al*. Optimized approaches for generation of integration-free iPSCs from human urine-derived cells with small molecules and autologous feeder. *Stem Cell Reports*. 2016; 6:717-728.
 58. Sternecker JL, Reinhardt P, Scholer HR. Investigating human disease using stem cell models. *Nat Rev Genet*. 2014; 15:625-639.
 59. Chen Y, Luo R, Xu Y, Cai X, Li W, Tan K, Huang J, Dai Y. Generation of systemic lupus erythematosus-specific induced pluripotent stem cells from urine. *Rheumatol Int*. 2013; 33:2127-2134.
 60. Zhou J, Wang X, Zhang S, Gu Y, Yu L, Wu J, Gao T,

- Chen F. Generation and characterization of human cryptorchid-specific induced pluripotent stem cells from urine. *Stem Cells Dev.* 2013; 22:717-725.
61. Jia B, Chen S, Zhao Z, *et al.* Modeling of hemophilia A using patient-specific induced pluripotent stem cells derived from urine cells. *Life Sci.* 2014; 108:22-29.
 62. Zhang SZ, Li HF, Ma LX, Qian WJ, Wang ZF, Wu ZY. Urine-derived induced pluripotent stem cells as a modeling tool for paroxysmal kinesigenic dyskinesia. *Biol Open.* 2015; 4:1744-1752.
 63. Hildebrand L, Rossbach B, Kühnen P, Gossen M, Kurtz A, Reinke P, Seemann P, Stachelscheid H. Generation of integration free induced pluripotent stem cells from fibrodysplasia ossificans progressiva (FOP) patients from urine samples. *Stem Cell Res.* 2016; 16:54-58.
 64. Cai J, Orlova Valeria V, Cai X, Eekhoff Elisabeth MW, Zhang K, Pei D, Pan G, Mummery Christine L, ten Dijke P. Induced pluripotent stem cells to model human fibrodysplasia ossificans progressiva. *Stem Cell Reports.* 2015; 5:963-970.
 65. Foresta C, Zuccarello D, Garolla A, Ferlin A. Role of hormones, genes, and environment in human cryptorchidism. *Endocr Rev.* 2008; 29:560-580.
 66. Bay K, Main KM, Toppari J, Skakkebaek NE. Testicular descent: INSL3, testosterone, genes and the intrauterine milieu. *Nat Rev Urol.* 2011; 8:187-196.
 67. Thatava T, Armstrong AS, De Lamo JG, Edukulla R, Khan YK, Sakuma T, Ohmine S, Sundsbak JL, Harris PC, Kudva YC, Ikeda Y. Successful disease-specific induced pluripotent stem cell generation from patients with kidney transplantation. *Stem Cell Res Ther.* 2011; 2:48.
 68. Roth DA, Tawa NE, Jr., O'Brien JM, Treco DA, Selden RF. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med.* 2001; 344:1735-1742.
 69. Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. *Lancet.* 2003; 361:1801-1809.
 70. Liao B, Bao X, Liu L, *et al.* MicroRNA cluster 302-367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J Biol Chem.* 2011; 286:17359-17364.
 71. Saha K, Jaenisch R. Technical challenges in using human induced pluripotent stem cells to model disease. *Cell Stem Cell.* 2009; 5:584-595.
 72. Billings PC, Fiori JL, Bentwood JL, O'Connell MP, Jiao X, Nussbaum B, Caron RJ, Shore EM, Kaplan FS. Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP). *J Bone Miner Res.* 2008; 23:305-313.
 73. Kaplan FS, Chakkalakal SA, Shore EM. Fibrodysplasia ossificans progressiva: Mechanisms and models of skeletal metamorphosis. *Dis Model Mech.* 2012; 5:756-762.
 74. Kim HS, Bernitz JM, Lee DF, Lemischka IR. Genomic editing tools to model human diseases with isogenic pluripotent stem cells. *Stem Cells Dev.* 2014; 23:2673-2686.
 75. Sternecker JL, Reinhardt P, Schöler HR. Investigating human disease using stem cell models. *Nat Rev Genet.* 2014; 15:625-639.
 76. Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, Okita K, Yamanaka S. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature.* 2009; 460:1132-1135.
 77. Li H, Collado M, Villasante A, Strati K, Ortega S, Canamero M, Blasco MA, Serrano M. The *Ink4/Arf* locus is a barrier for iPS cell reprogramming. *Nature.* 2009; 460:1136-1139.
 78. Hamasaki M, Hashizume Y, Yamada Y, Katayama T, Hohjoh H, Fusaki N, Nakashima Y, Furuya H, Haga N, Takami Y, Era T. Pathogenic mutation of ALK2 inhibits induced pluripotent stem cell reprogramming and maintenance: Mechanisms of reprogramming and strategy for drug identification. *Stem Cells.* 2012; 30:2437-2449.
 79. Hino K, Ikeya M, Horigome K, Matsumoto Y, Ebise H, Nishio M, Sekiguchi K, Shibata M, Nagata S, Matsuda S, Toguchida J. Neofunction of ACVR1 in fibrodysplasia ossificans progressiva. *Proc Natl Acad Sci U S A.* 2015; 112:15438-15443.
 80. Liang P, Lan F, Lee AS, Gong T, Sanchez-Freire V, Wang Y, Diecke S, Sallam K, Knowles JW, Wang PJ, Nguyen PK, Bers DM, Robbins RC, Wu JC. Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. *Circulation.* 2013; 127:1677-1691.
 81. Park CY, Kim J, Kweon J, Son JS, Lee JS, Yoo JE, Cho SR, Kim JH, Kim JS, Kim DW. Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs. *Proc Natl Acad Sci U S A.* 2014; 111:9253-9258.
 82. Park CY, Kim Duk H, Son Jeong S, Sung Jin J, Lee J, Bae S, Kim JH, Kim DW, Kim JS. Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9. *Cell Stem Cell.* 2015; 17:213-220.
 83. Kim BY, Jeong S, Lee SY, Lee SM, Gweon EJ, Ahn H, Kim J, Chung SK. Concurrent progress of reprogramming and gene correction to overcome therapeutic limitation of mutant *ALK2*-iPSC. *Exp Mol Med.* 2016; 48:e237.
 84. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods.* 2005; 2:185-190.
 85. Barruet E, Hsiao EC. Using human induced pluripotent stem cells to model skeletal diseases. *Methods Mol Biol.* 2016; 1353:101-118.
 86. Matsumoto Y, Ikeya M, Hino K, Horigome K, Fukuta M, Watanabe M, Nagata S, Yamamoto T, Otsuka T, Toguchida J. New protocol to optimize iPS cells for genome analysis of fibrodysplasia ossificans progressiva. *Stem Cells.* 2015; 33:1730-1742.
 87. Jiang Y, Cowley SA, Siler U, Melguizo D, Tilgner K, Browne C, Dewilton A, Przyborski S, Saretzki G, James WS, Seger RA, Reichenbach J, Lako M, Armstrong L. Derivation and functional analysis of patient-specific induced pluripotent stem cells as an *in vitro* model of chronic granulomatous disease. *Stem Cells.* 2012; 30:599-611.
 88. Flynn R, Grundmann A, Renz P, Hänseler W, James WS, Cowley SA, Moore MD. CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells. *Exp Hematol.* 2015; 43:838-848.e833.
 89. Gao F, Shen XZ, Jiang F, Wu Y, Han C. DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute. *Nat Biotechnol.* 2016; 34:768-773.
 90. Drozd AM, Walczak MP, Piaszkowski S, Stoczynska-Fidelus E, Rieske P, Grzela DP. Generation of human iPSCs from cells of fibroblastic and epithelial origin by

- means of the oriP/EBNA-1 episomal reprogramming system. *Stem Cell Res Ther.* 2015; 6:122.
91. Kim K, Zhao R, Doi A, Ng K, Unternaehrer J, Cahan P, Huo H, Loh YH, Aryee MJ, Lensch MW, Li H, Collins JJ, Feinberg AP, Daley GQ. Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. *Nat Biotechnol.* 2011; 29:1117-1119.
92. Kim K, Doi A, Wen B, *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature.* 2010; 467:285-290.
93. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ. Disease-specific induced pluripotent stem cells. *Cell.* 2008; 134:877-886.
94. Kim C. Disease modeling and cell based therapy with iPSC: Future therapeutic option with fast and safe application. *Blood Res.* 2014; 49:7-14.
95. Cai J, Zhang Y, Liu P, *et al.* Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. *Cell Regen (Lond).* 2013; 2:6.
96. Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Teo S, Olsen KM, Gregg A, Noggle S, Tessier-Lavigne M. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature.* 2016; 533:125-129.
97. Bilic J, Izpisua Belmonte JC. Concise review: Induced pluripotent stem cells versus embryonic stem cells: Close enough or yet too far apart? *Stem Cells.* 2012; 30:33-41.

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Perceptions regarding a range of work-related issues and corresponding support needs of individuals with an intractable disease

Kumiko Imahashi^{1,*}, Reiko Fukatsu¹, Yasoichi Nakajima¹, Megumi Nakamura¹, Tateo Ito², Mariko Horigome³, Yuichiro Haruna⁴, Tatsuya Noda⁵, Yasuto Itoyama⁶

¹National Rehabilitation Center for Persons with Disabilities, Saitama, Japan;

²Japan Patients Association, Tokyo, Japan;

³Tokyo Colony Welfare Corp, Tokyo, Japan;

⁴National Institute of Vocational Rehabilitation, Chiba, Japan;

⁵Department of Public Health, Nara Medical University, Nara, Japan;

⁶International University of Health and Welfare, Fukuoka, Japan.

Summary

A number of persons with an intractable disease (ID) experience work-related problems that could lead to job loss. The aim of this study was to ascertain perceptions regarding a range of work-related issues and corresponding support needs of individuals with an ID. Potential participants were people ages 15 to 64 with one of the 130 intractable chronic diseases designated in the Act to Comprehensively Support the Daily and Social Activities of Persons with Disabilities (Comprehensive Support for the Disabled Act). Participants completed a self-administered questionnaire. With the assistance of patients' organizations, 3,000 questionnaires were mailed to potential participants. Questions included demographic characteristics, family concerns, employment/supported employment, work accommodations, and other aspects of life. Responses were received from 889 (29.6%) participants, and respondents had 57 IDs. Forty-six-point-seven percent of respondents reported being unemployed due to fatigue and/or long-term treatment. Nearly half of the unemployed respondents reported that they had been unable to work despite their willingness to do so. Common requests for accommodation included flexible work hours, working at home, and job/workplace modifications. Only 30% of respondents knew about job training programs and supported work available for persons with disabilities. The results of the study are relevant for employees, employers, and occupational health/human resource professionals. The issue of reasonable accommodations for persons with an ID needs to be addressed in future research in order to promote continued work by those persons.

Keywords: Intractable disease, chronic disease, employment, supported, social welfare

1. Introduction

Intractable diseases (IDs) are defined in the Principles of Policy for Intractable Diseases issued in 1972 by

the Ministry of Health, Labour, and Welfare of Japan as: *i*) diseases that have resulted from an unidentifiable cause and, without a clearly established treatment, have a considerably high risk of disability, or *ii*) diseases that chronically develop and that require a significant amount of patient care, causing a heavy burden on their family members both financially and mentally. Some of the characteristic problems of IDs include development of multiple disorders in addition to the main symptoms of a specific ID (*1,2*), an unstable general condition as symptoms worsen over several years, and variance in symptoms depending on patient's physical condition

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*Address correspondence to:

Dr. Kumiko Imahashi, National Rehabilitation Center for Persons with Disabilities, Namiki 4-1, Tokorozawa, Saitama 359-8555, Japan.

E-mail: imahashi-kumiko@rehab.go.jp

on a given day as well as the medication the patient is taking (3,4). In addition to these problems, some patients have symptoms that preclude them from living a regular life, such as fatigue (5,6), pain (7), and diarrhea (8). That said, many IDs have become chronic but manageable conditions because of advances in treatment, rehabilitation, and preventive medicine.

The needs of patients with an ID vary significantly since their treatments continue for years and their physical and mental status changes with age. These patients' need for employment has become an important issue in recent years (9) since patients need to lead a life with dignity in the community (10). Employment support (ES), including support provided *via* social welfare services, has therefore become an urgent issue for Japan to address.

An ES system for patients with an ID in Japan has yet to be fully implemented (11), but patients with an ID are clearly categorized as persons with a disability under the Act to Comprehensively Support the Daily and Social Activities of Persons with Disabilities (Comprehensive Support for the Disabled Act), which was implemented in 2013. People who use welfare services will now presumably increase. In 2013, a study examined the use of employment-related welfare services (EWS) by persons with an ID in Japan (12). Major findings of that study were that ES services under the Comprehensive Support for the Disabled Act had not adequately reached the public and that only 16% of service providers had provided services to patients with an ID. However, patients, *i.e.* users of those services, were not included in that study, so those findings only depict part of the situation. The current study should be able to depict the rest of the situation. As a complement to the previous study, the current study has sought to ascertain perceptions regarding a range of work-related issues and corresponding support needs of individuals with an ID.

2. Materials and Methods

Potential participants in this study were patients with one of the 130 intractable chronic diseases (Supplementary Table S1, <http://www.irdrjournal.com/docindex.php?year=2016&kanno=3>) designated in the Comprehensive Support for the Disabled Act. According to the Act, the 130 designated chronic diseases must meet 3 requirements: the lack of an established cure, the need for long-term treatment, and the existence of diagnostic criteria. Therefore, an intractable chronic disease may not necessarily be a rare disease. Since this study focused on work-related issues, potential participants were patients between the ages of 15 and 64, the legal working age and the general retirement age in Japan, respectively.

Potential participants were sent an anonymous self-administered questionnaire. The participants were provided with instructions that including the following: an explanation of this study and its purpose,

contact information, a statement on anonymity and confidentiality, and a note indicating that returning the questionnaire by mail would constitute consent to participation in this study. The questionnaire asked participants to report their status as of Oct 1, 2014. Since the mailing addresses of patients are nonpublic personal information, prefectural patients' associations were asked for their cooperation. These associations are organized by region, not by disease type. With the assistance of those patients' associations, 3,000 questionnaires were mailed. The questionnaire included questions regarding demographic characteristics, family concerns, employment/supported employment, work accommodations, and other aspects of life.

The Ethics Committee of the National Rehabilitation Center for Persons with Disabilities approved this study.

3. Results

3.1. Demographic data

Responses were received from 889 (29.6%) participants with 57 IDs (Table 1) living in 41 of 47 prefectures in Japan. The most frequently reported diseases were connective tissue diseases such as systemic lupus erythematosus and takayasu arteritis, followed by nervous system disorders such as Parkinson's disease and myasthenia gravis, digestive system disorders such as Crohn's disease and ulcerative colitis, and visual disorders such as retinitis pigmentosa. The demographic characteristics of respondents are shown in Table 2.

3.2. Employment status

Of the 889 respondents, 459 (51.6%) reported that they were employed, 415 (46.7%) were unemployed, and 15 (1.7%) were did not respond. The most frequently reported reasons for unemployment were "physical decline" such as fatigue, mobility problems, and "Time commitments for treatment" such as long-term treatment and frequent hospital appointments (Table 3A).

Asked about their willingness to work (Table 3B), over half (56.6%) of the respondents who reported that they were "unemployed" also reported that they had been "unable to work despite [their] willingness to do so." Typical comments included "I want get a job when my physical strength has improved," "I would like to consider getting a job when my systemic pain is relieved," and "I would like to work at home so I can work at my own pace."

Common requests for accommodation included flexible working hours, working at home, and job/workplace modifications. The most common needs at work (Table 3C) were "a workplace that understands my condition" and "employment support." Furthermore, many of the comments on needs at work concerned working/the workplace such as "working conditions

Table 1. The number of respondents and percentage of 889 participants with 57 intractable chronic diseases

Diseases	Number of respondents	%
Systemic lupus erythematosus	195	21.9
Parkinson's disease	101	11.4
Myasthenia gravis	81	9.1
Takayasu arteritis	80	9.0
Sjogren's syndrome	63	7.1
Malignant rheumatoid arthritis (rheumatoid vasculitis)	56	6.3
Retinitis pigmentosa	48	5.4
Spinocerebellar degeneration	44	4.9
Polymyositis-dermatomyositis	35	3.9
Multiple sclerosis	29	3.3
Mixed connective-tissue disease	29	3.3
Ulcerative colitis	23	2.6
Scleroderma, dermatomyositis, or polymyositis	22	2.5
Crohn's disease	20	2.2
Behcet's disease	16	1.8
Ossification of the posterior longitudinal ligament	13	1.5
Periarthritis nodosa	11	1.2
Antiphospholipid syndrome	11	1.2
Moyamoya disease	9	1.0
Chronic inflammatory demyelinating polyneuropathy	9	1.0
Adult-onset Still's disease	7	0.8
Primary pulmonary hypertension	7	0.8
Allergic granulomatous angiitis	5	0.6
Idiopathic chronic pulmonary thromboembolism with pulmonary hypertension	5	0.6
Idiopathic necrosis of the femoral head	4	0.4
Idiopathic osteonecrosis of femoral head	4	0.4
Ossification of the ligamentum flavum	3	0.3
Primary biliary cirrhosis	3	0.3
Hypopituitarism	3	0.3
Shy-Drager syndrome	2	0.2
Amyotrophic lateral sclerosis	2	0.2
Syringomyelia	2	0.2
Striatonigral degeneration	2	0.2
Aplastic anemia	2	0.2
Mitochondrial disease	2	0.2
Autoimmune hepatitis	2	0.2
Wegener's granulomatosis	2	0.2
HTLV-1-associated myelopathy	2	0.2
Guillain-Barré syndrome	1	0.1
Spinal muscular atrophy	1	0.1
Spinal and bulbar muscular atrophy	1	0.1
Lysosomal storage diseases	1	0.1
Subacute sclerosing panencephalitis	1	0.1
Ossification of the anterior longitudinal ligament	1	0.1
Age-related macular degeneration	1	0.1
Sudden sensorineural hearing loss	1	0.1
Idiopathic bilateral sensorineural hearing loss	1	0.1
Syndrome of abnormal secretion of prolactin	1	0.1
Syndrome of abnormal secretion of antidiuretic hormone	1	0.1
Addison's disease	1	0.1
IgA nephropathy	1	0.1
Refractory nephrotic syndrome	1	0.1
Dilated cardiomyopathy, congestive cardiomyopathy	1	0.1
Severe acute pancreatitis	1	0.1
Neurofibromatosis type 1	1	0.1
Neurofibromatosis type 2	1	0.1
Epidermolysis bullosa	1	0.1

where I can feel free to request a short break when I don't feel well," "a workplace where a protocol is in place in case I unexpectedly fall sick," and "I am hesitant to use employment support services because coworkers might begin to resent my occasionally taking time off for treatment and doctor's visits."

