ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 5, Number 4
November, 2016

IRCA BSSA

IACMHR

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

Intractable & Rare Diseases Research is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published quarterly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA, Shandong Academy of Medical Sciences, and Shandong Rare Disease Association.

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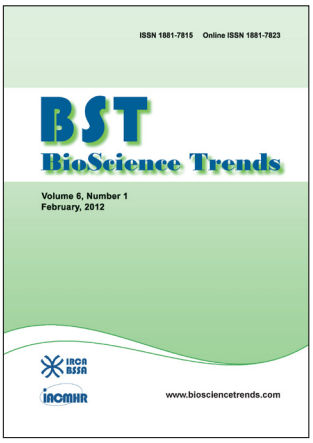
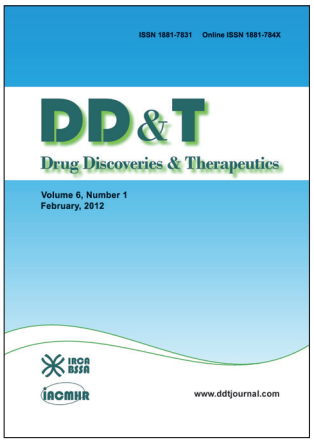
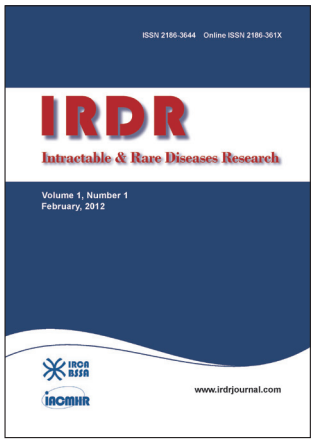


ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.biosciencetrends.com



ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.ddtjournal.com



ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.
www.irdrjournal.com

Intractable & Rare Diseases Research

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
E-mail: office@irdrjournal.com
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Editorial Board

CONTENTS

Volume 5, Number 4, 2016

Policy Forum

Reviews

Original Article

Brief Reports

Case Reports

CONTENTS (*Continued*)

(*This journal was partially supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.*)

Policy Forum

DOI: 10.5582/irdr.2016.01037

The Government's role in regulating, coordinating, and standardizing the response to Alzheimer's disease: Anticipated international cooperation in the area of intractable and rare diseases

Qi Tang[1,2], Peipei Song[3], Lingzhong Xu[1,*]

[1] *Department of Social Medicine and Medical Service Management, School of Public Health, Shandong University, Ji'nan, Shandong, China;*
[2] *Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;*
[3] *Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa-shi, Chiba, Japan.*

Summary **The World Health Organization (WHO) has emphasized that aging of the population is inextricably linked to many other global public health issues, such as universal health coverage, non-communicable diseases, and disability. However, Alzheimer's Disease International (ADI) estimates that 46.8 million elderly people worldwide were living with dementia in 2015. Alzheimer's disease (AD), the most common form of dementia, is one of the most common neurodegenerative diseases and is the main cause of cognitive impairment. AD will affect 5-7 out of every 100 older adults who are age 60 years or over. In response to the serious challenge posed by AD, governments are expected to play an important role in the prevention, diagnosis, and treatment of AD. As specific examples, *i*) the Japanese Government has instituted and supported regulations to encourage the development of AD drugs in order to accelerate research and development of innovative drugs; *ii*) the United States Government has cooperated with multiple partners such as non-governmental organizations in the response to AD; *iii*) Chinese governmental measures have standardized clinical diagnosis and treatment as part of the response to AD, including eligible patients, diagnostic criteria, therapeutic schedules, drug selection, and required inspections; *iv*) with political support from member governments, the European Union has issued guidelines and conducted clinical studies on medicines for the treatment of AD in order to ascertain the various stages of the disease and the relevance of biomarkers. AD is an intractable disease, so different countries need to share clinic trial information and cooperate in the conduct of those trials. International cooperation will play a key role in the response to other intractable and rare diseases.**

Keywords: Alzheimer's disease, dementia, accelerated regulation, cooperation with multiple partners, clinical pathway

1. Introduction

As a result of aging of the population, there were 900 million people (12.2% of the population) worldwide age 60 years or over in 2015 (*1*). The World Health Organization (WHO) has emphasized that aging of the population is inextricably linked to many other global public health issues, such as universal health coverage, non-communicable diseases, and disability (*2*). Alzheimer's Disease International (ADI) estimates that 46.8 million elderly people worldwide were living with dementia in 2015, which included 22.9 million in Asia, 10.5 million in Europe, 9.4 million in the United States (US), and 4.0 million in Africa (*3*). Alzheimer's disease (AD), a common form of dementia, is one of the most common neurodegenerative diseases and is the

Released online in J-STAGE as advance publication October 24, 2016.

**Address correspondence to:*
Dr. Lingzhong Xu, Department of Social Medicine and Medical Service Management, School of Public Health, Shandong University, No.44, Wenhuaxi Road, Ji'nan 250012, Shandong, China.
E-mail: lzxu@sdu.edu.cn

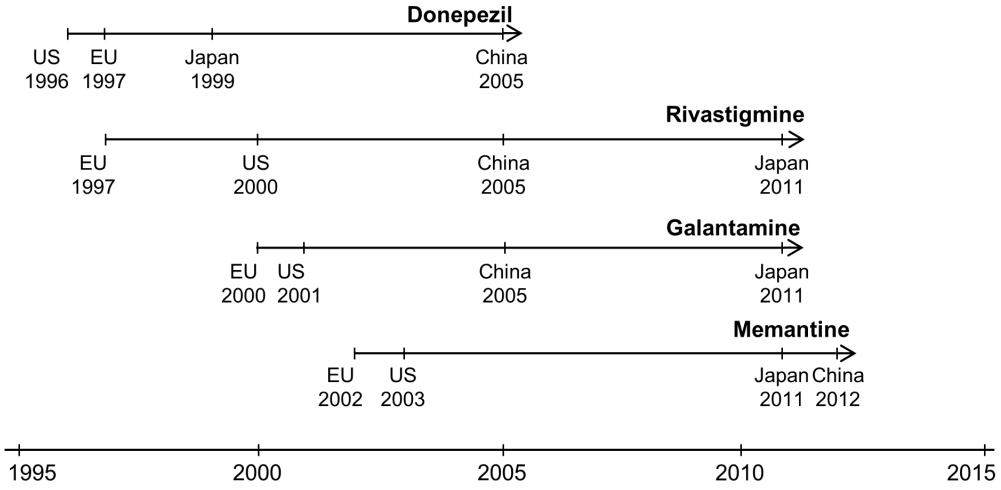

Figure 1. The four drugs approved for the treatment of Alzheimer's disease in Japan, the US, the EU, and China. Donepezil, rivastigmine, galantamine, and memantine are four conventional drugs for the treatment of AD. The official release date of AD drugs in Japan lagged behind that in the EU and the US, resulting in the issue of a "lag in AD drugs." Indeed, the year of approval for Galantamine was far later China, mainly because of the lag in the experimental stage and the prolonged application process. In response, the Pharmaceuticals and Medical Devices Agency (PMDA) put forward appropriate countermeasures, such as increase in examinants, gradually reducing the "lag in AD drugs." AD: Alzheimer's disease; EU: European Union; US: United States.

main cause of cognitive impairment (*4*). AD will affect 5-7 out of every 100 older adults who are age 60 years or over (*5*).

AD is an incurable disease, and a major symptom of AD is progressive dementia that eventually results in disruption of daily life (*6*). Current drugs for AD target cholinergic and glutamatergic neuro transmission, thus improving symptoms, but their neuroprotective activity is still debated (*7*). Much effort is directed towards identifying disease-modifying therapies, as indicated by the fact that several compounds in different phases of development. Since AD can start 10-15 years before it becomes full-blown, once a person is displaying classic symptoms of dementia may be too late, so drug treatment during its early stages remains one hope to stem the disease's course (*8*).