3.3. Awareness of employment-related welfare services (EWS)

Providers of EWS offer transition support, *i.e.* job

training/placement, or continued support, *i.e.* job opportunities, for persons with a disability in Japan. Of 889 respondents, 260 (29.2%) reported that they knew of EWS while 611 (68.7%) reported that they have never heard of EWS (Table 4A). Approximately 20% of the respondents who reported that they had "never heard of EWS" were asked if they wanted to know about those services but responded that they had "no need" or "no opinion" (Table 4B). In contrast, more than half of the respondents had a favorable view of EWS. Typical comments were that "Although I don't intend to get a job,

Table 2. Demographics and baseline characteristics of 889 respondents

Characteristics	n = 889	%
Age (mean: 49.5, S.D.: 10.7)		
Sex		
Male	249	28.0
Female	635	71.4
N/A	5	0.6
Families and Living Arrangements (multiple answers allowed)		
living alone	121	13.6
living with spouse or partner	490	55.1
living with parents	265	29.8
living with children, and children-in-law	265	29.8
living with siblings	62	7.0
living with grandparents	18	2.0
living with grandchildren	15	1.7
living with others	31	3.5
Primary caregivers (multiple answers allowed)		
living independently	555	62.4
spouse or partner	172	19.3
parents	87	9.8
children, and children-in-law	33	3.7
siblings	20	2.2
grandparents	1	0.1
grandchildren	0	0.0
home care services (public)	57	6.4
home care services (private)	15	1.7
other	30	3.4
there is no one to ask for help	16	1.8
Ability to go out (multiple answers allowed)		
able to go out alone	736	82.8
need an attendant	145	16.3
need to be dropped off and picked up	104	11.7
other	40	4.5
Current residence		
self/family-owned housing	686	77.2
rented public/private housing	176	19.8
housing for company/government employees	14	1.6
hospital	2	0.2
group home/welfare facility	1	0.1
other	6	0.7
no response	4	0.4
Primary source of income (multiple answers allowed)		
salary, wages, or fees for labor	454	51.1
pension	285	32.1
benefits	47	5.3
welfare payment	24	2.7
allowance from family	20	2.2
business/assets	40	4.5
other	156	17.5

knowing about those services is a good thing," "I don't need the information at this moment, but I may want it if I need it in the future," and "I want to use EWS to find out how long I can work and how much work I can manage."

4. Discussion

This study analyzed 889 responses from individuals with an ID. Responses were collected from patients with 57 different diseases out of designated 130 IDs. To survey patients with the remaining 73 diseases,

Table 3. Reasons for unemployment (A), willingness to work (B), and needs at work (C) among 415 respondents who answered that they were "unemployed"

(A) Response	n = 415*	%
Physical decline	219	52.8
Time commitments for treatment	151	36.4
Unable to find a right job	107	25.8
Concentrating on studying/housekeeping	102	24.6
No need to work	46	11.1
Need ongoing care	39	9.4
Aged	35	8.4
Other	74	17.8
*Multiple answers allowed.		
(B) Response	n = 415*	%
I am unable to work despite my willingness to do so	235	56.6
I don't want to work/I don't need to work	78	18.8
I am seeking a job	44	10.6
Other	25	6.0
No response	33	8.0
(C) Response	n = 415*	%
A workplace that understands my condition: Exemption from physically demanding tasks such as handling heavy objects and after-hours work	232	56
Employment support: Support to find a job that meets my requirements or employers/co-workers are informed about my condition	193	47
Flexible work hours and break time: Permission to adjust work hours, Time off for hospital appointments or care	166	40
Right career/Rewarding career	118	28
Telecommuting	111	27
Barrier-free workplace	87	21
Assistance with travel to work	77	19
Inclusion in the employment quota system	54	13
Work-sharing	44	11
Attendant care in the workplace: suction	17	4
Self-care accommodations in the workplace: stoma care	10	2
Other	20	5
*Multiple answers allowed.		

Table 4. Awareness of employment-related welfare services (EWS) among 889 participants (A), and the desire to know about EWS among 611 respondents who answered that "I've never heard of EWS" (B)

(A) Response	n = 889	%
I knew of EWS	260	29.2
I've never heard of EWS	611	68.7
No response	18	2.0
(B) Response	n = 611	%
I want to know about those services	341	55.8
No need	130	21.3
No opinion	120	19.6
Other	9	1.5
No response	9	1.5

other questionnaire distribution channels, such as medical institutions or academic societies, need to be explored separately. According to representatives of some of the patients' associations, lists of patients' mailing addresses do usually not include patient ages, so the questionnaire could have been unintentionally mailed to patients outside the targeted age range, thus leading to the low response rate.

Approximately half of the respondents were employed at the time of the survey, and half of the unemployed respondents reported difficulties in getting a job despite their willingness to work. The main reasons for their unemployment were "physical decline" and "time commitments for treatment." Analysis of the reasons for unemployment indicated that some of the respondents' perceptions stemmed from a lack of information on the support services available to them. Respondents reported several areas where they desired special accommodations, such as working hours, job tasks, the workplace, and time off for hospital visits/care. These areas coincided with the areas where providers of EWS made special arrangements, as indicated by the survey of those providers in 2013 (12).

Results of the current survey indicated that awareness of EWS is as low as 30%, and respondents were also not sufficiently aware of general welfare services for the disabled, either. Since half of the respondents who had been unaware of those services reported that they wanted to know more about those services, medical/welfare institutions need to have a system that reliably informs patients of available welfare services during their diagnosis and treatment.

Detailed interviews need to be conducted to ascertain the needs of individuals with an ID and the accommodations that providers of EWS actually make in order to recommend what welfare services individuals with an ID need to facilitate their employment in Japan.

In conclusion, an understanding of the perceptions regarding work-related issues and the corresponding support needs of patients with an ID is essential not only for people providing support but for all relevant parties. The results of this study, therefore, are relevant for employees, employers, and occupational health/human resource professionals. Placing an excessive burden on co-workers and employers would result in the loss of employment opportunities for patients with an ID. Thus, the issue of reasonable accommodations for persons with an ID needs to be addressed in future research in order to promote continued work by those persons.

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References

1. Riboldi P, Gerosa M, Luzzana C, Catelli L. Cardiac involvement in systemic autoimmune diseases. *Clin Rev Allergy Immunol.* 2002; 23:247-261.
2. Scherer JR. Inflammatory bowel disease: Complications and extraintestinal manifestations. *Drugs Today (Barc).* 2009; 45:227-241.
3. Haahr A, Kirkevold M, Hall EO, Ostergaard K. Living with advanced Parkinson's disease: A constant struggle with unpredictability. *J Adv Nurs.* 2011; 67:408-417.
4. Doogan C, Playford ED. Supporting work for people with multiple sclerosis. *Mult Scler.* 2014; 20:646-650.
5. Westhoff G, Dörner T, Zink A. Fatigue and depression predict physician visits and work disability in women with primary Sjögren's syndrome: Results from a cohort study. *Rheumatology (Oxford).* 2012; 51:262-269.
6. Murphy R, Tubridy N, Kevelighan H, O'Riordan S. Parkinson's disease: How is employment affected? *Ir J Med Sci.* 2013; 182:415-419.
7. Shahrbanian S, Auais M, Duquette P, Andersen K, Mayo NE. Does pain in individuals with multiple sclerosis affect employment? A systematic review and meta-analysis. *Pain Res Manag.* 2013; 18:e94-100.
8. Ghosh S, Mitchell R. Impact of inflammatory bowel disease on quality of life: Results of the European Federation of Crohn's and Ulcerative Colitis Associations (EFCCA) patient survey. *J Crohns Colitis.* 2007; 1:10-20.
9. Banks P, Lawrence M. The Disability Discrimination Act, a necessary, but not sufficient safeguard for people with progressive conditions in the workplace? The experiences of younger people with Parkinson's disease. *Disabil Rehabil.* 2006; 28:13-24.
10. Kikuchi H, Mifune N, Niino M, Kira J, Kohriyama T, Ota K, Tanaka M, Ochi H, Nakane S, Kikuchi S. Structural equation modeling of factors contributing to quality of life in Japanese patients with multiple sclerosis. *BMC Neurol.* 2013; 13:10.
11. Nasu A, Yamada K, Morioka I. Difficulties at work and work motivation of ulcerative colitis sufferers. *Sangyo Eiseigaku Zasshi.* 2015; 57:9-18. (in Japanese)
12. Fukatsu R, Imahashi K, Nakajima Y, Ito T, Horigome M, Haruna Y, Noda T, Itoyama Y. Research on utilization of National Employment Welfare Service by persons with intractable diseases in Japan. *LIFE: Int J Health Sci.* 2015; S1:172-179.

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Alcohol use dependence in fragile X syndrome

María J Salcedo-Arellano^{1,2,3,*}, Reymundo Lozano⁴, Flora Tassone^{3,5}, Randi J Hagerman^{3,6}, Wilmar Saldarriaga^{1,2,7}

¹ School of Medicine, Universidad del Valle, Cali, Colombia;

² Research Group in Congenital & Perinatal Malformations, Dysmorphology and Clinical Genetics (MACOS), Universidad del Valle, Cali, Colombia;

³ Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, University of California, Davis Medical Center, Sacramento, CA, USA;

⁴ Seaver Autism Center. Departments of Genomic Sciences, Psychiatry and Pediatrics. Icahn School of Medicine, Mount Sinai Hospital, New York, NY, USA;

⁵ Department of Biochemistry and Molecular Medicine, University of California, Davis, USA;

⁶ Department of Pediatrics, University of California, Davis Medical Center, Sacramento, CA, USA;

⁷ Departments of Morphology and Obstetrics & Gynecology, Universidad del Valle, Hospital Universitario Del Valle, Cali, Colombia.

Summary

Alcohol use disorders (AUDs) have been reported in a limited number of individuals with cognitive impairment but rarely in those with fragile X syndrome (FXS). However, in Colombia, culturally, alcohol consumption is very common. Here, we report eight cases of patients with FXS who have frequent alcohol consumption in Ricaurte, Colombia. Some of these patients have also used tobacco and illegal substances, including cocaine, which use has not been previously reported in those with FXS. Alcohol and substance use dependence is associated with exacerbation of their behavioral problems, such as increased impulsivity and aggression, as well as of medical problems such as an increased frequency of seizures.

Keywords: Alcohol use disorders, fragile X syndrome

1. Introduction

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability (ID) and the most common single gene cause of Autism Spectrum Disorder (ASD) (1). The prevalence is approximately 1 per 5000 in the general population (2), although in Colombia, South America the prevalence is higher than in other parts of the world (3). A very high prevalence of FXS has been found in a small agricultural rural village, Ricaurte, which is close to Cali and this is thought to relate to a founder effect from the Conquistadores originally from Extremadura region of Spain. In Ricaurte, with a population of just under 1,500 people, there are approximately 20 adults with

FXS. There are only two main institutions in Ricaurte, the church and the bar where socialization occurs and the individuals with FXS often go to the bar. Many individuals with FXS also work in the fields where it is the custom to drink alcohol.

Alcoholism has rarely been described in those with FXS in the US and in Europe, although occasional cases have been reported in higher functioning individuals with FXS (4). Those with ID or ASD and FXS usually do not frequent bars and those that take care of individuals with FXS typically do not allow the use of alcohol. However, in Colombia particularly in this village those with FXS usually decide for themselves where to go and the social center of the village is the local bar. The use of alcohol is part of the culture in Colombia and the use of cocaine products is a frequent problem because it is easy to obtain. Thus, although alcoholism has only rarely been reported in individuals with FXS, it is common in Ricaurte, Colombia.

Alcohol use disorders (AUDs) are associated with an approximate 6-fold increase in all-cause mortality and is associated with behavior dysregulation including

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*Address correspondence to:

Dr. María J Salcedo-Arellano, School of Medicine, Universidad del Valle, Cali, Colombia.

E-mail: jimena.salcedo@gmail.com

an increase in aggression (5). In this study, we report eight cases of frequent alcohol consumption, six of them meeting criteria for alcoholic dependence syndrome in adult males with FXS. Three additional individuals with FXS that we encountered have had alcohol consumption problems in the past, however they do not consume at present because of family interventions.

2. Patients and Methods

This study was conducted from January, 2016 to May, 2016. The protocol was reviewed and approved by the Ethical Committee of Universidad del Valle; informed consent was provided by the legal guardian of each of the participants. All 20 FXS patients in Ricaurte had DNA testing for *FMR1* mutations as previously described (6). All of them had their medical history gathered and their physical examination performed by a member of the research team. We used the DSM-5 criteria to define the severity of ID on the basis of adaptive functioning and not IQ scores, classifying each patient into mild, moderate, severe or profound ID. By using a clinician-administered version of The Alcohol Use Disorders Identification Test (AUDIT), a screening tool developed by the World Health Organization (WHO) derived from a cross-national data set, we assessed three domains: hazardous alcohol use, dependence symptoms, and harmful alcohol use (7,8). We interviewed a family member that resides in the same house with each of the reported cases, since a self-report version of the AUDIT could not be used due to the patients' intellectual disabilities and lack of reading faculties. We found that 8 out of the 20 FXS in Ricaurte drink alcohol regularly; the data pertinent to substance use is included under each of the cases we report. Following the assessment of the AUDIT we applied the International Classification of Diseases (ICD-10) (WHO) criteria (9) for the alcohol dependence syndrome (ADS) to those scoring 8 or more points in the AUDIT obtaining that 6 of 8 met criteria for alcohol dependence syndrome. See Table 1 for results of the AUDIT.

Case 1, a 27-year-old Hispanic male with a full mutation of > 200 CGG repeats. He has a long history of significant alcohol, marijuana and bazuco (cocaine paste) abuse, starting approximately ten years ago. Cocaine paste is a crude extract of the coca leaf which contains cocaine sulfate along with coca alkaloids and varying quantities of benzoic acid, kerosene and methanol. It is inexpensive and therefore widely used by low-income populations in Colombia. He has also a medical history of behavioral disorders including severe anxiety, angry outbursts and impulsivity, experiencing repeated aggression episodes. At present he is managed with pipotiazine 100 mg, a long lasting antipsychotic, every two weeks. He has severe intellectual disability

and his verbal language is very limited. However, family members report him to also be involved in risky sexual encounters with males and homosexual tendencies. After alcohol and drug use he experiences episodes of uncontrollable rage and turns violent with family members. During his medical evaluation he reports hematochezia as a new symptom. He scored 22/40 on the AUDIT and met criteria for ICD-10 ADS (Table 2).

Case 2, a 40-year-old Hispanic male with a full mutation of > 200 CGG repeats. He has a long history of significant alcohol and tobacco abuse that started when he was 18 years old. He prefers to consume alcohol every other day to drunkenness and smokes two packs of cigarettes daily. He does not report any pathologic or surgical history, however reports to have experienced hematemesis once after an alcohol intake. He has a moderate ID, partially attended first grade only but he holds a fair verbal language skill. He works as a farmer. His family members state he is involved in unprotected sexual encounters with prostitutes associated with alcohol intake; he scored 17/40 on the AUDIT and met criteria for ICD-10 ADS (Table 2).

Case 3, a 26-year-old Hispanic male with a full mutation of > 200 CGG repeats. He experiences impulsivity, anxiety and hyperactivity. At present he takes risperidone 2 mg/day. However, his mother says that he sometimes refuses his medication. He has a moderate ID and good verbal communication skills; when interviewed he reported drinking alcohol two or three times a week. He does not smoke or consume illegal drugs. He has never shown aggression or apparent secondary effects associated to drinking, he scored 5/40 on the AUDIT which did not meet cutoff criteria for ADS of 8/40 (Table 2).

Case 4, a 32-year-old Hispanic male with a full mutation of > 200 CGG repeats. He has a history of seizures from childhood. He currently uses carbamazepine 200 mg/day and valproic acid 1 gram/day. He has a moderate ID and works as a helper in a cattle ranch. He had a long history of heavy alcohol consumption that started during his teenage years and stopped a year ago. His family had to intervene by contacting his friends and acquaintances and requesting them not to provide him with liquor since he experienced more frequent seizure episodes while under the influence of alcohol as well as angry outbursts and aggression. He scored 15/40 on the AUDIT and met criteria for ICD-10 ADS (Table 2).

Case 5, a 68-year-old Hispanic male with a full mutation of > 200 CGG repeats. He has a severe ID, meets criteria for ASD and has functional limitations in effective communication. Medical history does not report any pathology or surgical procedure. Family members describe moderate alcohol consumption, about twice a week. However, they cannot precisely say how many drinks he takes because he drinks the

Table 1. The alcohol use disorders identification test (AUDIT)

Items	C1	C2	C3	C4	C5	C6	C7	C8
1. How often do you have a drink containing alcohol? (0) Never (1) Monthly or less (2) 2 to 4 times a month (3) 2 to 3 times a week (4) 4 or more times a week	3	3	3	3	3	2	2	2
2. How many drinks containing alcohol do you have on a typical day when you are drinking? (0) 1 or 2 (1) 3 or 4 (2) 5 or 6 (3) 7, 8, or 9 (4) 10 or more	4	4	1	2	4	4	2	1
3. How often do you have six or more drinks on one occasion? (0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily	4	3	1	1	3	3	1	2
4. How often during the last year have you found that you were not able to stop drinking once you had started? (0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily	3	3	0	3	3	3	3	0
5. How often during the last year have you failed to do what was normally expected from you because of drinking? ** (0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily	-	-	-	-	-	-	-	-
6. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session? (0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily	0	0	0	0	0	0	0	0
7. How often during the last year have you had a feeling of guilt or remorse after drinking? (0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily	0	0	0	0	0	0	0	0
8. How often during the last year have you been unable to remember what happened the night before because you had been drinking? ** (0) Never (1) Less than monthly (2) Monthly (3) Weekly (4) Daily or almost daily	-	-	-	-	-	-	-	-
9. Have you or someone else been injured as a result of your drinking? (0) No (2) Yes, but not in the last year (4) Yes, during the last year	4	4	0	2	0	2	0	2
10. Has a relative or friend or a doctor or another health worker been concerned about your drinking or suggested you cut down? (0) No (2) Yes, but not in the last year (4) Yes, during the last year	4	4	0	4	0	2	0	0
Total score	22/40	21/40	5/40	15/40	13/40	16/40	8/40	7/40

**Questions 5 and 8 could not be answered. Question 5: the cases do not hold a steady job or other responsibilities to be measured by this question. Question 8: the patients were unable to understand the question and it could not be answered by a family member.

left overs of other people at the bar, and this behavior goes on for as long as the bar stays open during the weekend. He does not experience aggressive behavior with the consumption but he becomes more anxious

with alcohol intake. He scored 13/40 on the AUDIT and met criteria for ICD-10 ADS. At the time of the medical examination a heart arrhythmia was found and a mucosal wound that his family says he has had for

Table 2. Patients with FXS (> 200 CGG repeats)



Case 1. A 27-year-old male has severe intellectual disability and meets criteria for ICD-10 alcohol dependence syndrome. After alcohol and drug use he experiences increased aggression and impulsivity.



Case 2. A 40-year-old male with a long history of significant alcohol and tobacco abuse. He has a moderate intellectual disability and meets criteria for ICD-10 alcohol dependence syndrome. He experiences exacerbation of his behavioral problems and medical issues related to alcohol consumption.



Case 3. A 26-year-old male has a moderate intellectual disability and has never shown aggression or apparent secondary effects associated to drinking.



Case 4. A 32-year-old male with a history of epilepsy. He has a moderate intellectual disability and meets criteria for ICD-10 alcohol dependence syndrome. He experiences more frequent seizure episodes while under the influence of alcohol and increased aggression.



Case 5. A 68-year-old male has a severe intellectual disability, meets criteria for autism spectrum disorder and alcohol dependence syndrome. He experiences increased anxiety with alcohol intake.



Case 6. A 57-year-old male has a moderate intellectual disability and meets criteria for ICD-10 alcohol dependence syndrome. He has had two arm fractures as a result of falling while drinking.



Case 7. A 67-year-old male has a severe intellectual disability, meets criteria for autism spectrum disorder and alcohol dependence syndrome.



Case 8. A 47-year-old male has a moderate intellectual disability and a history of epilepsy. Currently seizures occur only with alcohol consumption. He does not meet criteria for alcohol dependence syndrome.

over a year (Table 2).

Case 6, a 57-year-old Hispanic male with a full mutation of > 200 CGG repeats. He has a moderate ID and meets criteria for ASD. His verbal language has limited sentence structure and is very repetitive. He is not followed by a physician and he has never been hospitalized. His mother describes moderate alcohol intake every weekend. He has had two arm fractures as a result of falling while drinking. During the medical examination he was found to have hypertension. He scored 16/40 on the AUDIT and met criteria for ICD-10 ADS (Table 2).

Case 7, a 67-year-old Hispanic male with a full mutation of > 200 CGG repeats. He has severe ID and meets criteria for ASD. His verbal language has very reduced vocabulary. He lacks family support and does not have any medical records. He works in agriculture. At medical examination a fungal skin infection, compatible with tinea versicolor, was found that covered approximately 50% of his body surface. Using basic language he says he drinks alcohol regularly and his friends confirm his statements. He scored 8/40 on the AUDIT and met criteria for ICD-10 ADS (Table 2).

Case 8, a 47-year old Hispanic male with a full mutation of >200 CGG repeats. He has moderate ID and deficits in the comprehension and verbal communication. He was diagnosed with epilepsy at the age of 17, currently using phenobarbital 3 mg/kg/day; keeping seizures under control, occurring only related to drinking. His medical records report divergent strabismus in his left eye and a cataract surgery performed in his right eye. In addition, he suffered an arm fracture while consuming alcohol two years ago. No symptoms were reported during the medical evaluation. He referred weekly low/moderate alcohol consumption. Scoring 7/40 on the AUDIT, which does not meet criteria for ADS (Table 2).

3. Results and Discussion

In this study, we report that 8 of 20 men with FXS, who were evaluated in Ricaurte, had significant alcohol consumption and 6 of them met criteria for alcohol dependence syndrome. The high rate of alcoholism in men with FXS in Ricaurte Colombia is likely related to several issues. The culture in this region includes the use of alcohol with social gatherings and the main area to gather is the bar in this small village or in the fields where many individuals work. There is a lack of medical care in this region so psychiatric medications are usually not available, whereas alcohol can dull the most common behavioral problem of FXS, specifically anxiety and hyperactivity. However, the consequences of alcohol use can include an increase in aggression which is a problem in approximately 30% of those with FXS and many of these cases have had aggression and falls with alcohol consumption.

Agriculture is the base of the economy in the region and most of the people of Ricaurte work in the fields, including those with intellectual disabilities who can function independently. Farmers have been associated with alcohol abuse in many rural regions of the world including Australia (10), India (11), Africa (12,13), China (14), North America (15) and the same is true in Colombia.

Childhood learning disorders or ID is also seen in fetal alcohol syndrome when the mother consumes alcohol during pregnancy which may have exacerbated some of the ID seen in the cases reported here although we did not obtain this history in the cases above. It is also known that individuals with ADHD tend abuse the use of alcohol and other substances (16) and typically all the cases of FXS have ADHD.

Slyater 2010 (17), reviewed Medicaid healthcare billing claims and concluded that 2.6% of all people with ID had a diagnosable substance abuse disorder. Other estimates using different methodologies vary widely, ranging as high as 26% (18). Individuals with ID who are substance abusers share some traits. They tend to begin drinking alcohol a couple years later than their peers without ID, they are less likely to be Caucasian (17) and they are less likely to seek help for their problem. Additionally, this group is at greater risk of complications from drinking because they tend to be prescribed medications for other conditions, such as seizures, metabolic disorders, and co-occurring mental illness that might negatively interact with alcohol and drugs. No previous reports have reported or reviewed the prevalence of alcoholism in FXS.

Kendler *et al.* 2016 observed that heritable factors contributed in AUD mortality in early to middle adulthood (5). Also, Wrase *et al.* 2008 observed alterations of cortical and limbic structures in individuals during the development of alcohol dependence (19); these structures are closely related with emotions and decision making (20,21). Such alterations combined with genetic behavioral factors found in individuals with FXS, such as impulsivity, could contribute to drug dependence (22) as well as the involvement in risk-taking sexual behavior observed in some of the cases we report. In addition, elevated rates of alcohol-associated aggression are found in individuals with AUD (23) and may lead to fatal outcomes. On the other hand, long-term alcohol use together with increasing age, tobacco use, poor nutrition and absent health care are furnishing factors for high alcohol-associated mortality rates in late adulthood (5,24).

In FXS, the silencing of the *FMR1* gene results in the absence of FMRP causing the over expression of many post synaptic proteins and upregulation of the mGluR5 pathway which can also predispose to substance abuse (25,26). The lack of inhibition of the glutamatergic signaling, especially the dysfunction of mGluR1 and mGluR5 in the absence of FMRP, has been

also related to abnormal dendritic morphology, reduced seizure threshold, excess hippocampal and cerebellar long term depression (LTD) and excess a-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptor internalization found in *fmr1* knockout animal model (27). This glutamatergic dysfunction is proposed to be involved in the pathophysiology of FXS. Many of the clinical findings in FXS patients including ID, anxiety, seizures and repetitive behaviors among other characteristics can be secondary to diminished inhibitory control of mGluR (27,28,29). Furthermore, mGluR5 signaling is involved in behavioral actions of ethanol including alcohol-induced withdrawal (AW), sedative effect and impaired motor activity (30).

In this report we describe several cases of FXS who are alcoholic likely because of social factors but also predisposed to alcoholism from intrinsic behavioral and molecular factors. Since alcoholism is associated with many health problems (5) in addition to exacerbating behavioral problems such as aggression and impulsivity leading to self-injury as described here. Then it is critical to treat this problem. Although an alcohol treatment program can be effective (31) and there is an urge to find improved medications to treat alcohol dependence (32) this may not be available in a rural, poor setting such as seen in Ricaurte. Acamprosate, an FDA-approved drug for the maintenance of abstinence from alcohol use in adults is also a targeted treatment for FXS (33). Acamprosate has pleiotropic effects impacting glutamate and GABA neurotransmission (34). Its mGluR5 antagonist effect has been demonstrated in animal models of alcoholism (30,35,36) followed by two pilot clinical trials conducted by Erickson *et al.* 2011 and 2013 in patients with FXS + ASD finding that 75% of the sample responded to treatment exhibiting improvement in social behavior, hyperactivity and communication skills (33). This medication should be studied further not only in those with FXS but also to treat the alcoholism that can occur in this disorder.

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References

1. Hagerman R, Hoem G, Hagerman P. Fragile X and autism: Intertwined at the molecular level leading to targeted treatments. *Mol Autism*. 2010; 1:12.
2. Tassone F, Iong KP, Tong TH, Lo J, Gane LW, Berry-Kravis E, Nguyen D, Mu LY, Laffin J, Bailey DB, Hagerman RJ. *FMR1* CGG allele size and prevalence

- ascertained through newborn screening in the United States. *Genome Med.* 2012; 4:100.
3. Saldarriaga W, Lein P, González Teshima LY, Isaza C, Rosa L, Polyak A, Hagerman R, Girirajan S, Silva M, Tassone F. Phenobarbital use and neurological problems in FMR1 premutation carriers. *Neurotoxicology.* 2016; 53:141-147.
 4. Loesch DZ, Sherwell S, Kinsella G, Tassone F, Taylor A, Amor D, Sung S, Evans A. Fragile X-associated tremor/ataxia phenotype in a male carrier of unmethylated full mutation in the FMR1 gene. *Clin Genet.* 2012; 82:88-92.
 5. Kendler KS, Ohlsson H, Sundquist J, Sundquist K. Alcohol use disorder and mortality across the lifespan: A longitudinal cohort and co-relative analysis. *JAMA Psychiatry.* 2016; 73:575-581.
 6. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, Hagerman PJ, Stenzel TT, Hadd AG, Latham GJ, Tassone F. A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. *Clin Chem.* 2010; 56:399-408.
 7. Babor TF, Biddle-Higgins JC, Saunders JB, Monteiro MG. AUDIT: The Alcohol Use Disorders Identification Test: Guidelines for Use in Primary Health Care. Geneva, Switzerland: World Health Organization. 2001. <http://www.who.int/iris/handle/10665/67205> (accessed January 23, 2016).
 8. Saunders JB, Aasland OG, Babor TF, de la Fuente JR, Grant M. Development of the Alcohol Use Disorders Screening Test (AUDIT). WHO collaborative project on early detection of persons with harmful alcohol consumption II. *Addiction.* 1993; 88:791-804.
 9. World Health Organization. The ICD-10 Classification of Mental and Behavioral Disorders: Diagnostic criteria for research 1993. <http://www.who.int/iris/handle/10665/37108> (accessed January 23, 2016).
 10. Brumby SA, Kennedy AJ, Mellor D, McCabe MP, Ricciardelli LA, Head A, Mercer-Grant C. The Alcohol Intervention Training Program (AITP): A response to alcohol misuse in the farming community. *BMC Public Health.* 2011; 11:242.
 11. Shidhaye R, Gangale S, Patel V. Prevalence and treatment coverage for depression: A population-based survey in Vidarbha, India. *Soc Psychiatry Psychiatr Epidemiol.* 2016. DOI: 10.1007/s00127-016-1220-9.
 12. Chasimpha S, McLean E, Chihana M, Kachiwanda L, Koole O, Tafatatha T, Mvula H, Nyirenda M, Crampin AC, Glynn JR. Patterns and risk factors for deaths from external causes in rural Malawi over 10 years: A prospective population-based study. *BMC Public Health.* 2015; 15:1036.
 13. Akangbe JA, Asiyambi AO, Ibraheem AF, Animashaun JO, Sola KE. An assessment of health practices among small-scale farmers in Kwara State, Nigeria. *Ann Agric Environ Med.* 2015; 22:488-490.
 14. Wang L, Wheeler K, Bai L, Stallones L, Dong Y, Ge J, Xiang H. Alcohol consumption and work-related injuries among farmers in Heilongjiang Province, People's Republic of China. *Am J Ind Med.* 2010; 53:825-835.
 15. Hsieh HH, Cheng SC, Sharma A, Sanders RA, Thiessen C. The relation of rural alcoholism to farm economy. *Community Ment Health J.* 1989; 25:341-347.
 16. Rhodes SS, Jasinski DR. Learning disabilities in alcohol-dependent adults: A preliminary study. *J Learn Disabil.* 1990; 23:551-556.
 17. Slayter EM. Demographic and clinical characteristics of people with intellectual disabilities with and without substance abuse disorders in a Medicaid population. *Intellect Dev Disabil.* 2010; 48:417-431.
 18. Strain EC, Buccino DL, Brooner RK, Schmidt CW, Bigelow GE. The triply diagnosed: Patients with major mental illness, cognitive impairment, and substance abuse. *J Nerv Ment Dis.* 1993; 181:585-587.
 19. Wrase J, Makris N, Braus DF, *et al.* Amygdala Volume Associated With Alcohol Abuse Relapse and Craving. *Am J Psychiatry.* 2008; 165:1179-1184.
 20. Bechara A, Damasio H, Damasio AR, Lee GP. Different contributions of the human amygdala and ventromedial prefrontal cortex to decision-making. *J Neurosci.* 1999; 19:5473-5481.
 21. Winstanley CA, Theobald DE, Cardinal RN, Robbins TW. Contrasting roles of basolateral amygdala and orbitofrontal cortex in impulsive choice. *J Neurosci.* 2004; 24:4718-4722.
 22. Makris N, Gasic GP, Seidman LJ, *et al.* Decreased absolute amygdala volume in cocaine addicts. *Neuron.* 2004; 44:729-740.
 23. Heinz AJ, Beck A, Meyer-Lindenberg A, Sterzer P, Heinz A. Cognitive and neurobiological mechanisms of alcohol-related aggression. *Nat Rev Neurosci.* 2011; 12:400-413.
 24. Heinz A, Beck A, Rapp MA. Alcohol as an environmental mortality hazard. *JAMA Psychiatry.* 2016; 73:549-550.
 25. Kumaresan V, Yuan M, Yee J, Famous KR, Anderson SM, Schmidt HD, Pierce RC. Metabotropic glutamate receptor 5 (mGluR5) antagonists attenuate cocaine priming- and cue-induced reinstatement of cocaine seeking. *Behav Brain Res.* 2009; 202:238-244.
 26. Quintero GC. Role of nucleus accumbens glutamatergic plasticity in drug addiction. *Neuropsychiatr Dis Treat.* 2013; 9:1499-1512.
 27. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. *Trends Neurosci.* 2004; 27:370-377.
 28. Erickson CA, Mullett JE, McDougale CJ. Brief report: Acamprosate in fragile X syndrome. *J Autism Dev Disord.* 2010; 40:1412-1416.
 29. Saldarriaga W, Tassone F, González-Teshima LY, Forero-Forero JV, Ayala-Zapata S, Hagerman R. Fragile X syndrome. *Colomb Med (Cali).* 2014; 45:190-198.
 30. Blednov YA, Harris RA. Metabotropic glutamate receptor 5 (mGluR5) regulation of ethanol sedation, dependence and consumption: Relationship to acamprosate actions. *Int J Neuropsychopharmacol.* 2008; 11:775-793.
 31. Hagedorn HJ, Brown R, Dawes M, Dieperink E, Myrick DH, Oliva EM, Wagner TH, Wisdom JP, Harris AH. Enhancing access to alcohol use disorder pharmacotherapy and treatment in primary care settings: ADaPT-PC. *Implement Sci.* 2016; 11:64.
 32. Heilig M, Goldman D, Berrettini W, O'Brien CP. Pharmacogenetic approaches to the treatment of alcohol addiction. *Nat Rev Neurosci.* 2011; 12:670-684.
 33. Erickson CA, Wink LK, Ray B, Early MC, Stieglmeier E, Mathieu-Frasier L, Patrick V, Lahiri DK, McDougale CJ. Impact of acamprosate on behavior and brain-derived neurotrophic factor: An open-label study in youth with fragile X syndrome. *Psychopharmacology (Berl).* 2013; 228:75-84.
 34. Mann K, Kiefer F, Spanagel R, Littleton J. Acamprosate:

- Recent findings and future research directions. *Alcohol Clin Exp Res.* 2008; 32:1105-1110.
35. Harris BR, Prendergast MA, Gibson DA, Rogers DT, Blanchard JA, Holley RC, Fu MC, Hart SR, Pedigo NW, Littleton JM. Acamprosate inhibits the binding and neurotoxic effects of trans-ACPD, suggesting a novel site of action at metabotropic glutamate receptors. *Alcohol Clin Exp Res.* 2002; 26:1779-1793.
36. Gupta T, Syed YM, Revis AA, Miller SA, Martinez M, Cohn KA, Demeyer MR, Patel KY, Brzezinska WJ, Rhodes JS. Acute effects of acamprosate and MPEP on ethanol Drinking-in-the-Dark in male C57BL/6J mice. *Alcohol Clin Exp Res.* 2008; 32:1992-1998.

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Novel *SLC16A2* mutations in patients with Allan-Herndon-Dudley syndrome

Keiko Shimojima¹, Koichi Maruyama², Masahiro Kikuchi³, Ayako Imai³, Ken Inoue⁴, Toshiyuki Yamamoto^{1,*}

¹ Tokyo Women's Medical University Institute for Integrated Medical Sciences, Tokyo, Japan;

² Department of Pediatrics, Central Hospital, Aichi Human Service Center, Kasugai, Japan;

³ Department of Pediatrics, Hitachi General Hospital, Hitachi, Japan;

⁴ National Institute of Neuroscience, National Center for Neurology and Psychiatry, Kodaira, Japan.

Summary

Allan-Herndon-Dudley syndrome (AHDS) is an X-linked disorder caused by impaired thyroid hormone transporter. Patients with AHDS usually exhibit severe motor developmental delay, delayed myelination of the brain white matter, and elevated T3 levels in thyroid tests. Neurological examination of two patients with neurodevelopmental delay revealed generalized hypotonia, and not paresis, as the main neurological finding. Nystagmus and dyskinesia were not observed. Brain magnetic resonance imaging demonstrated delayed myelination in early childhood in both patients. Nevertheless, matured myelination was observed at 6 years of age in one patient. Although the key finding for AHDS is elevated free T3, one of the patients showed a normal T3 level in childhood, misleading the diagnosis of AHDS. Genetic analysis revealed two novel *SLC16A2* mutations, p.(Gly122Val) and p.(Gly147Arg), confirming the AHDS diagnosis. These results indicate that AHDS diagnosis is sometimes challenging owing to clinical variability among patients.

Keywords: Thyroid function, delayed myelination, monocarboxylate transporter 8 (MCT8)

1. Introduction

Allan-Herndon-Dudley syndrome (AHDS; MIM #300523) is an X-linked disorder caused by impaired thyroid hormone transporters (monocarboxylate transporter 8: MCT8) (1). Patients with this syndrome exhibit severe motor developmental delay and radiological examination using magnetic resonance imaging (MRI) reveals delayed maturation of brain white matter (2,3). An abnormal thyroid hormone profile is another key finding for clinical diagnosis of AHDS (1). However, thyroid hormone abnormalities are not common among AHDS patients (4). The severity of delayed myelination in patients is also variable (5).

Therefore, clinical diagnosis of patients is sometimes challenging, and the disorder is often under-diagnosed. A final and complete diagnosis requires detection of a mutation in the solute carrier family 16 member 2 gene (*SLC16A2*) (5). This requirement also contributes to under-diagnosis.

Previously, we identified some *SLC16A2* mutations (6,7). In this study, we identified a new *SLC16A2* mutation in additional patients.

2. Patients and Methods

2.1. Patients

Case report 1 A 19-year-old male patient is a third-born child and had a birth weight of 2,940 g (25-50th centile). His elder brother (II-2) showed developmental delay from infancy, and required a tracheotomy at 12 years of age due to respiratory dysfunction (Figure 1A). He died suddenly at 14 years of age owing to bleeding from a trachea brachiocephalic artery fistula. The patient's elder sister is healthy and has a healthy son. The

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*Address correspondence to:

Dr. Toshiyuki Yamamoto, Tokyo Women's Medical University Institute for Integrated Medical Sciences, 8-1 Kawada-cho, Shinjuku-ward, Tokyo 162-8666, Japan.