In response to the serious challenge posed by AD, governments are expected to play an important role in the prevention, diagnosis, and treatment of AD. The purpose of this paper is to explore the government's role in regulation, coordination, and standardization in order to provide a reference for government support of the response to AD and to put forward several suggestions.

2. Government regulations to encourage the development of AD drugs

The Japanese Government has instituted and supported regulations to encourage the development of AD drugs in order to accelerate research and development of innovative drugs. In Japan, the elderly age 60 years or over account for 33.2% of the total population, which is the highest proportion in the world, thus underscoring the urgent need for discovery of AD drugs (*9*).

Overcoming the "lag in AD drugs" with government initiatives. The issue of a "lag in AD drugs" refers to the fact that the official release date of AD drugs in Japan lagged behind that in Europe and the US, mainly because of a lag in the experimental stage and the prolonged application process. In response, the Pharmaceuticals and Medical Devices Agency (PMDA), a Japanese regulatory agency, put forward appropriate countermeasures, such as increase in examinants, gradually reducing the "lag in AD drugs" (Figure 1). As a specific example, donepezil is used to improve cognition and behavior. Donepezil was the first drug approved for the treatment of AD in Japan in 1999. In 2011, 2 cholinesterase inhibitors (galantamine and rivastigmine) and an N-methyl-D-aspartate receptor inhibitor (memantine) were approved as symptomatic drugs, and the prescription of 3 drugs to patients with AD thus became possible in clinical settings, thereby overcoming the issue of the "lag in AD drugs" (*10*).

Accelerating research. The Japanese Government has implemented measures to accelerate research. According to international trends in drug development and research on AD drugs, the following three topics have passed review by the Ministry of Health, Labor and Welfare (MHLW) and will be implemented by the PMDA and University of Tokyo Hospital: *i*) trials examining AD drug dosage in comparison to a placebo; *ii*) differences in results of AD research and medical settings in Japan and overseas; *iii*) clinical trials of proof of concept (POC) and first in human (FIH) trials in Japan. In order to facilitate the implementation of these projects, a "Strategic discussion of pharmaceutical

affairs" was instituted in 2011 with support from the MHLW, guidance from the PMDA and involvement of colleges, research institutes, and companies (*11*). The main purposes include *i*) announcement of applications for AD drugs and devices and *ii*) routine communication between academia and the PDMA and guidance on clinical trials.

Facilitating the development of innovative drugs. In 2012, Japan's MHLW launched a novel project entitled "Accelerating Regulatory Science Initiatives" to facilitate the development of innovative drugs by developing a guideline for innovative drugs and by promoting personnel exchanges by the PMDA and research institutions (*12*). The PMDA, in cooperation with the University of Tokyo Hospital, implemented a research project to establish two research groups to develop a guideline for the clinical evaluation of drugs for AD: *i*) the Biomarker and Clinical Evaluation Group will establish biomarker-based criteria for the clinical evaluation of drugs for AD, and *ii*) the Modeling and Simulation Group will create disease models of AD using these techniques. Based on the support from the MHLW and collaboration between the PMDA and University of Tokyo Hospital, issues in clinical evaluation and development have been identified for the first time, including the use of biomarkers in inclusion criteria, efficacy endpoints, and clinical data required for application in Japan.

3. Government emphasis on cooperation with multiple partners in the response to AD

The US Government cooperates with multiple partners in the response to AD. As the sixth leading cause of death in the US, AD is a terrible progressive disease that destroys brain cells. The primary federal agency engaged in AD research, the National Institute on Aging (NIA) is a division of the US National Institutes of Health (NIH). The NIA is leading a broad scientific effort to understand the nature of aging and to extend the healthy, active years of life. The NIA emphasizes cooperation with multiple partners such as non-governmental organizations (NGOs).

Global clinical trials of AD drugs. Currently, there are two types of medications approved by Food and Drug Administration (FDA), cholinesterase inhibitors (Aricept, Exelon, Razadyne, and Cognex) and memantine (Namenda), to treat cognitive symptoms such as memory loss, confusion, and problems with thinking and reasoning of early AD (*13*). In order to test the effectiveness of these drugs, the Alzheimer's Disease Neuroimaging Initiative (ADNI), a global research project started in the US, actively supports the investigation and development of treatments that slow or stop the progression of AD, elucidating various disease biomarkers that reflect and even predict the progression of disease (*14*). In addition, three clinical trials are being conducted by the Alzheimer's Prevention Initiative (API), the Dominantly Inherited Alzheimer's Network (DIAN), and the Alzheimer's Therapeutic Research Institute (ATRI) to examine the preventive effects of AD drugs in the preclinical phase (*15*).

The NIA and the Alzheimer's Association. The Alzheimer's Association is the leading voluntary health organization in Alzheimer's care, support, and research. The Association works at the global, national, and local level to enhance care and support for all those affected by Alzheimer's and other dementias. With the encouragement of the NIA, the Alzheimer's Association was founded on April 10, 1980 (*16*). The Alzheimer's Association made new investments of over $17 million in more than 80 scientific investigations, part of the over 350 ongoing research projects funded by the Association in 21 countries, totaling over $80 million. These include grant awards to 68 projects through the Association's International Research Grant Program, representing proposals ranked highest by peer reviewers from an extremely competitive field of over 1,000 proposal ideas (540 invited applications). Since 1982, the Association has invested over $350 million in more than 2,300 scientific investigations. Advancing AD research remains a core element of the Association's identity and a key facet of its mission (*17*).

Cooperation with other research institutes. Cooperation with other research institutes, grant partnerships, and even joint studies led by government are crucial to a project working successfully (*18*). As specific examples, *i*) Biomarkers Across Neurodegenerative Diseases (BAND) is a program to stimulate analyses across AD, to perform further analysis of existing research information to advance biomarker discovery, and to standardize assays, genetic profiles, and imaging modalities; *ii*) Mechanisms of Cellular Death in Neurodegeneration (MEND) is a program to discover and understand mechanisms and pathophysiological processes by which brain cell loss is mediated in AD and thereby seek insights and potential targets for therapeutic interventions that would sustain healthy brain function, and *iii*) Imaging Dementia Evidence for Amyloid Scanning (IDEAS) is a program to determine clinical usefulness of diagnosing AD with a brain PET scan that detects amyloid plaques, a core feature of AD.

4. Government standardization of AD treatment

4.1. *The Chinese Government has implemented a clinical pathway for AD*

As of the end of 2011, there were 185 million people age 60 years or over in China, accounting for 13.7% of the total population. Aging is a key issue in China nowadays, given the serious challenge posed by AD (*19*). AD has a similar level of prevalence in China, Japan, the US, and Europe (Table 1) (*9,20-23*). Although the

Table 1. The prevalence of Alzheimer's disease in China, Japan, the US, and Europe

Year	Location	Population	Prevalence (%)	Reference
2008	Spain	*n* = 2,170	6.6	Tola-Arribas MA, 2013 (*5*)
2008-2009	China	*n* = 10,276; Age ≥ 65	5.14	Jia J, 2014 (*6*)
2010-2012	Japan	*n* = 2,922	5.7	Yasue M, 2015 (*7*)
2013	Portugal	*n* = 160,287; Age ≥ 60	5.91	Santana I, 2015 (*8*)
2015	US	Meta-Analysis	5.5	Steenland K, 2015 (*9*)

Table 2. Government support for treatment of Alzheimer's disease in Japan, the US, China, and the EU