E-mail: yamamoto.toshiyuki@twmu.ac.jp

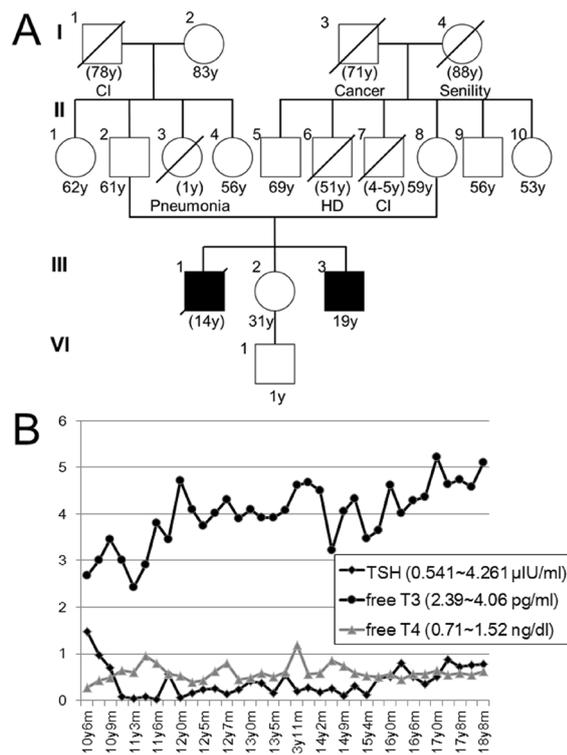


Figure 1. Clinical information for patient 1. (A) Family tree of patient 1. CI; cerebral infarction, HD; heart disease. **(B)** Serial results of thyroid hormone examinations in patient 1. Normal ranges are shown on the left. Free T3 gradually increased.

paternal aunt and the maternal uncle died in childhood from unknown etiology.

At 5 months of age, developmental delay was suggested due to head lagging. Subsequently, he showed failure to thrive as evidenced by his weight, which was consistently under the 3rd centile (7,150 g at 12 months old, 7,950 g at 3 years old, 8,420 g at 6 years old, 8,435 g at 9 years old, 10.2 kg at 12 years old, and 12.0 kg at 19 years old). Gross motor development was severely delayed and he was bedridden. His maximum motor ability was rolling over to the right lateral decubitus position. Developmental deterioration was not noted. At the age of 9 years, tube feeding was initiated owing to insufficient water intake. At 19 years old, he received a tracheostomy owing to recurrent respiratory infections. At night, he used a respirator. At this age, a gastric fistula was constructed.

At 10 years, a low free T4 level of 0.28 ng/dL (normal range: 0.71-1.52 ng/dL) was noted. Thyroid stimulating hormone (TSH) of 1.478 μIU/mL (normal range: 0.541-4.261 μIU/mL) and free T3 of 2.67 pg/mL (normal range: 2.39-4.06 pg/mL) were within normal limits. Then, thyroid hormone was prescribed and TSH level was decreased. Free T3 level was gradually increased from 12 years old (Figure 1B). Brain MRIs were regularly examined (Figures 2A-F). At 2 years and 6 months of age, subcortical white matter showed a high T2 signal (Figures 2A and 2B), indicating delayed myelination.

Since then, dilatation of the lateral ventricles and extracerebral spaces were observed (Figure s2E and 2F).

At present, his height is 129 cm (< 3rd centile) and his weight is 12 kg (< 3rd centile). He shows emotional reactions, characterized by various expressions, to environmental stimuli, but does not convey meaningful words or gestures. He occasionally nods in question and vocalizes during songs. He still shows head lagging. Neurological examination indicates generalized hypotonia. His upper and lower extremities show flexion and extension with scoliosis and knee contractures.

Case report 2 A 6-month-old male baby is the first born to healthy parents by emergency caesarian section at 41 weeks of gestation, with a birth weight of 3,820 g (> 97th centile). Apgar score was 8/8 at 1/5 minutes. His mother had a past history of spontaneous abortion. Family history was not remarkable. In early infancy, poor sucking was noted. At 6 months, owing to uncontrolled head, he was referred to the hospital. At that time, his weight was 7.75 kg (25-50th centile), length was 66.2 cm (10-25th centile), and occipitofrontal circumference was 44.2 cm (50-75th centile). Neurological examination showed generalized hypotonia. There was no nystagmus. Blood examination showed elevated TSH (4.720 μIU/mL) and free T3 (10.74 pg/mL) and a low level of free T4 (0.59 ng/dL). Conventional G-banding showed normal male karyotype of 46,XY. Brain MRI showed remarkable hypomyelination and reduced volume of the cerebrum (Figures 2G, H). Auditory brainstem response showed normal latency time. Due to poor sucking, tube feeding was initiated.

2.2. Genetic examination

A genetic study was performed in accordance with the Declaration of Helsinki and approval by the Ethics Committee at the Tokyo Women's Medical University. Based on clinical and radiological examinations, we suspected AHDS as a candidate diagnosis for both patients. After obtaining written informed consent from the patient's family, Sanger sequencing of the *SLC16A2* coding exons was performed and single nucleotide alterations were identified; for patient 1 in exon 1: NM_006517.4 (*SLC16A2_v001*):c.365G>T [p.(Gly122Val)] and for patient 2 in exon 2: NM_006517(*SLC16A2_v001*):c.439G>A [p.(Gly147Arg)]. Both variants were not reported in the Human Genetic Variation Database (<http://www.genome.med.kyoto-u.ac.jp/SnpDB>). The affected amino acids were conserved among species (Figures 3A and 3B). Functional effects of these variants were predicted using Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (http://sift.jcvi.org/www/SIFT_help.html), CADD (<http://cadd.gs.washington.edu/info>), and MutationTaster (<http://www.mutationtaster.org/>). All scores predicted a damaging effect. Because the identified two mutations were not reported previously, those were considered as

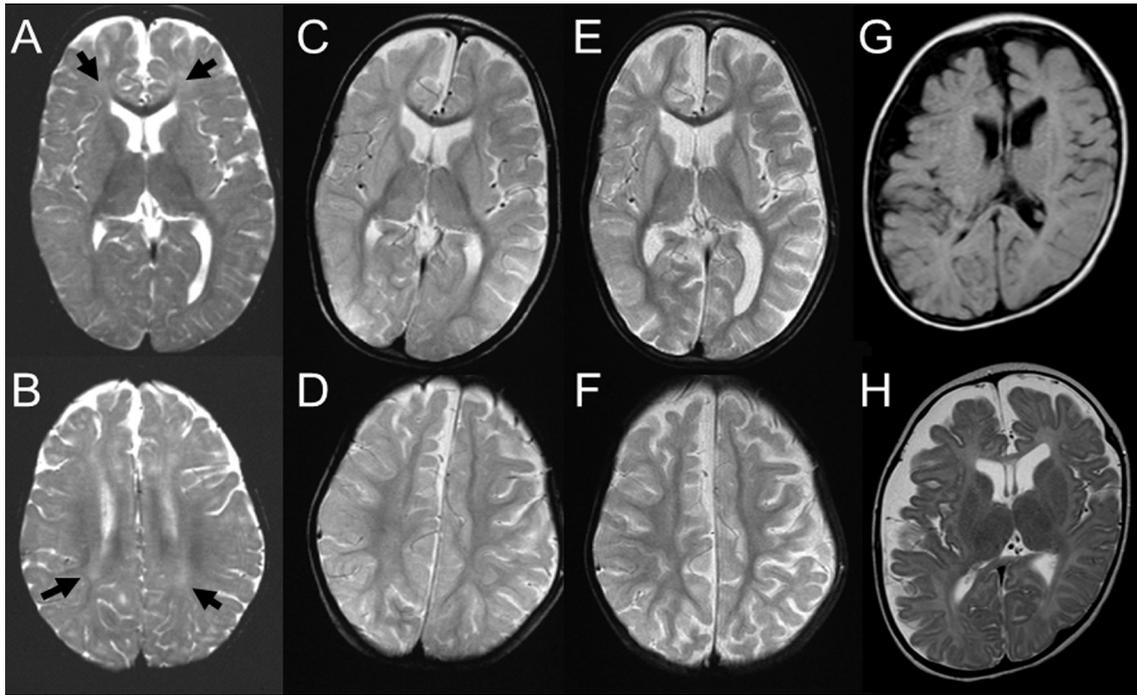


Figure 2. Brain magnetic resonance imaging. (A-F) Serial T2-weighted axial images for patient 1. Delayed myelination is demonstrated at 2 years and 6 months for the deep white matter (A, B; arrows). At 6 years and 1 month, myelination is mature (C, D). Diffuse brain atrophy is exhibited at 7 years and 8 months (E, F). (G, H) Axial images for patient 2 examined at 6 months. T1(G)- and T2(H) axial images demonstrate hypomyelination and brain volume loss.

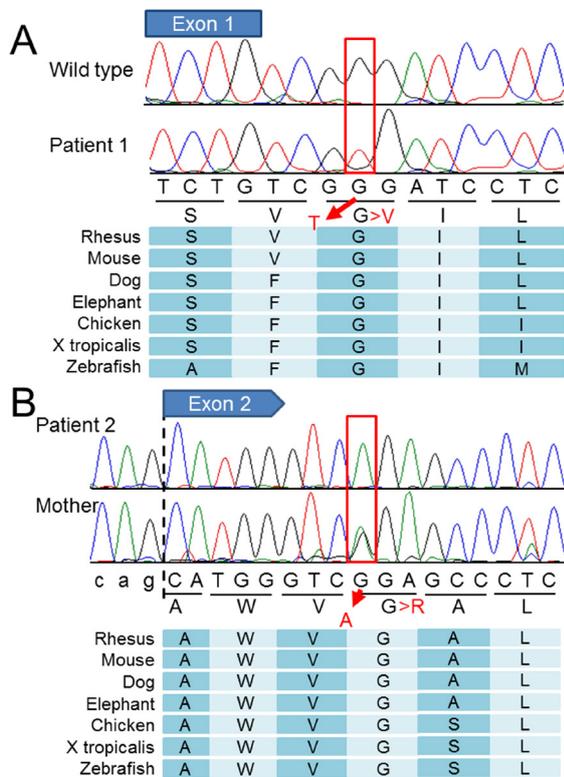


Figure 3. Results of Sanger sequencing and conservation among species. (A) Electropherogram demonstrates hemizygous G>T nucleotide change in patient 1. The affected codon is conserved among species (below). (B) Electropherogram demonstrates hemizygous G>A mutation in patient 2. His mother is heterozygous for the mutation, indicating an obligate carrier. The affected codon is conserved among species (bottom).

novel mutations (8).

The mother of patient 2 was heterozygous for the mutation, indicating an obligate carrier. The mother of patient 1 declined to be genotyped.

3. Results and Discussion

In this study, neurological findings of both patients were mainly generalized hypotonia, not paresis. Nystagmus and dyskinesia were not observed. Delayed myelination was observed during childhood. The difference between the two patients was the result of thyroid functions. The key finding for AHDS is an elevated free T3 level (1). Patient 2 showed the typical free T3 elevation in infancy. Thus, AHDS was easily suspected as a tentative diagnosis. On the other hand, patient 1 did not show free T3 elevation in childhood. Rather, free T3 was gradually elevated in adolescence.

Familial occurrence in patient 1 suggested an X-linked recessive disorder; however, there are many X-linked disorders associated with intellectual disability. Thus, we could not list AHDS as a candidate diagnosis for this patient. A final diagnosis of AHDS was eventually suspected for this patient based on gradually elevated free T3 levels and retrospective MRI evaluations suggesting transient delay of white matter maturation. Finally, a novel mutation in *SLC16A2* was identified at 19 years of age.

Tsurusaki *et al.* reported a patient with white matter abnormalities (9). Because an elevated T3 level was

not detected in the patient, they did not suspect AHDS as a candidate diagnosis. The exome sequencing identified an *SLC16A2* mutation in this case, and a final diagnosis of AHDS was made (9). This finding supports our inference that AHDS diagnosis is sometime challenging. Patterns of thyroid dysfunction vary among patients with AHDS (4). Although an elevated T3 level is listed as a necessary finding (8), we should consider AHDS as a candidate diagnosis when patients show generalized hypotonia and developmental delay, irrespective of thyroid function abnormalities.

Generally, growth impairment is not observed in early childhood, but later in patients with AHDS (1). Only a few reported children with AHDS showed growth impairment in early childhood (6,10). In this study, severe growth impairment and psychomotor developmental delay were the main symptoms in patient 1.

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References

- Schwartz CE, May MM, Carpenter NJ, Rogers RC, Martin J, Bialer MG, Ward J, Sanabria J, Marsa S, Lewis JA, Echeverri R, Lubs HA, Voeller K, Simensen RJ, Stevenson RE. Allan-Herndon-Dudley syndrome and the *monocarboxylate transporter 8 (MCT8)* gene. *Am J Hum Genet.* 2005; 77:41-53.
- Tonduti D, Vanderver A, Berardinelli A, Schmidt JL, Collins CD, Novara F, Genni AD, Mita A, Triulzi F, Brunstrom-Hernandez JE, Zuffardi O, Balottin U, Orcesi S. MCT8 deficiency: Extrapyramidal symptoms and delayed myelination as prominent features. *J Child Neurol.* 2013; 28:795-800.
- Matheus MG, Lehman RK, Bonilha L, Holden KR. Redefining the pediatric phenotype of X-linked monocarboxylate transporter 8 (MCT8) deficiency: Implications for diagnosis and therapies. *J Child Neurol.* 2015; 30:1664-1668.
- Boccone L, Dessi V, Meloni A, Loudianos G. Allan-Herndon-Dudley syndrome (AHDS) in two consecutive generations caused by a missense *MCT8* gene mutation. Phenotypic variability with the presence of normal serum T3 levels. *Eur J Med Genet.* 2013; 56:207-210.
- Dumitrescu AM, Liao XH, Best TB, Brockmann K, Refetoff S. A novel syndrome combining thyroid and neurological abnormalities is associated with mutations in a monocarboxylate transporter gene. *Am J Hum Genet.* 2004; 74:168-175.
- Kobayashi S, Onuma A, Inui T, Wakusawa K, Tanaka S, Shimojima K, Yamamoto T, Haginoya K. Clinical course and images of four familial cases of Allan-Herndon-Dudley syndrome with a novel monocarboxylate transporter 8 gene mutation. *Pediatr Neurol.* 2014; 51:414-416.
- Yamamoto T, Shimojima K, Umemura A, Uematsu M, Nakayama T, Inoue K. *SLC16A2* mutations in two Japanese patients with Allan-Herndon-Dudley syndrome. *Hum Genome Var.* 2014; 1:14010.
- Friesema EC, Visser WE, Visser TJ. Genetics and phenomics of thyroid hormone transport by MCT8. *Mol Cell Endocrinol.* 2010; 322:107-113.
- Tsurusaki Y, Osaka H, Hamanoue H, Shimbo H, Tsuji M, Doi H, Saito H, Matsumoto N, Miyake N. Rapid detection of a mutation causing X-linked leucoencephalopathy by exome sequencing. *J Med Genet.* 2011; 48:606-609.
- Biebermann H, Ambrugger P, Tarnow P, von Moers A, Schweizer U, Grueters A. Extended clinical phenotype, endocrine investigations and functional studies of a loss-of-function mutation A150V in the thyroid hormone specific transporter MCT8. *Eur J Endocrinol.* 2005; 153:359-366.

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Malignant McKittrick-Wheelock syndrome as a cause of acute kidney injury and hypokalemia: Report of a case and review of literature

Sarthak Malik¹, Bipadabhanjan Mallick¹, Kunaal Makkar², Vivek Kumar³, Vishal Sharma^{1,*}, Surinder Singh Rana¹

¹Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Chandigarh, India;

²Department of Internal Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India;

³Department of Nephrology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Summary Colonic polyps are usually asymptomatic, and are commonly detected during screening colonoscopy. Severe hypokalemia secondary to secretory diarrhea is a rare presentation of rectal polyps. We present a 70 years old female with hypokalemia and acute kidney injury secondary to secretory diarrhea due to moderately differentiated adenocarcinoma of rectum, all of which is syndromically sometimes referred to as McKittrick and Wheelock syndrome. The case is presented because McKittrick-Wheelock syndrome is still more uncommon with malignancy. The syndrome may be associated with other features of hypersecretory diarrhea. Though very rare, clinical suspicion would often lead to diagnosis and appropriate management. We also review the previously published reports of this entity.

Keywords: Diarrhea, rectal adenoma, colorectal cancer, hypokalemia

1. Introduction

Colonic polyps are usually asymptomatic or lead to nonspecific gastrointestinal complaints. They are mostly diagnosed during screening programmes for prevention of colon cancer. McKittrick and Wheelock syndrome is a rare disorder characterised by a rectal villous adenoma leading to chronic secretory diarrhea which in turn leads to severe dehydration, hypokalemia, hyponatremia and acute kidney injury (1). Occasional reports also describe the presence of underlying malignancy or other histologic entities as a cause of this syndrome. We present a 70 years old female who was admitted with hypokalemia and acute kidney injury, and finally diagnosed to have rectal malignancy.

2. Case Report

A 70 years old female presented to the emergency clinic with generalized weakness and reduced urine output for three days. She also complained of diarrhea for 7 months. The patient described frequency of diarrhea to be five to eight episodes per day, large volume, non-bloody, not improving with fasting and often led to nocturnal awakenings. She also gave a history of tenesmus and crampy lower abdominal pain. She received multiple courses of antibiotics from local practitioners with no relief in diarrhea. She denied any nausea or vomiting. Her symptoms worsened over last 1 week prior to presentation. On examination, she had signs of volume depletion; her pulse rate was 106/min and regular, with dry mucous membranes and blood pressure was 96/60 mm of Hg at presentation. Laboratory tests showed serum creatinine as 2.6 (normal: 0.5-1.2) with blood urea being 70 mg/dL. Serum potassium was 2.4 mmol/L (normal: 3.6-5 mmol/L), sodium 142 mmol/L (normal: 135-143 mmol/L), phosphate 1.4 mg/dL (normal: 2.4-4.1 mg/dL) and magnesium levels were 1.53 mg/dL (normal: 1.7-2.2 mg/dL). Her gastroduodenoscopy was normal. Serum IgA-tissue transglutaminase antibody was negative. Stool workup did not show any pus cells, RBC's, ova or cysts. Stool culture was negative. Urine examination did not reveal any active sediment

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*Address correspondence to:

Dr. Vishal Sharma, Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

E-mail: docvishalsharma@gmail.com

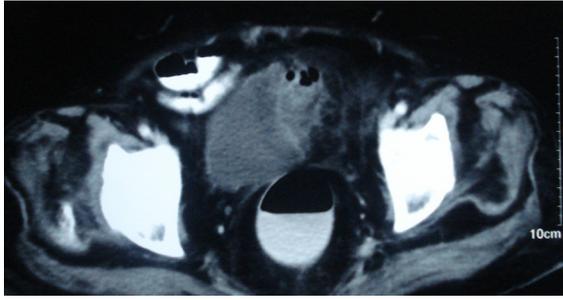


Figure 1. Asymmetrical mural thickening in the recto-sigmoid.

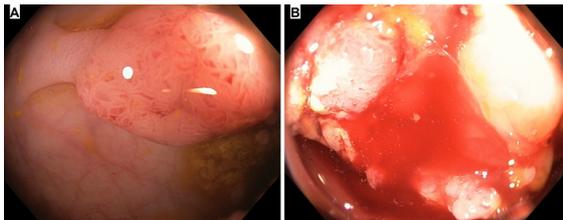


Figure 2. 2A Rectal polyp 2B Polypoid malignant appearing lesion.

and the ultrasound for kidneys was normal. After her renal functions improved a contrast enhanced CT scan of abdomen and pelvis revealed asymmetrical mural thickening along with polypoid growth involving recto-sigmoid colon (Figure 1). Sigmoidoscopic examination revealed a small polyp at 15 cm and a large polypoid friable lesion at 18 cm from anal verge (Figure 2A and 2B). Rest of the colonic mucosa was normal and free of polyps. Biopsy taken from the larger lesion was consistent with adenocarcinoma. Hypokalemia was corrected by oral and intravenous correction. The patient was referred to surgical services for further management.

3. Discussion

Rectal growth presenting as chronic diarrhea with hypokalemia is a rare cause of hypokalemia which was first described by McKittrick and Wheelock in 1954 (1). The disease classically presents as a triad of chronic diarrhea, dyselectrolynemias, and renal failure (2-7). We did an extensive PubMed search for all the cases of McKittrick and Wheelock syndrome reported in the English language up to March 2016. We used search terms "McKittrick", "AND" "Wheelock". Of the 44 results, four results were irrelevant and of the other 40 we excluded 9 because they were not in English language. The other 31 papers reported about 35 patients. We report the data on 35 cases of this syndrome in English literature (Table 1).

The mean age of presentation of these patients was 65.8 ± 10.11 years. The youngest age of presentation was a 30 years old male and the oldest case was an 82 years old female (8-9). There have been 17 female patients and 18 males reported indicating that the phenomenon is equally common in either gender. The

Table 1. Features of 35 cases of McKittrick-Wheelock syndrome reviewed

Feature	Result
Mean Age (years)	65.8 ± 10.11
Gender (Male: Female)	18:17
Etiology	Villous adenoma - 21 Carcinoma - 7 Tubulovillous adenoma - 5 Neuroendocrine tumor - 1 Hyperplastic polyp - 1
Duration of symptoms (months)	22.9 ± 33.9
Pattern of diarrhea	Watery - 28 Bloody - 7
Renal failure	30
Hypokalemia	Present - 26/27 Data NA - 8
Hyponatremia	Present - 22/23 Data NA - 12
Shock	7
Outcome	Recovery - 34 Data not available - 1
Complications	6

mean duration of symptoms prior to presentation was 22.9 ± 33.9 months with a minimum and maximum interval prior to presentation ranged between one month and ten years (1).

Our patient had all the features of the classical triad. Although the diarrhea is classically watery in nature, seven cases out of all the 35 reported cases had associated blood in stools (10-14). This can be expected as rectal bleeding is an important clinical feature of rectal cancer and polyps. Of seven patients with bleeding, only two patients had underlying malignancy while five had rectal villous adenoma. Renal failure was found to be present in 30 out of 35 reported cases at presentation with 3 patients not having renal injury and data was not available for 2 patients (15-17). The mean blood urea nitrogen and creatinine values at presentation were 106.1 ± 60 mg/dL and 5.1 ± 2.96 mg/dL respectively. The maximum value of BUN and creatinine reported were 220 and 11.7 respectively (18-19). Most of the patients achieved complete recovery with adequate hydration and surgical/endoscopic removal of lesions. Twenty nine cases achieved full recovery from renal failure, one patient did not undergo treatment and her outcome was not known (19). Our patient's renal failure also improved with hydration. Dyselectrolynemias is generally universal and was seen in all the patients. The mean values of potassium and sodium were 2.57 ± 0.78 meq/L and 118 ± 11 meq/L respectively. All the patients improved with medical and later surgical management. Most patients had chronic diarrhea and are fluid replete with increased compensatory fluid intake, however, a few cases presented with hypovolemic shock. Seven out these

35 cases were in hypovolemic shock at presentation (8,11,18-21).

The pathogenesis of diarrhea is due to the secretory activity of villous adenoma. Around 3% of villous adenoma has secretory activity and it is proportional to its size (22). The reason why only rectal (distal colonic) lesions are implicated in hypokalemia is because the distal location of lesion prevents compensatory colonic absorption leading to diarrhea, which even persists with fasting. Rectal location was the most common seen in 30 cases. Four patients had a large adenoma extending into sigmoid colon (7,16,23,24) and one patient had an extremely large adenoma extending from rectum into descending colon (11). The electrolyte depletion causes hypokalemia, hyponatremia, dehydration and metabolic acidosis. Our patient had severe hypokalemia at presentation which required a high dose of potassium correction daily. Prostaglandins (PGE2) has been postulated as a secretagogue responsible for this diarrhea (25). A study demonstrated markedly increased levels in diarrheal fluid (26). In a recently reported case, strong COX-2 expression was shown in goblet glands using immunohistochemical staining (23).

Majority of reported cases are due to a villous adenoma, however, rarely it can be secondary to an adenocarcinoma or even a neuroendocrine tumor (7). Out of 35 cases, 22 had a villous adenoma, eight cases had an underlying adenocarcinoma (8,11,14,21-22,27-29) one each had a hyperplastic polyp (30) and neuroendocrine tumor of rectum with liver metastasis (7). Histological details were not available for 3 cases (15). Management includes correction of dehydration and electrolyte abnormalities followed by a definitive therapy for removal of polyp. Medical therapy with indomethacin and somatostatin have been tried to reduce secretion from polyps (25). Endoscopic and surgical methods of polyp removal can be used. Our patient had an underlying adenocarcinoma which has so far been reported only in eight cases. Out of the 36 cases, one patient refused all treatment modalities (19); only one was managed with an endoscopic method using endoscopic submucosal dissection (29). Two patients refused surgery and were managed with medical therapy alone (7,15). The other 32 patients underwent surgery. Seven patients underwent laparoscopic anterior resection and the rest were managed with open surgery. It is likely that in the future many patients will be managed without surgery due to advancements in the field of endoscopic resection with use of endoscopic mucosal resection and endoscopic submucosal dissection.

Some of the patients suffer from underlying comorbidities and complications making management difficult. The various comorbidities reported so far are Cronkhite Canada syndrome (22), thalassemia (8), hypothyroidism (16), cirrhosis (9), deep vein thrombosis (31), diabetes mellitus (29), dermatomyositis (13) and even monoclonal gammopathy of undetermined

significance (24). The role of the above comorbidities in being the causative factor or just chance association is unclear. The reported complications in the literature included associated endocarditis (19), pseudoobstruction (32), rhabdomyolysis (20), clostridium difficile induced diarrhea (33), intussusception and rectal prolapse (12). Postoperative complications such as postoperative stricture (29) have been reported which require multiple sessions of rectal dilatations.

To conclude, McKittrick and Wheelock syndrome although uncommon, is an important differential diagnosis in patients presenting with electrolyte abnormalities, renal failure and secretory diarrhea. If promptly diagnosed and treated, it is a reversible illness. Definitive treatment requires excision of adenoma after correction of electrolyte abnormalities which can be lifesaving.

References

1. McKittrick LS, Wheelock FC Jr. Carcinoma of the colon. Charles C Thomas Springfield, IL, 1954; pp.61-63.
2. Nicholson P, Naugler C. McKittrick-Wheelock syndrome may represent the extreme of a normally distributed continuum of secretory activity in colorectal villous adenomas. *Clin Chim Acta*. 2014; 436:9-10.
3. Kapoor S. McKittrick-Wheelock syndrome: An often, overlooked complication of rectal adenomas. *Surg Endosc*. 2014; 28:2247.
4. Bozkurt N, Omer Özütemiz A, Akgün E, Doganavsargil B, Sezak M. Adenoma with rectal villous diarrhoea and severe hypokalaemia (McKittrick-Wheelock syndrome). *Br J Hosp Med (Lond)*. 2013; 74:648-649.
5. Fernández-López F, Paredes-Cotore JP. McKittrick-Wheelock syndrome- Prolapsed giant villous adenoma of the rectum. *Rev Esp Enferm Dig*. 2013; 105:309-310.
6. Winstanley V, Little MA, Wadsworth C, Cohen P, Martin NM. The McKittrick-Wheelock syndrome: A case of acute renal failure due to neoplastic cholera. *Ren Fail*. 2008; 30:469-473.
7. Nakhla SG, Murakami TT, Sundararajan S. Poorly differentiated neuroendocrine tumor of the rectum coexistent with giant rectal villous adenoma presenting as McKittrick-Wheelock syndrome. *Case Rep Oncol Med*. 2015; 2015:242760.
8. Lee YS, Lin HJ, Chen KT. McKittrick-Wheelock syndrome: A rare cause of life-threatening electrolyte disturbances and volume depletion. *J Emerg Med*. 2012; 43:e171-e173.
9. Podestà MA, Cucchiari D, Merizzoli E, Elmore U, Angelini C, Badalamenti S. McKittrick-Wheelock syndrome: A rare cause of acute renal failure and hypokalemia not to be overlooked. *Ren Fail*. 2014; 36:811-813.
10. Sanchez Garcia S, Villarejo Campos P, Manzanares Campillo Mdel C, Gil Rendo A, Muñoz Atienza V, García Santos EP, Ruescas García FJ, Bertelli Puche JL. Hypersecretory villous adenoma as the primary cause of an intestinal intussusception and McKittrick-Wheelock syndrome. *Can J Gastroenterol*. 2013; 27:621-622.
11. Choi WH, Ryuk J, Kim HJ, Park SY, Park JS, Kim JG, Choi GS. A case of giant rectal villous tumor with severe

- fluid-electrolyte imbalance treated by laparoscopic low anterior resection. *J Korean Surg Soc.* 2012; 82:325-329.
12. Galanis IN, Dragoumis DM, Christopoulos PN, Galanis NN, Atmatzidis KS. Giant villous adenoma and McKittrick-Wheelock syndrome in an incarcerated rectal prolapse. *Colorectal Dis.* 2010; 12:382-384.
 13. Koning GG, Rensma PL, van Milligen de Wit AW, van Laarhoven CJ. In-one-continuity rectal excision and anal mucosectomy of a giant villous adenoma: An alternative surgical approach. *Case Rep Gastroenterol.* 2008; 2:175-180.
 14. Lepur D, Klinar I, Mise B, Himbele J, Vranjican Z, Barsić B. McKittrick-Wheelock syndrome: A rare cause of diarrhoea. *Eur J Gastroenterol Hepatol.* 2006; 18:557-559.
 15. Dagan A, Reissman P. Giant secretory villous adenoma of the rectum and sigmoid presenting as McKittrick-Wheelock syndrome. *Int J Colorectal Dis.* 2010; 25:909-910.
 16. Caliskan C, Makay O, Firat O, Uğuz A, Akgün E, Korkut MA. McKittrick-Wheelock syndrome: Is it really rare? *Am J Emerg Med.* 2010; 28:105-106.
 17. Targarona EM, Hernandez PM, Balague C, Martinez C, Hernández J, Pulido D, Berindoague R, Trias M. McKittrick-Wheelock syndrome treated by laparoscopy: Report of 3 cases. *Surg Laparosc Endosc Percutan Tech.* 2008; 18:536-538.
 18. Martins HS, Brandão-Neto RA, de Carvalho AL, Santana AN, Aguiar FJ, Scalabrini-Neto A, Velasco IT. McKittrick-Wheelock syndrome: A cause of severe hydro-electrolyte disorders in ED. *Am J Emerg Med.* 2007; 25:1083.e1-e3.
 19. Dahya V, Dahya Z, Cua Y. McKittrick-Wheelock syndrome complicated by *Enterococcus faecalis* endocarditis: A unique combination. *Int J Colorectal Dis.* 2015; 30:285-286.
 20. Pucci G, Rondelli F, Avenia N, Schillaci G. Acute renal failure and metabolic alkalosis in a patient with colorectal villous adenoma (McKittrick-Wheelock syndrome). *Surgery.* 2013; 154:643-644.
 21. Tuță LA, Boșoteanu M, Deacu M, Dumitru E. McKittrick-Wheelock syndrome: A rare etiology of acute renal failure associated to well-differentiated adenocarcinoma (G1) arising within a villous adenoma. *Rom J Morphol Embryol.* 2011; 52:1153-1156.
 22. Watari J, Sakurai J, Morita T, *et al.* A case of Cronkhite-Canada syndrome complicated by McKittrick-Wheelock syndrome associated with advanced villous adenocarcinoma. *Gastrointest Endosc.* 2011; 73:624-626.
 23. Hsieh MC, Chen CJ, Huang WS. McKittrick-Wheelock syndrome. *Clin Gastroenterol Hepatol.* 2016; 14:e41-e42.
 24. Hashash JG, Holder-Murray J, Aoun E, Yadav D. The McKittrick-Wheelock syndrome: A rare cause of chronic diarrhoea. *BMJ Case Rep.* 2013; 2013. pii: bcr2013009208.
 25. Smelt AH, Meinders AE, Hoekman K, Noort WA, Keirse MJ. Secretory diarrhea in villous adenoma of rectum: Effect of treatment with somatostatin and indomethacin. *Prostaglandins.* 1992; 43:567-572.
 26. Popescu A, Orban-Schiopu AM, Becheanu G, Diculescu M. McKittrick-Wheelock syndrome - a rare cause of acute renal failure. *Rom J Gastroenterol.* 2005; 14:63-66.
 27. Bruno A, Chimienti D, Montanaro A, Prete F, Libutti P, Lisi P, Basile C. The McKittrick-Wheelock syndrome: A rare cause of severe hydroelectrolyte disorders and acute renal failure. *Case Rep Nephrol.* 2011; 2011:765689.
 28. Raphael MJ, McDonald CM, Detsky AS. McKittrick-Wheelock syndrome. *CMAJ.* 2015; 187:676-678.
 29. Ohara Y, Toyonaga T, Watanabe D, Hoshi N, Adachi S, Yoshizaki T, Kawara F, Tanaka S, Ishida T, Okuno T, Ikehara N, Morita Y, Umegaki E, Yokozaki H, Azuma T. Electrolyte depletion syndrome (McKittrick-Wheelock syndrome) successfully treated by endoscopic submucosal dissection. *Clin J Gastroenterol.* 2015; 8:280-284.
 30. Vercauteren C, Van de Putte, Pattyn P. McKittrick-Wheelock syndrome: Acute renal failure by a colorectal adenoma. A case study. *Acta Chir Belg.* 2012; 112:226-228.
 31. Khalife M, Eloubeidi MA, Hosn MA. McKittrick-Wheelock syndrome presenting with dermatomyositis and rectal prolapsed. *Clin Exp Gastroenterol.* 2013; 6:85-89.
 32. Miles LF, Wakeman CJ, Farmer KC. Giant villous adenoma presenting as McKittrick-Wheelock syndrome and pseudo-obstruction. *Med J Aust.* 2010; 192:225-227.
 33. Learney RM, Ziprin P, Swift PA, Faiz OD. Acute renal failure in association with community-acquired clostridium difficile Infection and McKittrick-Wheelock syndrome. *Case Rep Gastroenterol.* 2011; 5:438-444.

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Coffin-Siris syndrome with café-au-lait spots, obesity and hyperinsulinism caused by a mutation in the *ARID1B* gene

Fatma Mujgan Sonmez^{1,*}, Eyyup Uctepe², Mehmet Gunduz^{3,4}, Zeliha Gormez⁵, Seval Erpolat⁶, Murat Oznur³, Mahmut Samil Sagiroglu⁵, Huseyin Demirci⁵, Esra Gunduz³

¹Department of Child Neurology, Turgut Özal University Faculty of Medicine, Ankara, Turkey;

²Department of Medical Genetics, Health Sciences University, Diskapi Yildirim Beyazit Training and Research Hospital, Ankara, Turkey;

³Department of Medical Genetics, Turgut Özal University Faculty of Medicine, Ankara, Turkey;;

⁴Department of Otolaryngology, Turgut Özal University Faculty of Medicine, Ankara, Turkey;

⁵Advanced Genomics and Bioinformatics Research Center, The Scientific and Technological Research Council of Turkey (TUBITAK-BILGEM), Kocaeli, Turkey;

⁶Department of Dermatology, Turgut Özal University Faculty of Medicine, Ankara, Turkey.

Summary

Coffin-Siris syndrome (CSS) (MIM 135900) is characterized by developmental delay, severe speech impairment, distinctive facial features, hypertrichosis, aplasia or hypoplasia of the distal phalanx or nail of the fifth digit and agenesis of the corpus callosum. Recently, it was shown that mutations in the *ARID1B* gene are the main cause of CSS, accounting for 76% of identified mutations. Here, we report a 15 year-old female patient who was admitted to our clinic with seizures, speech problems, dysmorphic features, bilaterally big, large thumb, café-au-lait (CAL) spots, obesity and hyperinsulinism. First, the patient was thought to have an association of neurofibromatosis and Rubinstein Taybi syndrome. Because of the large size of the *NF1* gene for neurofibromatosis and *CREBBP* gene for Rubinstein Taybi syndrome, whole exome sequence analysis (WES) was conducted and a novel *ARID1B* mutation was identified. The proband WES test identified a novel heterozygous frameshift mutation c.3394_3395insTA in exon 13 of *ARID1B* (NM_017519.2) predicting a premature stop codon p.(Tyr1132Leufs*67). Sanger sequencing confirmed the heterozygous c.3394_3395insTA mutation in the proband and that it was not present in her parents indicating de novo mutation. Further investigation and new cases will help to understand this phenomenon better.

Keywords: *ARID1B* gene, café-au-lait spots, Coffin-Siris syndrome, phenotypic expansion

1. Introduction

Coffin-Siris syndrome (CSS, MIM 135900) is characterized by mild-to-moderate intellectual disability (ID), severe speech impairment, coarse facial features, hypertrichosis, hypoplastic or absent fifth fingernails or toenails and agenesis of the corpus callosum (1,2).

Recently, mutations in the genes *ARID1A*, *ARID1B*,

SMARCA4, *SMARCB1*, and *SMARCE1*, all of them encoding proteins belonging to the BAF complex which is one of the ATP-dependent chromatin remodeling complexes, were determined to be causative of CSS (3,4). It is now clear that mutations in different genes cause the differences in the phenotypes of patients with CSS. Among these genes, it was shown that mutations in *ARID1B* are the main cause of CSS, accounting for 68%, 83%, and 76% of identified mutations in different studies (3,5). Before the molecular basis of CSS was known, the diagnosis of CSS was based rigorously on clinical findings. After molecular diagnosis of the patients with CSS, the phenotypic spectrum of CSS is becoming wider especially in patients with *ARID1B* mutations (6).