Role	State	Measures	Results
Regulation	Japan	*i*) The PMDA put forward appropriate countermeasures, such as an increase in examinants, gradually reducing the "lag in AD drugs"; *ii*) Three topics have passed review by the MHLW and have been implemented by the PMDA and University of Tokyo Hospital; *iii*) A "Strategic discussion of pharmaceutical affairs" was instituted in 2011 with support from the MHLW, guidance from the PMDA, and involvement of colleges, research institutes, and companies; *iv*) In 2012, Japan's MHLW launched a novel project entitled "Accelerating Regulatory Science Initiatives" to facilitate the development of innovative drugs.	*i*) The prescription of 4 drugs to patients with AD thus became possible in clinical settings, thereby overcoming the issue of the "lag in AD drugs"; *ii*) Announcement of applications for AD drugs and devices and *ii*) routine communication between academia and the PDMA and guidance on clinical trials; *iii*) Issues in clinical evaluation and development have been identified for the first time, including the use of biomarkers in inclusion criteria, efficacy endpoints, and clinical data required for application in Japan.
Coordination	US	*i*) In order to test the effectiveness of drugs, ADNI, which was supported by the NIA, actively supports the investigation and development of treatments that slow or stop the progression of AD; *ii*) With the encouragement of the NIA, the Alzheimer's Association was founded on April 10, 1980; *iii*) The NIA cooperates with multiple partners such as BAND, MEND, IDEAS, and other NGOs in the response to AD.	*i*) Clinical trials by the API, DIAN, and ATRI are underway; *ii*) Since 1982, the Association has invested over $350 million in more than 2,300 scientific investigations; *iii*) Communication and cooperation on advance biomarker discovery, pathophysiological processes, and clinical usefulness.
Standardization	China	*i*) Peking University develops CPAD in 2013; *ii*) The NHFPC implements a clinical pathway for AD in China in April 2016; the CMA oversees the administrators of health care and medical facilities that manage the quality of medical care.	CPAD has standardized clinical diagnosis and treatment as part of the response to AD, including eligible patients, diagnostic criteria, therapeutic schedules, drug selection, and required inspections
	EU	*i*) In 2008, the CHMP issued the "*Guideline on Medicinal Products for the Treatment of Alzheimer's Disease and other Dementias*"; *ii*) In 2014, the CHMP released a paper entitled "*Discussion Paper on the Clinical Investigation of Medicines for the Treatment of Alzheimer's Disease and Other Dementias*".	*i*) Impact of new diagnostic criteria for AD; *ii*) Potential use of biomarkers and their temporal relationship with the different phases of AD in different stages of drug development; *iii*) Evaluation of the efficacy and safety of AD drugs.

ATRI: Alzheimer's Therapeutic Research Institute; ADNI: Alzheimer's Disease Neuroimaging Initiative; API: Alzheimer's Prevention Initiative; CHMP: the Committee for Medicinal Products for Human Use; CMA: Chinese Medical Association; CPAD: the Clinical Pathway for Alzheimer's Disease in China; DIAN: Dominantly Inherited Alzheimer's Network; EU: European Union; MHLW: the Ministry of Health, Labor, and Welfare; NGO: non-governmental organization; NHFPC: the National Health and Family Planning Commission; NIA: the National Institute on Aging; PMDA: the Pharmaceuticals and Medical Devices Agency; US: United States.

prevalence rates are roughly the same in these countries, China accounts for the major share of AD prevalence worldwide because of China's huge population. Chan *et al*. estimated that there were 5.69 million people with AD (3.85-7.53) in 2010 (*19*).

The Chinese Government has focused on a clinical pathway for AD. Evidence that provides the basis for guidelines is mostly from trials conducted in other countries due to very limited Chinese data available for local systematic review. Thus, more local evidence on AD care is needed to develop an evidence-based guideline appropriate for people in China. The inadequate implementation of the current AD guideline has resulted in a low rate of diagnosis and high rate of missed diagnosis, representing a further obstacle for patients with AD to receive dementia care in different areas nationwide. With this in mind, Peking University developed the Clinical Pathway for Alzheimer's Disease in China (CPAD) in 2013. The pathway was developed by determining how AD was clinically diagnosed and treated by physicians in routine practice, and the pathway should help address the low rate of AD diagnosis and the low rate of prescription of anti-dementia drugs and to support guideline development (*24*).

Based on previous studies, the National Health and Family Planning Commission (NHFPC) implemented a clinical pathway for AD in China in April 2016; the Chinese Medical Association (CMA) oversees the administrators of health care and medical facilities that manage the quality of medical care (*25-26*). This pathway has standardized clinical diagnosis and treatment as part of the response to AD, including eligible patients, diagnostic criteria, therapeutic schedules, drug selection, and required inspections.

4.2. *The clinical guideline on AD medicines in the European Union (EU)*

In 2008, the Committee for Medicinal Products for Human Use (CHMP), a center of the European Medicines Agency (EMA), issued the "*Guideline on Medicinal Products for the Treatment of Alzheimer's Disease and other Dementias*". In 2014, the CHMP released a paper entitled "*Discussion Paper on the Clinical Investigation of Medicines for the Treatment of Alzheimer's Disease and Other Dementias* (EMA/CHMP/539931/2014 Corr.)" (*27*). Given the current uncertainties regarding the pathophysiology of AD, the draft Guideline is intended to identify the various stages of the disease and the relevance of biomarkers. The draft Guideline aims at integrating the requirements for development programs which start earlier in the disease course with the necessary adaptations to the distinct manifestations of the illness at these stages.

The draft Guideline addresses: *i*) the impact of new diagnostic criteria for AD (including early and even symptomatic stages of disease) on clinical trial design; *ii*) the potential use of biomarkers and their temporal relationship to the different phases of AD in different stages of drug development (mechanism of action, target engagement, use as a diagnostic test, enrichment of study populations, stratification for subgroups, safety and efficacy markers, *etc.*); *iii*) evaluation of the efficacy and safety of AD drugs by assembling data on their long-term safety, the speed of their anticipated action, and the duration of the trial.

5. Perspectives for the future

As indicated by the specific regulations to accelerate research, cooperative efforts with multiple partners, and standardization of diagnosis and treatment, government plays an important role in the response to AD (Table 2). As specific examples, *i*) the Japanese Government has instituted and supported regulations to encourage the development of AD drugs in order to accelerate research and development of innovative drugs; *ii*) the US Government cooperates with multiple partners such as the Alzheimer's Association, BAND, MEND, IDEAS, and other NGOs in the response to AD; *iii*) Chinese governmental measures have standardized clinical diagnosis and treatment as part of the response to AD, including eligible patients, diagnostic criteria, therapeutic schedules, drug selection, and required inspections; and *iv*) with political support from member governments, the EU has issued guidelines and conducted clinical studies on medicines for the treatment of AD in order to ascertain the various stages of the disease and the relevance of biomarkers.

The advantage of government support for AD research is that it accelerates new drug research and it reduces the nonstandard diagnosis of intractable diseases such as AD through regulation, coordination, and standardization. Once AD diagnosis and treatment has been nationally standardized, a clinical pathway covers all stages of AD diagnosis and treatment will guide physicians in caring for patients with AD. In addition, government support is also essential to facilitate the global establishment of common standards and guidelines for AD. AD is an intractable disease, so different countries need to share clinic trial information and cooperate in the conduct of those trials. International cooperation will play a key role in the response to other intractable and rare diseases.

Acknowledgements

This work is granted by the National Natural Science Foundation of China (71673169).

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(Received July 13, 2016; Revised September 21; Accepted September 30, 2016)

Review

DOI: 10.5582/irdr.2016.01054

An overview of Compassionate Use Programs in the European Union member states

Gayathri Balasubramanian[1,*], Suman Morampudi[1], Pankdeep Chhabra[1], Arun Gowda[1], Behsad Zomorodi[2]

[1] *FSRC, Part of phamax, Bangalore, India;*
[2] *Phamax AG, Basel, Switzerland.*

Summary **The past decade witnessed rapid development of novel drugs and therapeutic biological agents. The marketing authorization for novel therapies is often time consuming and distressing for patients. Earlier clinical trials were the only way to access new drugs under development. However, not every patient meets the enrolment criteria, and participation is difficult for patients with life-threatening, long-lasting or seriously debilitating diseases like rare diseases. Early access programs like "Compassionate Use Program (CUP)" have generated alternative channels for such patients. The European Medical Agency provides regulations and recommendations for compassionate use, upon which every European Union (EU) member state has developed its own rules and regulations. Despite previous reviews and studies, the available information is limited and gaps exist. This literature review explores CUP in 28 EU member states. Data was collected through literature review and use of country-specific search terms from the healthcare domain. Data sources were not limited to databases and articles published in journals, but also included grey literature. The results implied that CUP was present in 20 EU member states (71%). Of 28 EU states, 18 (~64%) had nationalized regulations and processes were well-defined. Overall, this review identified CUP and its current status and legislation in 28 EU member states. The established legislation for CUP in the EU member states suggest their willingness to adopt processes that facilitate earlier and better access to new medicines. Further research and periodic reviews are warranted to understand the contemporary and future regulatory trends in early access programs.**

Keywords: Compassionate use, early access, special access, rare diseases, orphan drugs, European Union, European Union member states

1. Introduction

The past decade has seen rapid development in the field of novel drugs and therapeutic biological agents. Despite the remarkable innovations that took place in the field of novel therapeutics, marketing authorization for promising novel therapies is time consuming, which can be at times distressing for some patients, particularly, those with severe diseases (*1*). This implies a serious bearing on the overall quality of life in such patients, because treatment can be challenging and at times inadequate with currently authorized medicines (*1*). Hence, new drugs that are unauthorized or in the late phase of clinical trials are the only hope for a plausible successful treatment in such patients.

In the past, clinical trials were the only way for patients in many countries to access drugs under development (*2*). However, clinical trials are time-consuming and expensive, and not every patient meets enrolment criteria for specific clinical trials (*1*). Also, participation in clinical trials is a difficult choice for patients with life-threatening, long-lasting or seriously debilitating diseases. Nonetheless, in recent times, early access programs have opened doors to more

Released online in J-STAGE as advance publication October 4, 2016.

**Address correspondence to:*
Dr. Gayathri Balasubramanian, FSRC, Part of phamax, #19, KMJ Ascend, 5th Block Koramangala, Bangalore 560095, India.
E-mail: gayathri.balasubramanian@fs-researchcenter.com

possibilities for such patients, of which "Compassionate Use Program (CUP)" is one. There are approximately 7,000 different types of rare diseases (*3*). Globally, it is estimated that approximately 350 million people suffer from rare diseases of which around 8.6% are from Europe (*3*). Since 2010, the number of compassionate use requests has risen by nearly 25% and a major proportion of these requests are from rare disease patient populations, owing to highly engaged patients and caregivers connected to advocacy groups (*4*). Many pharmaceutical companies are developing new therapies for patients with rare, life-threatening genetic diseases and are also facilitating access to such drugs (*5*). Lately, Genzyme Corporation has donated imiglucerase to hundreds of severely affected patients with Gaucher's disease in three large-scale international CUP (*6,7*). Such efforts by pharma majors to improve early access to life-threatening disease drugs makes it essential to understand CUP and the processes involved.

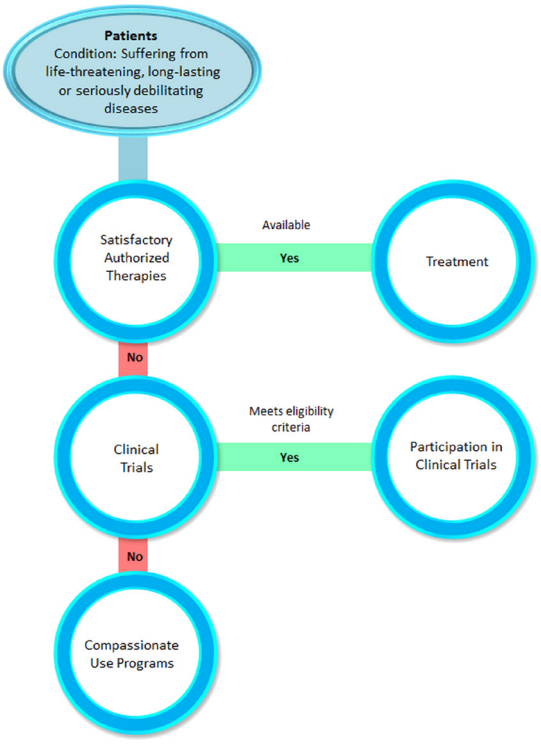

Figure 1. Pathway to compassionate use program. This figure depicts the pathway to access new medicines through compassionate use program for a patients suffering from severe or enervating diseases.

CUP are early access programs intended to facilitate the availability of new medicines to patients suffering with life threatening disorders or diseases in the EU member states (Figure 1) (*6*). In general, CUP are considered in the early stages of the product development cycle where patients get pre-launch access to the investigational drugs or drugs not yet authorized in the country. Unlike clinical trials, which are protocol driven and where participants have to meet certain inclusion and exclusion criteria, CUP allows patients without considering any criteria. But, CUP enrolls patients as per the laws and regulations outlined for the program (*2*). The European Medicines Agency (EMA) defines "compassionate use" as a treatment option that allows the use of an unauthorized medicinal product which is under development (*8*).

The EMA provides recommendations for compassionate use through the Committee for Medicinal Products for Human Use (CHMP). Also, laws and regulations are set by the EMA for compassionate use in the European Union (EU) (Table 1) (*8-11*). Every EU member state has developed its own legislation for CUP based on the EMA recommendations and legal framework. Therefore, it is necessary for stakeholders such as, health professionals, patients and patient organizations, pharmaceutical companies and policy makers to be informed of legislation and processes that facilitate or gain access to innovative medicines at the earliest.

Despite the availability of previous reviews and studies on CUP and related processes in the EU member states, the available information is superficial and limited to selected countries in the EU (*2,6,12*). Besides, a few gaps exist in the literature, due to changes and revisions of regulations and pathways that happen over time (*13*). Hence, there is a dearth of information in the published literature on existing CUP and current trends in all EU member states. Therefore, through this literature review we explored the presence of CUP in 28 EU member states to bridge the gaps for a better understanding of the legislation and specifics on CUP in every state.

The objectives of this review are to appraise the existing structure and processes for CUP in 28 EU member states, consolidate the information and present it as a comprehensive overview of the program in the countries.

Table 1. Laws and regulations set by the European Medical Agency for compassionate use in the European Union (*Ref. 9*)

Article 6 of Directive 2001/83/EC1 of the EU requires that medicinal products be authorized before they are marketed in a community. Previously, a clinical trial was the only option for using unauthorized medicinal products. However, CUP created a treatment option for patients in the EU suffering from a disease without existing satisfactory authorized therapy alternatives or who could not be part of a clinical trial. The EMA recommends compassionate use through the Committee for Medicinal Products for Human Use (CHMP) and a legal framework.
Article 83 (1) of Regulation (EC) No 726/2004 introduces the legal framework for compassionate use in the EU for medicinal products eligible to be authorized via the Centralized Procedure, stating that "By way of exemption from Article 6 of Directive 2001/83/EC, MS may make a medicinal product for human use belonging to the categories referred to in Article 3(1) and 3(2) of Regulation (EC) No 726/2004 available for compassionate use".