Here, we report a child with a novel *ARID1B*

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*Address correspondence to:

Dr. Fatma Mujgan Sonmez, Department of Child Neurology, Turgut Ozal University Faculty of Medicine, Alparslan Turkes Caddesi No: 57 06510, Ankara, Turkey.

E-mail: mjgsonmez@yahoo.com

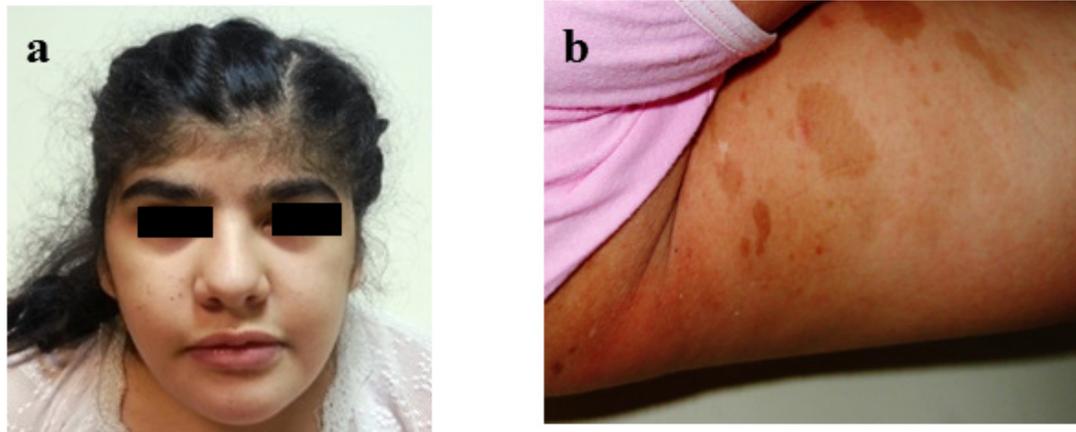


Figure 1. (a) Facial view of the patient (b) multiple café-au-lait spots on the left gluteal area.

mutation identified by whole-exome sequencing (WES) that presented with obesity and hyperinsulinism supporting earlier studies (6). Also she has café-au-lait (CAL) spots expanding the phenotypic spectrum of patients with CSS.

2. Case Report

A 15 year-old female patient was admitted to child neurology outpatient clinic with a speech problem and seizures. She was born to first-cousin parents as a second child after term pregnancy and normal delivery. At 2 months old, she had generalized tonic clonic seizure with fever during lung infection. She was able to sit at 1 year and walk independently at 2.5 years. She could speak single words at 10 years. Now, she can make sentences with two words. She had been evaluated at another clinic and had started a rehabilitation program for 5 years, also Ecocardiography (ECHO) revealed an atrial septal defect at that time. At the age of 13 years, afebrile generalized tonic-clonic seizures had started 3-4 times monthly and oxcarbazepine therapy was added. At that time, her weight was 32 kg (50-75%); height 150 cm (25-50%), head circumference was 54 cm. After the 6 month period, she has been seizure free for three years. At the age of 12 years, stereotypic movements have started. At 14 years old, she was operated for autism spectrum disorder (ASD).

At the age of 15: her weight was 68.5 kg (97%), height 150 cm (3%), BMI: 32.14. Physical examination showed craniofacial abnormalities including coarse face, low frontal hairline, synophrys, thick eyebrows, broad nose, thick, anteverted alae nasi, large mouth, thin upper vermillion, thick lower vermillion, short philtrum, short neck and large ears (Figure 1a). Other dysmorphic features included bilaterally big, large thumbs and short second finger on food, hallux valgus and pes planus. There are 9-10 numbers of CAL lesions on the left gluteal area and internal side of the femur

(with sizes between 2-6 centimeters) (Figure 1b). Also she has truncal obesity and excessive body hair especially on the lumbar and sacral region but all of the body. Neurological examination was normal except for mental and motor retardation. There are no other manifestations of NF in the girl and also there are not any other family members with CAL spots and Lisch nodules in the family.

Laboratory investigation including hemogram, biochemical and metabolic investigations including lactic acid, ammonia, urine and blood amino acids, and tandem MS were normal except for high TSH (6.46 IU/mL), insulin (29,4 mIU/mL, N: 2.6-24.9) levels and PTH levels (122.2 pg/mL, N:15-65) and low Vitamin D (5.1microgr/L). Cranial MRI showed small cerebellum, thin corpus callosum, and right lateral temporal periventricular (subependymal) nodular heterotopia. One year later, cranial MRI was repeated for evaluation of the cerebellum. There was no change in size. So, it was accepted as cerebellar hypoplasia. The Spinal MRI showed flat cervical lordosis. Renal doppler USG revealed bilateral Grade I increased echo. Pelvic ultrasonography was normal. Ophthalmological examination was first made at another center and Lisch nodules were reported on the left eye. According to these findings patient's prediagnosis was thought to be neurofibromatosis and Rubinstein Taybi syndrome. Because of the big size of the *NF1* gene for neurofibromatosis and *CREBBP* gene for Rubinstein Taybi syndrome, we decided to perform a whole exome sequence analyses.

3. Genetics

Cytogenetic analyses showed *46,XX*. There is no translocation or chromosomal rearrangement that involves both genes. After karyotype analyses, we decided to perform a WES analyses on this patient. We especially paid attention to *NF1* and *CREBBP*

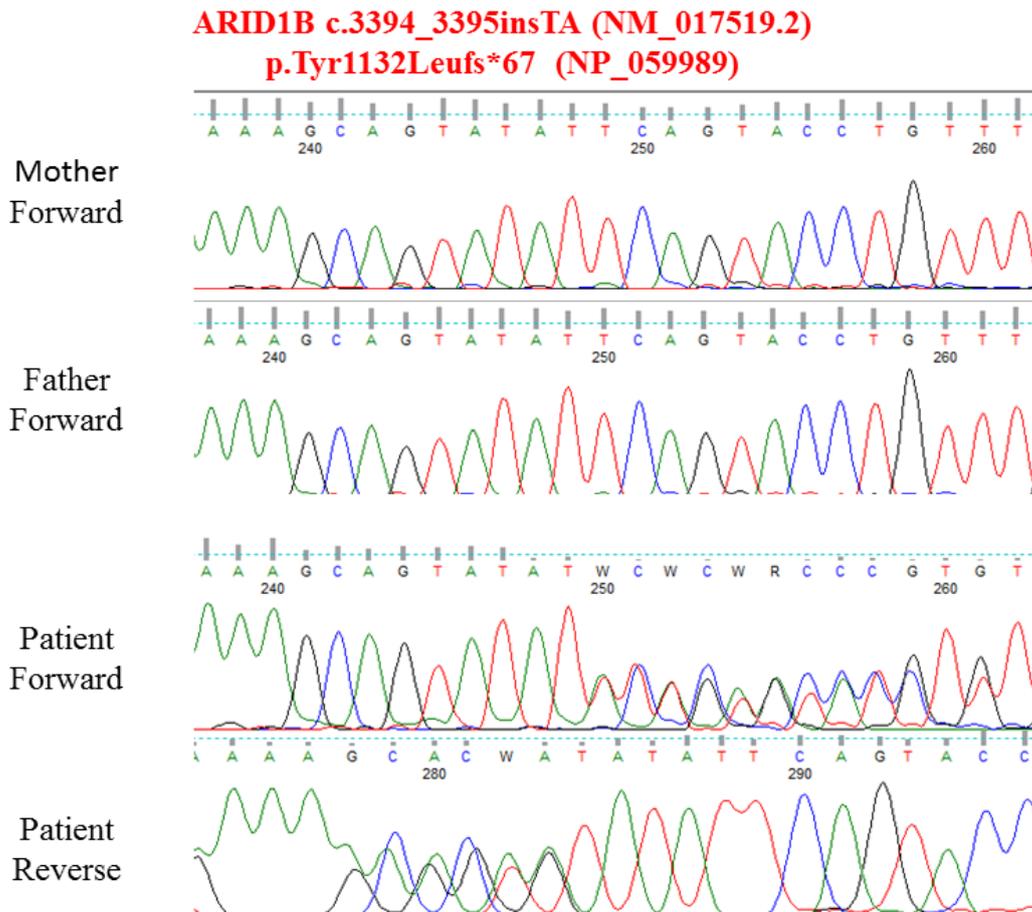


Figure 2. Electropherogram of the *ARID1B* mutation identified in our case compared with her parents electropherogram.

gene sequences during WES data analysis in terms of mutation. However we found nothing in these genes. After variant prioritization in the WES data, we identified the *ARID1B* (ARID-containing protein 1B [MIM 614556]) mutation, which was located on chromosome 6, involving a TA insertion (c.3394_3395insTA [NM_017519.2]) leading to p.(Tyr1132Leufs*67) (RefSeq NP_059989). Our case was heterozygous for this mutation, and the parents were normal indicating *de novo* mutation (Figure 2). This mutation caused the frameshift mutation which possibly caused the truncated protein formation. *ARID1B* mutation in this study was not annotated in databases of human variation [Exome Variant Server (<http://evs.gs.washington.edu/EVS/>), 1000 genomes (<http://www.1000genomes.org/>), dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>)]. Additionally, the mutation was not seen in the IGBAM in-house exome database ($n = 777$). To determine the predicted consequences of the mutations on *ARID1B* function, we used an in silico prediction method. Mutation Taster indicated that the mutation would be harmful. The *ARID1B* variant c.3394_3395insTA identified in this study was submitted to the Leiden Open Variation Database (LOVD) at www.lovd.nl/ARID1B. The mutation was confirmed by Sanger sequencing. We

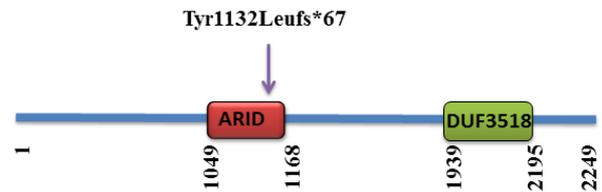


Figure 3. The protein domain organization of *ARID1B* and the localization of *ARID1B* mutation associated with Coffin-Siris syndrome.

also sequenced the mutation site for both parents and determined that mutation to be *de novo* (Figure 3).

We wanted to confirm Lisch nodules after molecular diagnosis in our hospital (*ARID1B* mutation) and it revealed that the patient has no Lisch nodules on her eyes. So it is especially important that molecular diagnosis changed from the first diagnosis. Also, so we believe that it is a phenotypic expansion rather than the probability of co-occurrence.

4. Discussion

CSS is characterized by developmental delay, severe speech impairment, distinctive facial features,

hypertrichosis, aplasia or hypoplasia of the distal phalanx or nail of the fifth digit and agenesis of the corpus callosum (2). This definition was based completely on clinical findings and was made before the molecular basis of CSS was well known. With the recent detection of truncated heterozygous mutations in the BAF complex in some individuals with CSS, it is an inevitable requirement that diagnostic criteria and definition for CSS must evolve.

In line with this view, most common features of CSS patients with an *ARID1B* mutation are determined to have intellectual disability, speech delay, coarse facial features, and hypertrichosis in a recent study. Other findings, present in a smaller subgroup of CSS patients are determined to have small 5th finger or toe nails, short fifth finger, feeding difficulties, agenesis of the corpus callosum, seizures, myopia, and growth delay in the same study (7).

Our case has no fifth digit involvement similar to some earlier CSS cases with *ARID1B* mutations (3,8,9). Although microcephaly has been mentioned as a feature of CSS (10), we did not observe it in our patient compared to some larger CSS cohort studies (11).

Our patient presented with obesity and hyperinsulinism supporting Vals *et al.* They suggest that these features may be associated with *ARID1B* gene mutations, further broadening the phenotypic spectrum of CSS, and they may be added to the list of clinical features of *ARID1B* mutations (6). These results reinforce the view that reevaluation of individuals with a broader phenotype is needed to determine the frequency of this finding in persons with molecularly confirmed CSS.

To the best of our knowledge this is the first report of a patient with CSS who also has CAL spots. CALs are present as well-circumscribed, evenly pigmented macules and patches that range in size from 1 to 2 mm to greater than 20 cm in greatest diameter. CAL macules are common in children. Although most CAL is present as 1 or 2 spots in an otherwise healthy child, the presence of multiple CAL, large segmental CAL, associated facial dysmorphism, other cutaneous anomalies, or unusual findings on physical examination should suggest the possibility of an associated syndrome. Neurofibromatosis type 1 is the most common syndrome seen in children with multiple CAL. Other associated multisystemic genetic disorders include McCune Albright Syndrome, Tuberous sclerosis, Fanconi anemia, Bloom syndrome, Ataxia telangiectasia, Russell-Silver Syndrome, Multiple endocrine neoplasia type 2b, Bannayan-Riley-Ruvalcaba syndrome, and Multiple lentiginos (LEOPARD) syndrome (12). Also the presence of multiple CAL has been described in patients with ring chromosome syndromes involving chromosomes 7, 11, 12, 15, and 17 (13).

As can be seen from the above discussion and earlier studies, there is a big variation in phenotype between patients with CSS. The underlying mechanism

of this variation has yet to be answered. Different functional effects on the BAF complex consists of over 25 core and interchangeable protein subunits by *ARID1B* haploinsufficiency and *SMARCB1/SMARCE1* missense mutations could give rise to broad variation in the clinical phenotypes (14). Also, the set of genes transcriptionally changed due to mutations in the BAF complex genes like *ARID1B* could modify the phenotype and determine how severely affected a patient with *ARID1B* haploinsufficiency will be.

Also our data supports the view by Dixon-Salazar *et al.* that not only is whole-exome sequencing a useful tool for identifying disease-causing genes, but it is also able to redefine or modify the diagnosis for some patients (15).

5. Conclusions

We report a patient with diagnosed CSS with WES. Clinically, in addition to classic features of CSS, the patient presented with CAL spots, obesity and hyperinsulinism. To the best of our knowledge this is the first report of a patient with CSS who has CAL spots. Further investigations and new cases will help to understand this phenomenon better.

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Note: We obtained a written informed consent from the families of the indexed individuals for participation in this study and accompanying images. The study was performed according to the Declaration of Helsinki protocols.

References

1. Coffin GS, Siris E. Mental retardation with absent fifth fingernail and terminal phalanx. *Am J Dis Child.* 1970; 119:433-439.
2. Fleck BJ, Pandya A, Vanner L, Kerkerling K, Bodurtha J. Coffin-Siris syndrome: Review and presentation of new cases from a questionnaire study. *Am J Med Genet.* 2001; 99:1-7.
3. Santen GW, Aten E, Sun Y, *et al.* Mutations in SWI/SNF chromatin remodeling complex gene *ARID1B* cause Coffin-Siris syndrome. *Nat Genet.* 2012; 44:379-380.
4. Tsurusaki Y, Okamoto N, Ohashi H, *et al.* Mutations affecting components of the SWI/SNF complex cause Coffin-Siris syndrome. *Nat Genet.* 2012; 44:376-378.
5. Tsurusaki Y, Okamoto N, Ohashi H, *et al.* Coffin-Siris syndrome is a SWI/SNF complex disorder. *Clin Genet.* 2014; 85:548-554.

6. Vals MA, Oiglane-Shlik E, Noukas M, Shor R, Peet A, Kals M, Kivistik PA, Metspalu A, Ounap K. Coffin-Siris Syndrome with obesity, macrocephaly, hepatomegaly and hyperinsulinism caused by a mutation in the *ARID1B* gene. *Eur J Hum Genet.* 2014; 22:1327-1329.
7. Santen GW, Clayton-Smith J, ARID1B-CSS consortium. The ARID1B phenotype: What we have learned so far. *Am J Med Genet C Semin Med Genet.* 2014; 166C:276-289.
8. Halgren C, Kjaergaard S, Bak M, *et al.* Corpus callosum abnormalities, intellectual disability, speech impairment, and autism in patients with haploinsufficiency of ARID1B. *Clin Genet.* 2012; 82:248-255.
9. Hoyer J, Ekici AB, Ende S, *et al.* Haploinsufficiency of ARID1B, a member of the SWI/SNF-a chromatin-remodeling complex, is a frequent cause of intellectual disability. *Am J Hum Genet.* 2012; 90:565-572.
10. Schrier SA, Bodurtha JN, Burton B, Chudley AE, Chiong MA, D'Avanzo M G, Lynch SA, Musio A, Nyazov DM, Sanchez-Lara PA, Shalev SA, Deardorff MA. The Coffin-Siris syndrome: A proposed diagnostic approach and assessment of 15 overlapping cases. *Am J Med Genet A.* 2012; 158A:1865-1876.
11. Santen GW, Aten E, Vulto-van Silfhout AT, *et al.* Coffin-Siris syndrome and the BAF complex: Genotype-phenotype study in 63 patients. *Hum Mutat.* 2013; 34:1519-1528.
12. Tekin M, Bodurtha JN, Riccardi VM. Cafe au lait spots: The pediatrician's perspective. *Pediatr Rev.* 2001; 22:82-90.
13. Shah KN. The diagnostic and clinical significance of cafe-au-lait macules. *Pediatr Clin North Am.* 2010; 57:1131-1153.
14. Hargreaves DC, Crabtree GR. ATP-dependent chromatin remodeling: Genetics, genomics and mechanisms. *Cell Res.* 2011; 21:396-420.
15. Dixon-Salazar TJ, Silhavy JL, Udpa N, *et al.* Exome sequencing can improve diagnosis and alter patient management. *Sci Transl Med.* 2012; 4:138ra178.

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Hemophagocytic lymphohistiocytosis: A rare cause of recurrent encephalopathy

Raashda Ainuddin Sulaiman^{1,2,*}, Marwan Yassin Shaheen³, Hamad Al-Zaidan^{1,2}, Zuhair Al-Hassnan^{1,2}, Moenaldeen Al-Sayed^{1,2}, Zuhair Rahbeeni^{1,2}, Nasir Ahmed Bakshi⁴, Namik Kaya⁵, Mazhor Aldosary⁵, Mohammed Al-Owain^{1,2}

¹ Department of Medical Genetics, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia;

² College of Medicine, Alfaisal University, Riyadh, Saudi Arabia;

³ Department of Hematology, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia;

⁴ Department of Pathology, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia;

⁵ Department of Genetics, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia.

Summary

We report an unusual case of recurrent encephalopathy due to acquired hemophagocytic lymphohistiocytosis (HLH) in a patient with propionic acidemia (PA). PA is an inherited metabolic disorder in which patients often present with encephalopathy and pancytopenia during metabolic decompensation. However, these patients may rarely develop HLH with similar presentation. This case illustrates the need to distinguish HLH induced encephalopathy from the one secondary to metabolic decompensation in these patients, as early diagnosis and treatment of HLH improves prognosis. This case also highlights the importance of considering HLH in patients presenting with unexplained encephalopathy, as early diagnosis and treatment is lifesaving in this otherwise lethal condition. To our knowledge this is the first case report of acquired HLH presenting as recurrent encephalopathy followed by complete recovery, in a metabolically stable patient with PA.