2. Methodology

Data was collected through an extensive literature review process to present the consolidated information. First, search terms like "compassionate use," "expanded access," "patient access programs," "medicines/drugs regulations," and "access to new drugs" were defined and included for each member state. Data was extracted using the country-specific search terms from diverse fields of study such as: health policies, medicines for rare diseases, pricing and reimbursement, health care access, health services research, and Federal documents. Iterative database (PubMed MEDLINE) searches were conducted to retrieve articles related to CUP. Since the subject required a thorough and systematic search for literature on regulations and CUP details, the data sources were not limited to articles published in journals, but also included grey literature. The sources for grey literature included: *i*) Government websites of respective countries; *ii*) Institutional repositories like the EU Commission, EMA and Rare Diseases Europe (Eurordis); *iii*) The EUR-Lex for legal documents portraying the medical laws, acts and compassionate use; *iv*) Bielefeld Academic Search Engine (BASE); *v*) OpenGrey; *vi*) Google; and *vii*) Others (blogs, newsletters and forums).

Additionally, a reference list of relevant articles was reviewed to find other studies. Subsequently, after abstract sifting, relevant articles that described CUP, its regulations and other related information for each member state were retrieved for further study. In instances where the current CUP information in a member state could not be ascertained or was not retrievable through literature search, it was labeled as unavailable. Data were recorded based on a set of three key themes: *i*) The CUP and a brief overview of the program including its definition in 28 EU member states; *ii*) National regulations on CUP in each of the 28 EU member states; *iii*) National authorities responsible for CUP.

For the purpose of this review, the countries under study were stratified into six factions. The EU5 countries are the first faction as they are referred to the most. The rest of the five factions comprise countries classified based on United Nations Geoscheme namely: Western Europe, Eastern Europe, Northern Europe, Southern Europe and Asia. Specific insights from the literature are presented as tables and charts for a meaningful comparison of CUP legislation and processes among the EU member states.

3. Findings

3.1. *Early access program*

Most of the EU member states have special programs that facilitate early patient access to new medicines through a national authority (*14,15*). However, the prevailing early access programs are known by various names in each country such as CUP, special access program, Named Patient Program (NPP), managed access program *etc*. (*2,16-20*). Moreover, these terms vary based on geographic location and are often used interchangeably. Also, they can imply different ideas with respect to the geographic area (*18*). Nonetheless, all these programs make a drug available to a patient prior to authorization and commercial launch in the country (*2,18*).

3.2. *Regulations for CUP*

This literature review reports the most recent CUP related processes in the EU member states. The appraisal of available literature such as government websites and reports revealed a strong evidence of adoption of CUP. There was a significantly positive correlation between the EU and individual country laws and recommendations. The results from the reviewed literature on CUP are summarized in Table 2 to Table 7. The tables compile the definition and an overview of CUP along with the medicinal products under it. On the whole, CUP was in place in 20 EU member states (71%) (Figure 2). These countries were Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Finland, France, Germany, Hungary, Italy, Latvia, Malta, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden and United Kingdom (UK). The remaining eight countries (Cyprus, Estonia, Greece, Ireland, Lithuania, Luxembourg, Poland and Slovakia)

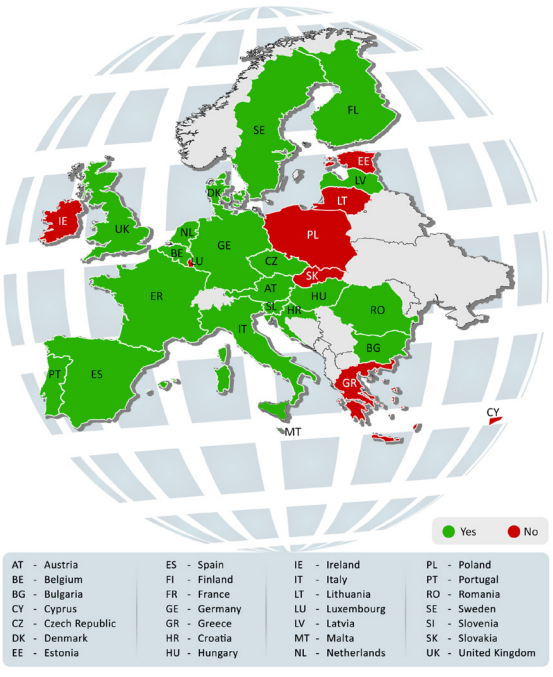

Figure 2. Compassionate use program in the European Union member states. This figure shows the presence of Compassionate use program in various European Union member states. The countries shaded in green have implemented the program and the ones in red have not.

Table 2. Compassionate use programs in EU5 countries (*Ref. 12,22-38*)

Items	France	Germany	Italy	Spain	UK
Authority involved	Agence nationale de sécurité du médicament et des produits de santé (ANSM)	German Federal Institute for Drugs and Medical Devices (BfArM) and the Paul-Ehrlich Institute (PEI)	Agenzia Italiana del Farmaco (AIFA)	Spanish Agency of Medicines and Health Products (AEMPS)	Medicines and Healthcare Products Regulatory Agency (MHRA)
Laws and regulations	Article L5121-12 and Article R5121-68	– Section 21 (2) no.6, German Medicinal Product Act (AMG), in conjunction with Article 83 of Regulation (EC) No. 726/2004 – Ordinance on Medicinal Products for Compassionate Use as per Section 80 of the German Medicinal Product Act was published on 21 July 2010 in the Federal Law Gazette 2010 part I No 37 and enforced on 22 July 2010	– A Ministerial Decree of 8 May 2003 Law no. 326 of 24 November 2003 on the price and reimbursement system – Also, the national legislation, Law 648/96, makes compassionate use possible at the National Health System's (SSN) expense	Real Decreto 1015 of 19th June 2009 Cohort or Nominative	– Human Medicines Regulations 2012 (SI 2012/1916) – The Guidance Note 14 on 'The supply of unlicensed relevant medicinal products for individual patients' Nominative
Overview	The conditions for ATU: – Specialties are to treat, prevent or diagnose serious or rare diseases – No proper treatment – Efficiency and job security are presumed as per the scientific knowledge Also, there are two types of ATU: – Cohort ATU - for a group or sub-group of registered patients – ATU – nominative - for named single patient	Medicinal products for human use can be used for specific groups of patients without marketing authorization or previous approval in Germany	An unauthorized medication can be included by AIFA in the official list to be prescribed at the NHS charge, if it is for a specific disease with no therapeutic choice. Three types of medications that can be included are: – Innovative drugs authorized for sale abroad, but not in Italy – Unauthorized drugs which underwent clinical trials – Drugs to be used for a therapeutic indication different from those authorized	– CUP exists in Spain, facilitating access to medicines under investigation as per the EU definition for compassionate use through temporary authorization – Both individuals and cohorts benefit. The regulations and process for CUP are well defined – The companies decide to supply free of charge or not, based on which negotiations happen	– The MHRA launched early access to medicines scheme (EAMS) scheme in April 2014 –MHRA gives a scientific opinion on the benefit/risk balance of the medicine, as per the data when the EAMS submission is made. The opinion lasts for a year and is renewable

belonged in one of these categories: *i*) Did not have a CUP regulation or process; *ii*) No information was available; *iii*) Lack of clarity in the retrieved and available information.

Among the 28 EU member states, 18 (~64%) had nationalized regulations in place and the processes for CUP were well-defined as per the information gathered (Figure 3). These countries are Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Hungary, Italy, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden and United Kingdom. The decisions were managed and approvals were granted by a competent National Agency in the countries where CUP existed (*12*).

According to this review, few countries lack official CUP and regulations. Every EU member state has nationalized regulations based on the EU framework. The national CUP makes medicinal products available either to individuals (NPP) or cohorts of patients governed by every member state's legislation. However, named patient basis is not CUP as per the EU regulations. So, the doctor responsible for the treatment contacts the manufacturer directly (*9,19-21*). CUP in 28 member states categorized by UN Geoscheme are summarized below.

CUP in EU5: France has an elaborate scheme for CUP called Temporary Authorizations for Use (ATU). It allows the exceptional use of medicinal products

Figure 3. Presence of regulations and well-defined processes in the European Union member states. This chart shows that 18 countries of the European Union member states have legislation and a well-defined process in place for Compassionate use program and the rest do not.

without a marketing authorization and not subject to a clinical trial. The program is well-defined with structured regulations and procedures in the rest of EU5 namely

Table 3. Compassionate use programs in Western European countries (*Ref. 12,31,39-46*)

Items	Austria	Belgium	Luxembourg	Netherlands
Authority involved	Austrian Federal Office for Safety in Health Care (Bundesamt für Sicherheit im Gesundheitswesen, BASG)	Federal Agency for Medicines and Health Products (FAMHP)	Direction de la Santé Villa Louvigny Division de la Pharmacie et des Medicaments	Medicines Evaluation Board
Laws and regulations	8a Austrian Medicinal Products Act (AMG)	– Belgian law 1 May 2006 Art.6 quater point 2 (modifications ofmodifications of the loi du 25 mars 1964 sur les médicaments) – Articles 106 and 107 in the Royal Decree executive measures of the Law 1 May 2006 (However, The Royal Decree of 25 April 2014 amending the Royal Decree of 14 December 2006 on medicinal products for human and veterinary use was published and is effective since July 2014)	Regulations specifically for CUP are absent. Further information unavailable	At national level, the legal requirements are implemented in the Medicines Act in Article 40 paragraph 3 (f) and the Ministerial Regulations Article 3.18
Overview	CUP in Austria covers: – Medicinal products developed biotechnologically – Veterinary products – Unauthorized medicinal products for human use with a new active substance, for which the therapeutic indication is the treatment acquired immune deficiency syndrome, viral diseases, cancer, neurodegenerative disorder, diabetes, auto-immune diseases and other immune dysfunctions – Medicinal products designated as orphan medicinal products	CUP permits use of medicinal products unauthorized in Belgium, to patients with a chronically or seriously debilitating or life threatening disease, and who cannot be treated satisfactorily by an authorized medicinal product.	– No CUP – All medicinal products require a prior authorization "Autorisation de Mise sur le Marché" - article 22 (1) of the "Code de la sécurité sociale". The only exception is the EMA authorization valid in all the member states	If there is no registered alternative available and new drugs for multiple patients (cohort) is deemed necessary before a marketing authorization, a CUP is applicable. NPP also exists.

Germany, Italy, Spain and UK (Table 2) (*12,22-38*).

CUP in Western European countries: Among Austria, Belgium, Luxembourg and Netherlands, Luxembourg did not have CUP (Table 3) (*12,31,39-46*).

CUP in North European countries: In Estonia and Ireland, although there is no CUP, NPP exists. Lithuania does not have the program. Denmark, Finland, Latvia and Sweden do have CUP with Latvia lacking regulations and a clear structure (Table 4) (*12,31,47-58*).

CUP in Southern European countries: There is minimal information about CUP in Greece and Malta. Though there is a regulatory procedure for CUP in Malta, there is no official legislation. Compassionate use of medicines in individual patients is documented, but there is a dearth of clarity whether this is NPP or CUP. Croatia, Portugal and Slovenia have CUP with national regulations (Table 5) (*12,31,59-68*).

CUP Eastern European countries: Czech Republic, Bulgaria, Hungary and Romania have CUP with legislation in place. There is no CUP in Poland. In Slovakia, there is no CUP, but participation of patients in international registries influences CUP (Table 6) (*12,31,69-82*).

CUP in Asia: There are no regulations allowing access to unauthorized medicinal products outside clinical trials in Cyprus (Table 7) (*83*).

3.3. *Recent changes*

The literature review deduces that countries like Hungary and Sweden, without national legislation previously, have now formulated them (*13*). Newly shaped policies and regulations have resulted due to higher demand for CUP, especially for orphan drugs for rare diseases. Clear regulations aid in systematizing CUP and providing better access to medicines. For instance, more than 20,000 patients were treated with over 200 products under French legislation for compassionate use by 2007 (*84*). A study on all ATUs with marketing authorization between 01 January, 2005, and 30 June, 2010, concluded that the licensing and public bodies' review time was shortened by a combined total of 36 months. Also, the French ATU program accelerated the availability of new drugs in spite of the longer standard administrative path (*85*). Since 2006, the EU member states submitted more than 50 CUP notifications to the EMA, of which around two-fifths were for orphan medicinal products (*6*).

3.4. *Benefits of CUP*

Implementing early access programs like CUP has multifold benefits, both to patients and pharmaceutical companies (*18,86-92*). First, CUPs benefit patients unable to participate in clinical trials due to mobility issues or who fail to fulfill the eligibility criteria (*13*). They are also preferred when no treatment options are available or access to investigational drug/biologics/ medical devices is the last resort for patients suffering from serious diseases/disorders (*13*). Through early access programs, patients have access to promising drugs at an earlier stage during the life cycle, for instance, post phase II. Otherwise, patients have to wait for a considerable amount of time until the drug

Table 4. Compassionate use programs in North European countries (*Ref. 12,31,47-58*)

Items	Estonia	Denmark	Ireland	Lithuania	Finland	Latvia	Sweden
Authority involved	State Agency of Medicines (SAM)	Lægemiddelstyrelsen -Danish Medicines Agency	Health Products Regulatory Authority (HPRA)	State Medicines Control Agency	Finnish Medicines Agency (Fimea)	State Agency of Medicines of Latvia	Läkemedelsverket/Medical Product Agency (MPA)
Laws and regulations	No CUP as in EMEA/27170/2006 but NPP processed by national regulations	Section 29 (1) of the Danish Medicines Act	– No specific legislation for CUP. However, it can fall under the Irish clinical trial regulations SI 190 of 2004 or SI 540 of 2007 – Also, named patient regime exists which transposes Article 5(1) of Directive 2001/83/EC for EU countries	– Regulations for CUP are absent. – Further information unavailable	Medicines Decree 693/1987, 1184/2002 and 868/2005	Regulations for CUP are absent.	§5 of the Medicine Act no 859 of 1992, recently updated the 28th August 2012 Läkemedelsverkets föreskrifter (LVFS) Nominative
Overview	– CUP not formally authorized as per national legislation – NPP exists	Under special conditions, after application, the sale or dispensing of medicinal products in limited amounts (not covered by marketing authorization or not marketed in Denmark) may be authorized	Named patient and clinical trial regimes exist, but not CUP. Further information unavailable.	Information unavailable	Compassionate use is permitted in exceptional cases where no other treatments are appropriate or produce the anticipated effect. Medicines available through CUP are prescription based only. Permission for CUP is needed for: – An individual patient in an outpatient care – individual applications for permission – A cohort of patients in an institution - health care institutions to apply for permission The permission is valid for one year, starting from the date of issue.	Compassionate use exists through the Latvian hospitals. However, it is unclear if it is on a named-patient basis or CUP. Further information unavailable.	An unauthorized medicinal product can be available for compassionate use in Sweden to increase patients' access to drugs being developed in the EU and to enable a common EU procedure. The MPA introduced CUP in 2012 for the Swedish health care as a complement to licensed prescription.