Keywords: Inherited metabolic disorders, propionic acidemia, pancytopenia

1. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a hyper-inflammatory condition caused by impaired function of natural killer cells (NKC) and cytotoxic T lymphocytes (CTL) leading to poor regulation of immune response. There is hypercytokinemia and abnormal proliferation of histiocytes and macrophages, which engulf hematopoietic cells (*1*). Diagnostic criteria for HLH includes fever, splenomegaly, pancytopenia, increased serum ferritin, triglycerides, soluble CD25, decreased fibrinogen levels and hemophagocytosis.

HLH can be familial with genetic etiology or acquired, associated with infections, autoimmune disorders, immune suppression and malignancies. It has been increasingly reported in various inherited metabolic disorders. Herein, we report a patient with propionic acidemia (PA) who presented with recurrent episodes of encephalopathy and pancytopenia. The presentation of encephalopathy was initially attributed to the metabolic decompensation. However once the diagnosis of acquired HLH was established, it became evident that encephalopathy and pancytopenia were related to HLH presentation and responded well to treatment.

2. Case Report

A 16-year-old boy, a known case of PA and mild cognitive impairment, presented to the emergency room in May 2014 with three days history of fever, vomiting, diarrhea, lethargy and confusion. On examination, he was drowsy, and febrile (38.5°C). Spleen was palpable 4 cm below the costal margin.

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*Address correspondence to:

Dr. Raashda A Sulaiman, Department of Medical Genetics, MBC: 75 King Faisal Specialist Hospital and Research Centre, PO Box No: 3354, Riyadh, 11211, Saudi Arabia.
E-mail: rsulaiman@kfshrc.edu.sa

Table 1. Correlation of the level of consciousness with blood ammonia levels and HLH markers

Items	Level of consciousness	Ammonia $\mu\text{mol/L}$ (ref: <50)	WBC (count $\times 10^9$)	Platelet (count $\times 10^9$)	Hemoglobin (g/L)	Ferritin mg/L (ref: 30-400)	CD25 pg/mL (ref: 458-1997)
First admission							
Day 1	Drowsy	66	1.4	123	83		
Day 3	Drowsy	77	2.0	14	72	1,845	> 5,000
Second admission							
Day 1	Alert	70	12.4	196	103		
Day 4	Drowsy	60	3.5	21	83	2,159	
Third admission							
Day 1	Alert	75	3.6	102	117		
Day 4	Drowsy	121	1.7	44	54	1,430	2,987
Day 26	Alert	134	4.28	271	78		

HLH, hemophagocytic lymphohistiocytosis; WBC, white blood cell.

He had pancytopenia and marginally raised plasma ammonia levels (Table 1). Blood tests including renal, liver function and venous blood gas (VBG) analysis were unremarkable. Computerized tomographic (CT) scan of brain did not show edema or intracranial bleeding. Clostridium difficile toxin was positive in stool. He received antibiotics and fluid resuscitation. His other medications were carnitine and sodium bicarbonate. On the third day of admission he had more marked pancytopenia and continued to be encephalopathic. However, his blood ammonia level was never high enough to account for the severity of the encephalopathy (Table 1), which raised suspicion of another underlying disorder like HLH causing reduced consciousness. He was found to have raised serum ferritin and soluble CD 25 levels (Table 1). A bone marrow aspiration showed active erythropoiesis, granulopoiesis and normal looking megakaryocytes. Histiocytes were significantly increased in number and many of them demonstrated hemophagocytosis (Figure 1B). No clonal chromosomal abnormality was detected by interphase fluorescence *in vitro* hybridization (FISH) DNA probes. Sequencing of the known familial HLH genes (*PRF1*, *MUNC13-4*, *STX11*, and *STXBP2*) was negative. This fulfilled the HLH 2004 diagnostic criteria (fever, pancytopenia, splenomegaly, low plasma fibrinogen, high serum ferritin, soluble CD25 levels and hemophagocytosis). He was treated according to the HLH 2004 protocol with methylprednisolone, vinblastine and gamma globulins. The patient recovered with complete resolution of encephalopathy and was discharged to home after 5 weeks.

He was readmitted three months later, with sepsis secondary to Port-a-Cath infection. He was alert on admission in spite of severe metabolic acidosis (VBG: pH 7.2; base excess -16.9 and bicarbonate 9 mmol/L). He had leukocytosis and blood ammonia level was 70 $\mu\text{mol/L}$ (Table 1). Blood culture grew *Escherichia coli*. He was treated with intravenous antibiotics and sodium bicarbonate. His metabolic acidosis resolved. On day 4 of admission, he became drowsy. A relapse of HLH was suspected as evident by pancytopenia and raised

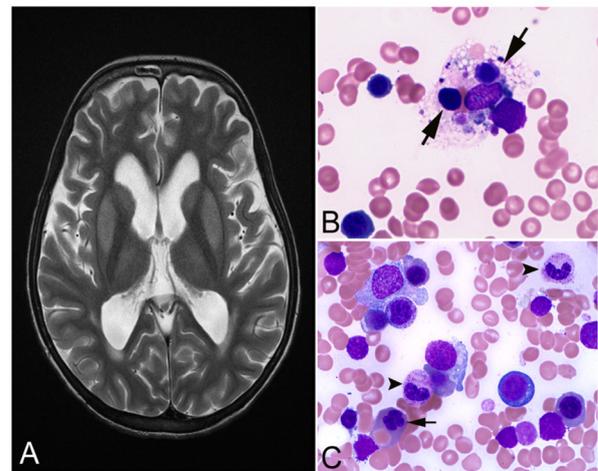


Figure 1. Radiological and hematological abnormalities. (A), Brain MRI showing T2 hyperintensity with edema of putamen; (B) Bone marrow aspirate showing a phagocytic histiocyte with ingested hematopoietic cells: a nucleated red cell precursor and a lymphocyte, highlighted by black arrows; (C) Bone marrow aspirate showing two dysplastic neutrophils, black arrow heads and one dysplastic erythroid precursor, black arrow. Wright-Giemsa staining $\times 500$.

serum ferritin concentrations (Table 1). He responded promptly to methylprednisolone with full recovery and was discharged in two weeks.

His third admission was with community acquired pneumonia in June 2015. He was fully conscious and metabolically stable on admission. Sputum culture grew *Streptococcus pneumoniae*. He received parenteral antibiotics. On day 4, he became confused, disoriented with severe pancytopenia (Table 1). He was treated with methylprednisolone. He remained encephalopathic requiring ventilator support over the next two weeks. A magnetic resonance imaging (MRI) of the brain showed edema of putamen and cerebellum (Figure 1A). An electroencephalogram revealed continuous generalized slow activity. Cerebrospinal fluid examination was unremarkable. In view of persistent pancytopenia, a bone marrow biopsy was performed. It showed morphological changes in all three cell lines consistent with myelodysplastic syndrome (MDS) with ring

sideroblasts and residual HLH (Figure 1C). No MDS related chromosomal anomaly was detected. He also received intermittent treatment with granulocyte colony-stimulating factor (G-CSF). He gradually recovered with no neurological sequelae and was discharged to home after seven weeks of hospitalization. His brain MRI scan repeated after eight weeks of discharge, showed complete resolution of the edema of putamen and cerebellum. His hemoglobin level was stable 114 g/L, white cell count $4.41 \times 10^9/L$, platelet count was $295 \times 10^9/L$.

3. Discussion

PA (OMIM 606054) is an inherited metabolic disorder caused by the deficiency of enzyme propionyl-CoA carboxylase. During acute metabolic decompensation which is mostly triggered by infections, these patients often develop encephalopathy due to hyperammonemia and or severe metabolic acidosis. Such patients also develop bone marrow suppression causing pancytopenia which is a common complication, seen not only during acute metabolic decompensation but is also observed during a metabolically stable state (2).

Although seen in patients with PA during metabolic decompensation, the encephalopathy in our patient was due to HLH. Central nervous system (CNS) involvement in HLH has been reported in 73% of cases at the time of diagnosis (3). It may also develop later during the course of the disease and is associated with poor prognosis (3-5). Neuropathological studies have shown perivascular infiltration of meninges and brain parenchyma by activated lymphocytes and macrophages with hemophagocytosis and active inflammation. This may progress to multifocal necrosis and astrogliosis (6). The neurological manifestations of HLH include varying degrees of encephalopathy, seizures, meningitis, hemiparesis, ataxia and cranial nerve palsy (3-5). CSF analysis may show raised proteins and or hemophagocytic cells. The role of cytokines in CSF remains to be elucidated.

Neuro-imaging in HLH shows a wide spectrum of abnormalities including periventricular T2 weighted hyperintensity, nodular parenchymal enhancement, leptomeningeal enhancement, hemorrhage and restricted diffusion. Gratton *et al.* in 2015 reported basal ganglia involvement in 4 out of 7 (57%) adult patients with acquired HLH (4). Interestingly, it is common for organic acidemias to involve basal ganglia as well. Indeed the MRI brain of our patient showed edema of putamen and cerebellum, which in the absence of other HLH features, could have been attributed to his primary metabolic disorder. Basal ganglia involvement in both disorders suggests that they may share a common mechanism of mitochondrial involvement leading to compromised aerobic respiration. Further research is required to explain the underlying mechanism.

CNS involvement in HLH is associated with poor prognosis (3-5), however, our patient responded well to the HLH treatment and recovered fully after each episode. During his third admission he had a prolonged period of encephalopathy when he had developed transient MDS, in addition to HLH. Although Sipahi *et al.* have previously reported isolated MDS in a patient with PA (7), our patient had a combination of HLH and MDS during his last admission.

The pathogenesis of acquired HLH in inherited metabolic diseases, remains unclear. It is hypothesized that the trigger, either a severe infection or accumulation of toxic metabolites would suppress the function of NK and CTL, producing HLH. Inflammasome activation by oxidative stress may also cause hyperinflammation (1). Moreover, these patients might have genetic mutations that have not yet been identified as related to HLH such as those controlling inflammation and regulating cytokine function (8). Recently whole exome sequencing of DNA from secondary HLH patients found variants in familial HLH related genes as well as new candidate genes (9).

Acquired HLH in PA has been reported earlier in younger children during metabolic crisis, with no evidence of infection (10,11). However, relapsing HLH has not been reported in organic acidemias, for example PA. In our patient, all three episodes of HLH with encephalopathy were triggered by severe systemic bacterial infection. He was metabolically stable as his plasma ammonia levels were not significantly raised and he did not have metabolic acidosis. To our knowledge this is the first case report of repeated episodes of acquired HLH secondary to systemic infection presenting as recurrent encephalopathy in a metabolically stable patient with PA, who showed remarkable responsiveness to the treatment. Acquired HLH has been reported in an increasing number of inherited metabolic disorders and other systemic illnesses. Awareness of this complication and its neurological manifestations should prompt early diagnosis, and timely treatment with a better disease outcome in this otherwise potentially lethal condition.

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References

1. Janka GE, Lehmborg K. Hemophagocytic syndromes – An update. *Blood Rev.* 2014; 28:135-142.
2. Grünert SC, Müllerleile S, De Silva L, *et al.* Propionic acidemia: Clinical course and outcome in 55 pediatric and adolescent patients. *Orphanet J Rare Dis.* 2013; 8:6.
3. Haddad E, Sulis ML, Jabado N, Blanche S, Fischer A, Tardieu M. Frequency and severity of central nervous

- system lesions in hemophagocytic lymphohistiocytosis. *Blood*. 1997; 89:794-800.
4. Gratton SM, Powell TR, Theeler BJ, Hawley JS, Amjad FS, Tornatore C. Neurological involvement and characterization in acquired hemophagocytic lymphohistiocytosis in adulthood. *J Neurol Sci*. 2015; 357:136-142.
 5. Jovanovic A, Kuzmanovic M, Kravljanac R, Micic D, Jovic M, Gazikalovic S, Pasic S. Central nervous system involvement in hemophagocytic lymphohistiocytosis: A single-center experience. *Pediatr Neurol*. 2014; 50:233-237.
 6. Henter JI, Nennesmo I. Neuropathological findings and neurologic symptoms in twenty three children with hemophagocytic lymphohistiocytosis. *J Paediatr*. 1997; 130:350-365.
 7. Sipahi T, Yilmaz D, Tavit B. Propionic acidemia with myelodysplasia and neutropenia in a Turkish child. *J Pediatr Hematol Oncol*. 2004; 26:154-155.
 8. Cetica V, Sieni E, Pende D, Danesino C, De Fusco C, Locatelli F, Micalizzi C, Putti MC, Brondi A, Fagioli F, Moretta L, Griffiths GM, Luzzatto L, Arico M. Genetic predisposition to hemophagocytic lymphohistiocytosis: Report on 500 cases from the Italian registry. *J Allergy Clin Immunol*. 2016; 137:188-196.
 9. Kaufman KM, Linghu B, Szustakowski JD, Husam A, Yang F, Zhang K, Filipovich AH, Fall N, Harley JB, Nirmala NR, Grom AA. Whole exome sequencing reveals overlap between macrophage activation syndrome in systemic juvenile idiopathic arthritis and familial hemophagocytic lymphohistiocytosis. *Arthritis Rheumatol*. 2014; 66:3486-3495.
 10. Gokce M, Unal O, Hismi B, Gumruk F, Coskun T, Balta G, Unal S, Cetin M, Kalkanoglu-Sivri HS, Dursun A, Tokatli A. Secondary hemophagocytosis in 3 patients with organic acidemia involving propionate metabolism. *Pediatr Hematol Oncol*. 2012; 29:92-98.
 11. Kasapkara CS, Kangin M, Oflaz Ozmen B, Ozbek MN, Demir R, Karatas M, Tumer L, Ezgu FS, Hasanoglu A. Secondary hemophagocytosis in propionic acidemia. *Iran J Pediatr*. 2015; 25:e339.
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Cystic adventitial disease of the common femoral vein: A case report

Jimei Yu, Chaojun Lu, Xiaohua Pan, Weihua Li*

Department of Operation, Huashan North Hospital, Fudan University, Shanghai, China.

Summary

Adventitial cystic disease (ACD) of the common femoral vein is a rare vascular disorder. It becomes more difficult to recognize preoperatively especially when the femoral vein is affected. We report the case of a 62-year-old female patient who presented with a one-month history of painless swelling in her right lower extremity. She had no specific past medical history and no history of trauma, and had a full coagulopathy profile that was negative for any hypercoagulable syndrome. On examination, her lower right leg was significantly swollen with a palpable mass in her right inguinal region. A computerized tomography (CT) with contrast was performed to provide more information and revealed an eccentric compression over the medial wall of the right common femoral vein. During surgical exploration, adventitial cystic mucinous disease was enucleated and the patient underwent femoral exploration, excision of the cysts and reconstruction of iliac femoral vein graft using an artificial blood vessel. The pathological examination confirmed the diagnosis. The patient continued to do well, and she had an unremarkable venous duplex evaluation at her 6-month follow-up. The presentation, investigation, treatment, and pathology of this condition are discussed with a literature review.

Keywords: Adventitial cystic disease (ACD), femoral vein, review

1. Introduction

Adventitial cystic disease (ACD) of the veins is a rare condition with an uncertain etiology in which a mucin containing cyst is formed in the walls of the veins. The arterial variety of ACD has often been described in the popliteal artery (1). Patients with this disease will have severe swelling, tenderness, and pain (2). In this report, we discuss the case of a 62-year-old woman who presented with a swollen lower leg secondary to obstruction of the common femoral vein. We performed a computerized tomography (CT) scan and ultrasound and this led to excision of a cyst and reconstruction of an iliac femoral vein graft using an artificial blood vessel. As a result, the patient made a full recovery. We

also discuss the pathology and the diagnostic methods for this condition.

2. Case Report

A 62-year-old female was referred to our vascular unit with a one month history of right lower extremity painless swelling. She had no specific past medical history and no history of trauma and had a full coagulopathy profile that was negative for any hypercoagulable syndrome. On examination, her right lower leg was obviously swollen – 10 cm larger in circumference than the left side, with signs of palpable masses in the right inguinal region. No other abnormality was found on physical examination. Ultrasonography showed a cystic mass containing hypoechoic materials attached to the right common femoral vein. Because the diagnosis was uncertain, CT with contrast was performed to provide more information and to exclude other causes such as unusual tumors. A contrast-enhanced computed tomography (CECT) scan also showed the presence of an intraluminal low-attenuating mass lesion (3.2 × 2.1 cm)

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*Address correspondence to:

Dr. Weihua Li, Department of Operation, Huashan North Hospital, Fudan university, No. 108, Luxiang Road, Baoshan District, Shanghai 201907, China.
E-mail: buanjingdelvtu@163.com

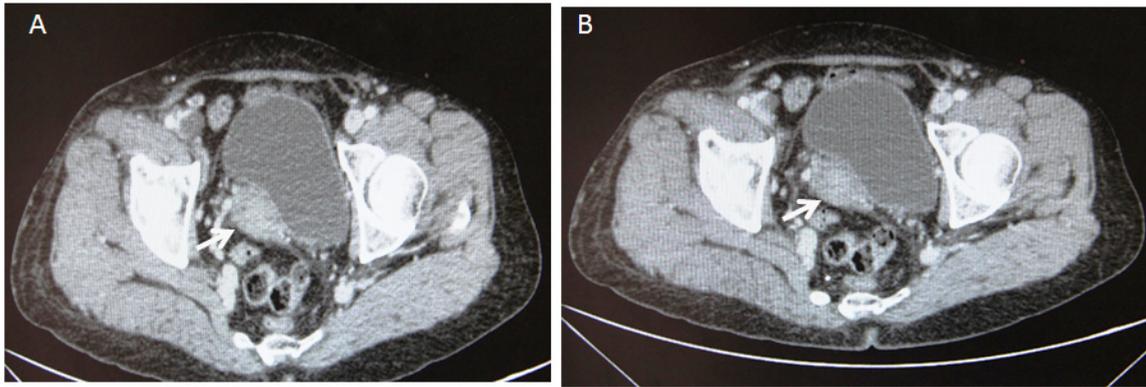


Figure 1. Contrast-enhanced computed tomography demonstrating distension of the left common femoral vein due to an intraluminal hypoattenuating mass lesion (arrow) attached to the bladder wall (A). The mass was presumed to be a deep vein thrombus (B).