Table 5. Compassionate use programs in Southern European countries (*Ref. 12,31,59-68*)

Items	Croatia	Greece	Malta	Portugal	Slovenia
Authority involved	Ministarstvo zdravlja (Ministry of Health) and The Agency for Medicinal Products and Medical Devices of Croatia (HALMED)	National Organization for Medicines	Malta Medicines Authority	Instituto Nacional da Farmácia e do Medicamento (INFARMED)	Javna agencija RS za zdravila in medicinske pripomočke - Public Agency of the Republic of Slovenia for Medicinal Products and Medical Devices (JAZMP)
Laws and regulations	Pursuant to Article 73 of the Medicinal Products Act (Official Gazette 71/07 and 45/09) ordinance on pharmacovigilance	Information unavailable (may be in progress)	– Though there is a pharmaceutical regulatory procedure, no official national legislation exists – Unlicensed medicinal products as directed in DH Circular 137/2004 (updated version DH Circular 270/06 - Health concerning the "Guidelines Governing the Use of Medicinal Products for Human Use without a Marketing Authorization" is different from CUP – Further information unavailable	– Articles 92-93 of the Decreto Lei no. 176 of 30th August 2006 – INFARMED'S Decision 105/CA/2007, of 1 March 2007 – Nominative	83 Article 6 Medicines Act (Official Gazette of RS, no. 17/14)
Overview	– It is possible to adopt compassionate use from diagnosis to the approval to use the drug – Compassionate use facilitates access to unauthorized medicinal products for chronic, serious, or a life-threatening diseases which cannot be treated satisfactorily by an authorized medicinal product	– Compassionate use of medicines for individual patients exists. However, information on access for a group of patients is unavailable – Literature shows that there are high implementation barriers such as lack of infrastructure to support early access programs in the country	– There is a regulatory procedure for CUP to use medicinal products consistent with EU regulations – The product applied for under CUP must be under evaluation in the centralized authorization procedure at the EMA	Medicines, including the ones for compassionate use without a marketing authorization, under special and exceptional circumstances, can be used to treat patients in always under an authorization granted with a temporary and transitory nature by the INFARMED	In Slovenia, in line with the EU regulation, CUP is: – The administration of a new active ingredient, constituting a significant therapeutic, scientific and technical innovation – For drugs obtaining marketing authorization or clinical testing of medicines – For a group of patients with a chronically or seriously debilitating disease that cannot be satisfactorily treated with medicines that have marketing authorization – The sponsor/manufacturer of a CUP must provide the product free of charge, which includes the costs of supply and wholesale distribution.

Table 6. Compassionate use programs Eastern European countries (*Ref. 12,31,69-82*)

Items	Czech Republic	Bulgaria	Hungary	Poland	Romania	Slovakia
Authority involved	State Institute for Drug Control (SÚKL)	Ministry of Health	Országos Gyógyszerészeti és Élelmezés-egészségügyi (OGYÉI) Intézet National Institute of Pharmacy and Nutrition	Office for Registration of Medicinal Products, Medical Devices and Biocidal Products	National Agency for Medicines and Medical Devices (NAMMD)	State Institute for Drug Control
Laws and regulations	Act on Pharmaceuticals No. 378/2007 – Section 8, paragraph 3 (b) (2) of the Act on Pharmaceuticals, unauthorized advanced-therapy medicinal products – Section 49 of the Act on Pharmaceuticals – Section 49a and 49b of the Act on Pharmaceuticals	Regulation N° 10/17 November 2011	Act XCV of 2005 on Medicinal Products for Human Use and on the Amendment of Other Regulations Related to Medicinal Products Section 25/C (effective since January 2016)	No CUP. Further information unavailable.	Ministry of Health, Order no. 1018 of 3 September 2014 on approval of Conditions for authorization of human medicinal products for compassionate use, in accordance with provisions of Article 83 of Regulation (EC) no. 726/2004 of EU	Information unavailable
Overview	– Unauthorized advanced-therapy medicinal products (only somatic cell therapy or tissue engineering medicinal products) may be used for a patient on the decision of a medicinal doctor, at the responsibility of the respective healthcare service provider (healthcare facility). – Medicines can be used for specific/special therapeutic programs – CUP is allowed where hospital Exemption has been allowed (this only applies to advanced-therapy medicinal products)	Medicinal products for human use without marketing authorization in Bulgaria are accessible through CUP. However, the current regulation concerns only medicines approved by the EMA	CUP is available for life-threatening or debilitating medical condition. The manufacturer is to provide the drug free for CUP. Further information unavailable.	– No CUP – However, life-saving treatment with medicines registered outside Poland is subject to individual decisions of the Minister of Health	The medicinal products possibly accessible with CUP are: – Medicinal products for human use manufactured biotechnologically. For example, recombinant DNA technology and controlled expression of gene – Medicinal products for human use with a new active substance unauthorized in the EU and whose therapeutic indication is a treatment for acquired immunodeficiency syndrome, cancer, neurodegenerative diseases, diabetes, autoimmune diseases and other malfunctions of the immune system and viral diseases – Medicinal product for human use designated as an orphan medicinal product as per Regulation (EC) no. 141/2000 of the European Parliament and of the Council of 16 December 1999 on orphan medicinal products – Advanced therapy medicinal product	Participation of the patients in Slovakia in the international registries influences CUP. Implementation and streamlining CUP is in progress. No further information available.

Table 7. Compassionate use program in Asia (*Ref. 83*)

Items	Cyprus
Authority involved	Pharmaceutical Services of the Ministry of Health
Laws and regulations	According to a report published in 2012, there are no regulations in place allowing access to unauthorized medicinal products outside clinical trials
Overview	Information unavailable

is authorized and is on the market, if not for the early access programs (*13*). Second, pharmaceutical and biotechnology companies provide a fast and efficient response to patient demand outside of traditional access routes due to such programs (*87,90*). Early access facilitates the smoother transition of a drug from clinical trials to the market and also prepares companies to develop global launch strategies based on global usage patterns and market landscape predictions (*86,88,89*). Besides, the market authorization holders get the opportunity to resolve any product related issues and can overcome challenges encountered by pre-approved drugs through early access (*19,88*). Above all, early access furnishes valuable information pertaining to real world evidence for practice and further research (*18,20*). Furthermore, patients, physicians and patient organizations are increasingly becoming aware of the possibilities to access a drug through such early access programs. Moreover, the existing framework for compassionate use in the EU member states can be effectively utilized to plan new access programs. For instance, the adaptive pathways approach, a scientific concept for medicine development and data generation, is part of the EMA's efforts to improve timely access for patients to new medicines that utilize the existing EU regulatory framework for medicines (*93,94*).

3.5. *Challenges in implementing CUP*

The governments and pharmaceutical companies are taking steps to implement CUP considering the benefits and importance of early access to drugs. However, it is highly challenging and complex for pharmaceutical companies to initiate early access programs like CUP. First, despite the existing EU Regulations, pharmaceutical companies face challenges as the regulations vary from country to country. This mandates regulators, policy makers and other key stakeholders to streamline processes and create transparency. Second, innovative drugs are relatively expensive and are becoming increasingly difficult for governments and payers to include them in their reimbursement schemes. This mandates for clearly set regulations and rationalized procedures to help patients, patient organizations and physicians for better access to drugs. Additionally, it is imperative for pharmaceutical companies to be updated on these regulations and processes on CUP for easier entrance into a market.

3.6. *Strengths and limitations of this study*

This review has its own strengths and limitations. Previously, the information available publicly on CUP, especially on legislation was limited and in many cases outdated when compared with the available grey literature (*6,12,13*). However, the current systematic search addresses these gaps by including data from grey literature and peer-reviewed journal articles. Despite the efforts by researchers to capture all the available data, there still exist a few gaps in the literature. For instance, information on CUP is not available online for certain member states where CUP is still in the initial stages.

4. Conclusion and recommendations

Overall, this review identified the presence of CUP and highlighted its current status and legislation in 28 EU member states. This review found that CUP has a positive impact and potential benefits for patients and pharmaceutical companies. The established legislation for CUP in the EU member states suggests that the EU countries are ready to adopt methods for early access to medicines. An implication of this is the possibility to make new medicines available within a shorter time span for patients. Furthermore, the existing framework for compassionate use in the EU member states can pave the way for more access programs.