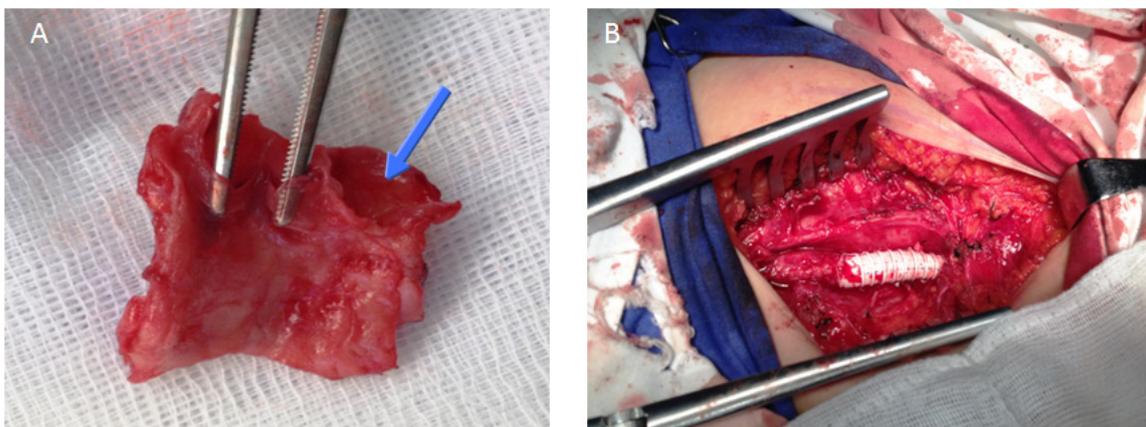


Figure 2. An adventitial cystic mass (Blue arrow) extends from the medial to the posterior surface of the right CFV (A). Operative image illustrates the excision of tumor and reconstruction of iliac femoral vein graft (B).

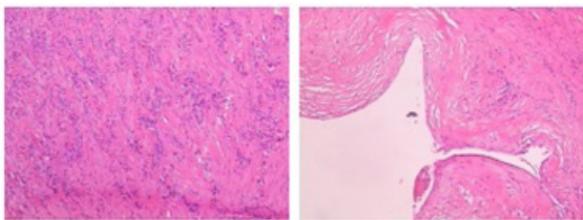


Figure 3. Postoperative photomicrograph indicated a cystic structure with layers of collagen separated by scanty elastic fibers and fibrosis in the wall of the vein and the excised tissue was connective tissue with infiltration of inflammatory cells.

involving the right CFV (Figure 1). The CT revealed obstruction of flow at the level of the CFV, with a presumed thrombus in the CFV and a long saphenous vein but no obvious extravascular mass. The clinical diagnosis was ACD.

A venotomy was made in the posterior wall to reveal thick gelatinous mucoid material lying within a cystic cavity formed by the vein wall. The cyst was excised, with reconstruction of the iliac femoral vein graft using an artificial blood vessel (Figure 2). Histology of the excised specimen revealed a cystic structure with

layers of collagen separated by scanty elastic fibers, fibrosis in the wall of the vein and the excised tissue was connective tissue with infiltration of inflammatory cells (Figure 3). Postoperatively, the patient received anticoagulation therapy with warfarin and made an uneventful recovery. At the 6-month follow-up, the swelling in the leg had resolved, and the common femoral vein was patent on color duplex imaging, with no mass effect.

3. Discussion

ACD is characterized by the accumulation of a gelatinous fluid containing mucoproteins and mucopolysaccharides within the adventitial layer of a blood vessel (1). ACD was first reported in 1947 by Atkins and Key (3), but ACD of the arteries is more frequent in men, it is predominantly located in the popliteal artery and it clinically presents with intermittent claudication (2). However, ACD of the venous system is a very rare condition, with fewer than 20 cases reported in the worldwide literature (4-8). One of the earliest reports of venous ACD was in the short saphenous vein, and in contrast to arterial ACD, venous

ACD rarely affects the popliteal segment. The venous variety occurs with an equal frequency in both sexes and it most often involves the common femoral vein and causes swelling of the affected limb (2).

The exact etiology of ACD remains unclear, but it can be explained in similar terms as that of arterial ACD (2,9-12): *i*) The repeated trauma theory (the adventitia undergoes cystic degeneration as a result of stretching and distortion near the joints); *ii*) Ectopic aganglionosis (synovial cells implant into adventitia usually related to arterial ACD near joints); *iii*) the systemic disorder theory (degeneration of the adventitia as a result of connective tissue diseases); and *iv*) the developmental theory (mesenchymal cells from nearby joints implant into the adventitia of the vessel during embryological development).

Histopathologically, the cyst may be uniloculated or multiloculated. The disease process produces an expanding cyst that destroys the elastic tissue between the medium and the adventitia of the vessel wall, and the elastic tissue is replaced with fibrous connective tissue. There is usually no acute or chronic inflammation. The cyst is lined with fibrous connective tissue and the cyst contains an eosinophilic mucoid gel that consists of mucoproteins and mucopolysaccharides (1,5).

The diagnosis of ACD of the vein can be suspected on the basis of patient history, results of a physical examination and image findings. As was the case in our patient, the diagnosis is rarely made before surgery owing to the rarity of the condition. First-line investigation should probably involve duplex ultrasound imaging, which is cheap, available in most centers, and noninvasive, to exclude aneurysms and synovial cysts and to localize the cyst to the vessel wall. Ultrasound imaging may show the presence of a typical, anechoic mass with a posterior acoustic window and may allow ultrasound-guided treatment (13,14). CT and magnetic resonance imaging (MRI) have also been advocated to localize the pathology to the vessel wall, exclude other pathologies (such as Baker's cyst), and allow guided drainage. MRI can reveal high-signal-intensity cysts with extrinsic compression of the vessel lumen. Venography in venous ACD may reveal the site and extent of obstruction to flow and may show a classic scalloped appearance or hourglass narrowing caused by extrinsic compression of the vessel lumen. CT venography is superior to traditional venography for making the diagnosis because the cystic mass can be directly observed regardless of the degree of obstruction. A CT venography can be successfully used in imaging ACD of the vein. When compared with venography, it has the advantage of a noninvasive examination that can directly image the surrounding parenchyma and aid in surgical or percutaneous treatment planning. Whatever imaging is used, it will be necessary to have a high index of suspicion to correctly diagnose this rare condition preoperatively (15-17).

Owing to the small number of reported cases, the ideal treatment is unknown and there are three options for venous ACD treatment: *i*) Most authors advocate transadventitial or transluminal evacuation of the cyst, with removal of the cyst wall to prevent recurrence, as in the case we have described. *ii*) Minimally invasive management has been reported with image-guided drainage of adventitial cysts, but incomplete evacuation of cysts secondary to high viscosity has resulted in high recurrence rates. *iii*) The use of needle aspiration of the fluid, guided by ultrasound or CT, has been tried successfully in some cases, although the fluid has a tendency to reaccumulate because the mucin-secreting mesenchymal cells are left in situ (1,4,13).

In summary, ACD of the vein is a rare malady, but it should be suspected for patients with symptoms of deep vein thrombosis, and especially when the diagnostic investigation indicates an extrinsic mass. Thus, ACD of the vein needs to be considered in the differential diagnosis of unexplained leg swelling. Furthermore, to ensure a successful outcome, close follow-up of the patient is necessary.

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References

1. Ksepka M, Li A, Norman S. Cystic Adventitial Disease. *Ultrasound Q.* 2015; 31:224-226.
2. Prasad NK, Desy NM, Amrami KK, Spinner RJ. How to explain cystic adventitial disease coexisting in an adjacent artery and vein. *Clin Anat.* 2015; 28:833-835.
3. Atkins HJB, Key JA. A case of myxomatous tumour arising in the adventitia of the left external iliac artery. *Br J Surg.* 1947; 34:426-427.
4. Kwun WH, Suh BY. Adventitial cystic disease of common femoral vein. *J Korean Surg Soc.* 2011; 80(Suppl 1):S75-S79.
5. Kim SH, Lee CE, Park HO, Kim JW, Choi JY, Lee JH. Adventitial cystic disease of the common femoral artery: A case report and literature review. *Korean J Thorac Cardiovasc Surg.* 2013; 46:150-152.
6. Mousa AY, Alhalbouni S, Abu-Halimah S, Gill G, Sadek B, Nanjundappa A, Hass SM, Aburahma AF. Cystic adventitial disease of the common femoral vein: A case report and review of the literature. *Vasc Endovascular Surg.* 2013; 47:569-572.
7. Scott MF, Gavin T, Levin S. Venous cystic adventitial disease presenting as an enlarging groin mass. *Ann Vasc Surg.* 2014; 28:489.e15-e18.
8. Chen Y, Sun R, Shao J, Li Y, Liu C. A contemporary review of venous adventitial cystic disease and three case reports. *Phlebology.* 2015; 30:11-16.
9. Allemang MT, Kashyap VS. Adventitial cystic disease of the popliteal artery. *J Vasc Surg.* 2015; 62:490.
10. Veraldi GF, Scudo G, Scorsone L, Mezzetto L, Castellani RL. Cystic adventitial disease of the popliteal artery: Report of two cases and review of the literature. *G Chir.*

- 2014; 35:229-234.
11. Lejay A, Ohana M, Delay C, Georg Y, Girsowicz E, Thaveau F, Scholey JW, Geny B, Chakfe N. Cystic adventitial pathology as an entity in peripheral arterial disease. *J Cardiovasc Surg (Torino)*. 2016; 57:282-291.
 12. Meecham L, Wright A, Atwal A. Cystic adventitial disease of popliteal artery: Case report. *Int J Angiol*. 2016; 25:68-69.
 13. Paravastu SC, Regi JM, Turner DR, Gaines PA. A contemporary review of cystic adventitial disease. *Vasc Endovascular Surg*. 2012; 46:5-14.
 14. Niizeki T, Ishino M, Kitahara T, Yamauchi S, Ikeno E, Kubota I. A case of cystic adventitial degeneration of the left popliteal artery diagnosed by intravascular ultrasound. *Clin Med Insights Case Rep*. 2016; 9:11-14.
 15. Johnson JM1, Kiankhooy A, Bertges DJ, Morris CS. Percutaneous image-guided aspiration and sclerosis of adventitial cystic disease of the femoral vein. *Cardiovasc Intervent Radiol*. 2009; 32:812e816.
 16. Seo JY, Chung DJ, Kim JH. Adventitial cystic disease of the femoral vein: A case report with the CT venography. *Korean J Radiol*. 2009; 10:89-92.
 17. Hai Z, Shao G. Images in vascular medicine. Cystic adventitial disease of the popliteal artery. *Vasc Med*. 2012; 17:283-284.

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Behavioral phenotype in a child with Prader-Willi syndrome and comorbid 47, XYY

Pooja Palkar, Anahid Kabasakalian, Bonnie Taylor, Ellen Doernberg, Casara Jean Ferretti, Genoveva Uzunova, Eric Hollander*

Autism and Obsessive Compulsive Spectrum Program, Department of Psychiatry, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, NY, USA.

Summary

We report a 12-year-old male with Prader-Willi syndrome (PWS) and 47, XYY syndrome. Genetic work up revealed 47, XYY karyotype. PWS diagnosis was made by polymerase chain reaction methylation and maternal uniparental disomy (mUPD) was determined to be the etiology. Review of distinct behavioral features, possible interplay between the two syndromes and considerations for diagnoses are presented. To our knowledge, this is the first report of behavioral features in PWS with comorbid 47, XYY.

Keywords: Prader-Willi syndrome, 47, XYY, autism spectrum disorder, attention deficit hyperactivity disorder

1. Introduction

Prader-Willi syndrome (PWS) and 47, XYY syndrome both are rare genetic conditions and their concurrence is even rarer. Each is characterized by discrete physical and behavioral features. PWS is a neurodevelopmental disorder resulting from loss of function or deletion of genes in a particular region of chromosome 15 (critical region 15q11-q13) (1). About 70% cases are due to deletion of the gene segment on paternal chromosome 15. About 25% cases are due to maternal uniparental disomy (mUPD) which means both copies of chromosome 15 are maternally inherited instead of one from each parent (1). Other cases are attributed to translocations and genomic imprinting defects. Clinical features of PWS include neonatal hypotonia, childhood onset hyperphagia with subsequent obesity, hypogonadism, short-stature, facial dysmorphism along with significant neurological, cognitive and behavioral abnormalities. Common behavioral features in PWS are

food seeking behavior, resistance to change, irritability, skin picking and temper tantrums. These are a source of significant impairment. Secondly, 47, XYY syndrome is caused by the presence of an extra Y chromosome in each cell due to nondisjunction i.e. an error during cell division. Patients may go undiagnosed and may be diagnosed during evaluation for tall stature. The XYY phenotype commonly includes tall stature, macrocephaly, macroorchidism, hypotonia, hypertelorism, and tremor (2). Behavioral abnormalities may not always be present and vary widely among affected individuals. When abnormalities do occur, patients may have learning disabilities, delayed development of speech, language and motor skills. Feature in common for the two syndromes is risk for autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD).

2. Clinical Report

We discuss a 12-year-old Caucasian male with diagnosis of PWS and 47, XYY syndrome who presented to our clinic. He was born full term to a healthy 27-year-old gravida 1, para 1 mother. Perinatal history was significant for reduced fetal movements and a three pound weight loss in mother around 38 weeks of gestation. Ultrasound showed diminished umbilical cord flow and the mother was induced but she failed to progress. Therefore, patient was delivered by emergency C-section after he showed non-reassuring heart tones. Birth weight was 2,700 g and

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*Address correspondence to:

Dr. Eric Hollander, Autism and Obsessive Compulsive Spectrum Program, Department of Psychiatry, Montefiore Medical Center, Albert Einstein College of Medicine, 111 East 210th Street, Bronx, NY- 10467, USA.
E-mail: eholland@montefiore.org

APGAR scores were 6 and 7. At delivery, he had PWS specific features such as hypotonia, feeding difficulties with poor respiratory effort and received resuscitation for the same. Subsequently, he required Neonatal Intensive Care Unit (NICU) admission and treatment with surfactant for several days. Patient had microphallus and bilateral undescended testes, both commonly seen in PWS. He showed global developmental delay and intellectual disability. He sat by himself at 6 months of age, crawled at 11-12 months of age and walked independently at 19 months of age. He started speaking words at 2 years of age. He has been receiving special education. Patient received growth hormone from age 6 months to 11 years. It was stopped at 11 years at the request of the parents because of attainment of optimum growth.

According to his parents, he relies on schedule and insists on sameness. They reported- "The schedule is comforting and rules really help him navigate". When given an advanced notice and/or explanation patient was better able to tolerate deviations from the schedule or anticipated events. He has a particular interest in sports and his conversations are focused on teams and players. He is a devoted fan of a sports team and may become quite upset if they lose. Parents denied food seeking behavior. They do not restrict the range of foods he eats, but monitor his calorie intake.

Past history is significant for a 20 pounds weight gain around age 10 years. He lost the weight during an eight week hospitalization at an inpatient program for Prader Willi patients. He also had periods of increased aggression and hitting at the time and was started on escitalopram by his doctor to address his anxiety. He had an adverse reaction to the medication as demonstrated by increased irritability, tantrums, aggressiveness, restlessness and disrupted sleep. Therefore, escitalopram was discontinued. Owing to his aggression, irritability and frustration he was started on a stimulant at the time and continues to take methylphenidate to date. He achieved better symptom control, ability to articulate his feelings and internal experiences by saying things he felt rather than acting out. It also improved his attention, memory and possibly contributed to the weight loss.

On psychiatric evaluation at our clinic, his major issue was anticipatory anxiety which was evident in his repeated questioning, particularly with regards to events in future. Questioning stopped once he was reassured and knew what to anticipate. Thus, difficulty tolerating changes in routine was noted. He also demonstrated anxiety around new things and frequently asked "why?" to requests during the clinical encounter. He became very anxious when his abdomen was exposed for physical exam and was noted to have an exaggerated emotional response to disappointment. He demonstrated repetitive behavior like rubbing his forehead. C-YBOCS (Children's Yale-brown Obsessive Compulsive Scale: Modified for ASD) score was 16 which is moderately

high. Preoccupation and restricted range of interest with regards to sports and his favorite teams was noted. He did not demonstrate hyperphagia, food related obsessions, skin picking, hoarding behaviors, self-injurious behavior, suicidal ideation, impulsivity, hyperactivity, psychotic features and seizures.

Neurological exam showed high threshold for pain, temperature dysregulation, low tone and clumsiness. Patient was not overweight at the time of evaluation. His weight and height have been trending in 75th and 80th percentile respectively. Laboratory reports showed slightly raised LDL and hematocrit levels.

3. PWS and 47, XYY syndrome

The clinical history and presentation of the patient identified anticipatory anxiety as a major behavioral issue. PWS has been shown to be a highly complex psychological disorder with multiple areas of disturbance and anxiety could be a part of this symptom complex (3,4). His repetitive behavior, resistance to change and temper outbursts can be attributed to behavioral phenotype of PWS (5,6). Patient demonstrates many but not all behavioral symptoms typically seen in PWS. He did not manifest hyperphagia, food seeking behavior and skin picking which are common in PWS. It would be interesting to seek if this is a result of interplay between the two genetic disorders.

The fact that patient responded well to stimulants may indicate an underlying component of ADHD. Patient did not manifest hyperactivity and impulsivity which are common in ADHD. This also raises the question whether patients with PWS and 47, XYY have atypical presentation for ADHD. Skokauskas *et al.* noted ADHD-like behavior in PWS patients in their research study (7). Bardsley *et al.* performed a cohort study that showed higher prevalence of ADHD and ASD in men with with XYY (2). Thus, patients with 47, XYY should be evaluated for both (8). This patient has PWS and XYY, so he is at increased risk of having ADHD and ASD. The case highlights the importance of evaluating patients with PWS and comorbid 47, XYY syndrome for ADHD and ASD. PWS is a genetic syndrome in which careful attention to comorbidities and details regarding all potential behavioral and somatic manifestations can lead to a significant improvement in health.

References

1. Bittel DC, Butler MG. Prader-Willi syndrome: Clinical genetics, cytogenetics and molecular biology. *Expert Rev Mol Med.* 2005; 7:1-20.
2. Bardsley MZ, Kowal K, Levy C, Gosek A, Ayari N, Tartaglia N, Lahlou N, Winder B, Grimes S, Ross JL. 47, XYY syndrome: Clinical phenotype and timing of ascertainment. *J Pediatr.* 2013; 163:1085-1094.
3. Whitman BY, Accardo P. Emotional symptoms in Prader-Willi syndrome adolescents. *Am J Med Genet.* 1987;

- 28:897-905.
4. Reddy LA, Pfeiffer SI. Behavioral and emotional symptoms of children and adolescents with Prader-Willi syndrome. *J Autism Dev Disord.* 2007; 37:830-839.
 5. Dykens EM, Kasari C. Maladaptive behavior in children with Prader-Willi syndrome, down syndrome, and nonspecific mental retardation. *Am J Ment Retard.* 1997; 102:228-237.
 6. Clarke DJ, Boer H, Whittington J, Holland A, Butler J, Webb T. Prader-Willi syndrome, compulsive and ritualistic behaviours: The first population-based survey. *Br J Psychiatry.* 2002; 180:358-362.
 7. Skokauskas N, Sweeny E, Meehan J, Gallagher L. Mental health problems in children with Prader-Willi syndrome. *J Can Acad Child Adolesc Psychiatry.* 2012; 21:194-203.
 8. Ross JL, Tartaglia N, Merry DE, Dalva M, Zinn AR. Behavioral phenotypes in males with XYY and possible role of increased NLGN4Y expression in autism features. *Genes Brain Behav.* 2015; 14:137-144.

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Editorial and Head Office:

Pearl City Koishikawa 603
2-4-5 Kasuga, Bunkyo-ku
Tokyo 112-0003, Japan
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