Further research is necessary to determine the specific phases involved in the CUP, pricing and reimbursement frameworks in countries. Case studies and success stories which reflect the benefits of such early access programs from both patients' and other stakeholders' perspectives can give the relevant impetus for education on such programs. Since inadequate information on healthcare access can often be a stumbling block for stakeholders, periodic reviews need to be taken to throw light on emerging changes within the regulatory structures, both at European and member state levels to discern market trends.

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(Received July 4, 2016; Revised August 25, 2016; Accepted August 31, 2016)

Review

DOI: 10.5582/irdr.2016.01082

The neurobiology of the Prader-Willi phenotype of fragile X syndrome

Zukhrofi Muzar[1,2,§], Reymundo Lozano[3,§,*], Alexander Kolevzon[3,c,e,f], Randi J. Hagerman[1,*]

[1 a)] *Medical Investigation of Neurodevelopmental Disorders MIND Institute, [b)] Department of Pediatrics, UC Davis Medical Center, Sacramento, CA, USA;*

[2] *Department of Histology, Universitas Muhammadiyah Sumatera Utara (UMSU) Faculty of Medicine, Medan, North Sumatera, Indonesia;*

[3 c)] *Seaver Autism Center for Research and Treatment, [d)] Departments of Genetics and Genomic Sciences, [e)] Psychiatry, and [f)] Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, USA.*

Summary **Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability and autism, caused by a CGG expansion to greater than 200 repeats in the promoter region of *FMR1* on the bottom of the X chromosome. A subgroup of individuals with FXS experience hyperphagia, lack of satiation after meals and severe obesity, this subgroup is referred to have the Prader-Willi phenotype of FXS. Prader-Willi syndrome is one of the most common genetic severe obesity disorders known and it is caused by the lack of the paternal 15q11-13 region. Affected individuals suffer from hyperphagia, lack of satiation, intellectual disability, and behavioral problems. Children with fragile X syndrome Prader-Willi phenotye and those with Prader Willi syndrome have clinical and molecular similarities reviewed here which will impact new treatment options for both disorders.**

Keywords: Fragile X syndrome (FXS), Prader-Willi phenotype, *FMR1* gene, Hyperphagia, Autism, IGF-1, Growth hormone

1. Introduction

Prader-Willi syndrome (PWS) is the most common cause of obesity and intellectual disability, occurring in about 1 in 15,000 in the general population (*1-3*). PWS is characterized by hyperphagia and lack of satiation after meals. Typical physical features include: a round face, narrow palpebral fissures, short stature, small genitalia, and short fingers and toes. Behavioral and cognitive features include mild intellectual disability (ID), poor attention, obsessive behavior particularly around food so that food hoarding and food stealing are common, excessive skin picking, and remarkably good ability with puzzles. PWS is caused by an absence of expression of imprinted genes in the paternally derived PWS/Angelman syndrome (AS) region (15q11.2-q13) of chromosome 15 by one of several genetic mechanisms (paternal deletion, maternal uniparental disomy and rarely an imprinting defect) (*2*).

Fragile X syndrome (FXS) is the most common inherited cause of ID and Autism Spectrum Disorder (ASD) and it is caused by a CGG trinucleotide expansion of over 200 repeats (full mutation) in the 5' region of the fragile X mental retardation 1 gene (*FMR1*) at Xq27.3. This full mutation leads to hypermethylation of *FMR1* and a subsequent lack of transcription and translation, which in turn results in a deficiency of the FMR1 protein (FMRP). The FMRP is an RNA binding protein that mainly negatively regulates the translation hundreds of genes, many of which are critical for synaptic plasticity (*4*). The prevalence of FXS is 1 in 5,000 males and 1 in 8,000 females (*5*). The features of FXS include hyperactivity, attentional problems, poor eye contact, hand-flapping, anxiety, hyperarousal and

Released online in J-STAGE as advance publication November 14, 2016.

[§]These authors contributed equally to this works.
**Address correspondence to:*
Dr. Reymundo Lozano, Seaver Autism Center for Research and Treatment, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1230, New York, NY 10025, USA.
E-mail: Reymundo.lozano@mssm.edu

Dr. Randi J. Hagerman, MIND Institute, UC Davis Health System, 2825 50th Street, Sacramento, CA 95817, USA.
E-mail: rjhagerman@ucdavis.edu

a lack of habituation to sensory stimuli. The physical features of FXS include a long face, prominent ears, hyperextensible finger joints and flat feet. Males with FXS have macroorchidism during and after puberty with a normal phallus. Although the clinical phenotype is very different between PWS and FXS there is a subgroup of individuals with FXS that develop hyperphagia, obesity, and hypogonadism or delayed puberty. Because this phenotype looks like those with Prader-Willi syndrome, it is described as Fragile X syndrome - Prader-Willi phenotype (FXS-PWP) (Figure 1A and 1B). This phenotype in FXS was first reported by Fryns *et al*. (1987) and has subsequently been described by others (*6-10*). This phenotype is not related to a deletion of 15q11-q13 nor due to maternal uniparental disorders, and it occurs in less than 10% of individuals with FXS (*11*). Although short stature and small fingers and toes can sometimes occur in the FXS-PWP this is not seen in the majority of cases. Nowicki *et al*. (2007) found lower cytoplasmic interacting FMR1 protein 1 (CYIP1) levels in the blood of patients with the FXS-PWP compared to those with FXS without the PWP (*6*). They also found a higher rate of ASD in those with the FXS-PWP compared to FXS alone (*6*).

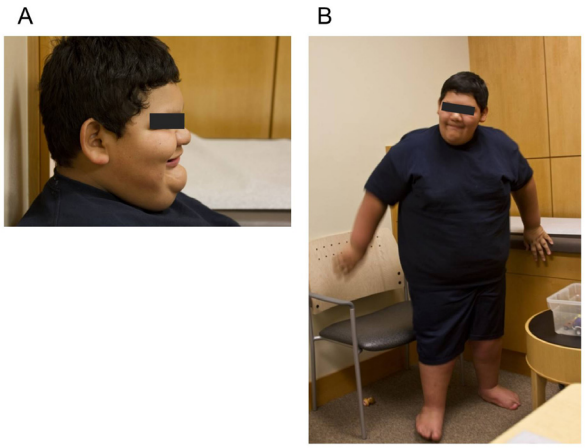

Figure 1. 11 year old with FXS and PWP, showing severe obesity, small hands and mild facial dysmorphic features

2. Neurobiology of FXS with PWP

Patients with the FXS-PWP have hyperphagia that arises in childhood (*6*), as is the case in PWS. Even though there is no clear molecular explanation underlying hyperphagia in FXS, it is hypothesized that it arises from dysregulation of gamma-aminobutyric acid (GABA) system in the hypothalamus. The lateral hypothalamus (LH) is a critical modulator of feeding (*12,13*). A previous study by Jennings *et al*. demonstrated that GABAergic (*Vgat*-expressing) neurons in the LH are responsible for producing appetitive and consummatory behaviors (*14*). FXS animal models have lowered GABA subunit receptors, synthesis of GABA, GABAergic input to many regions of the brain, and increased catabolism of GABA (*15,16*). Similarly, cerebral GABAA receptor expression is reduced in several brain regions of subjects with PWS (*17*).

There is a high rate of ASD (7 of 13, 54%) in the patients with FXS-PWP (*6*). This may be related to the reduction mRNA levels of the CYFIP1, which was found to be two to fourfold lower in the patients with FXS-PWP compared to individuals without FXS and patients with FXS without the PWP (*6*). CYFIP1 is localized to the critical region for PWS at 15q between breakpoint 1 and 2 (*18*). FMRP binds to CYFIP1 (*19*) in the execution of its role as a transporter and regulator of translation of mRNAs (*20-22*). CYFIP1 expression levels are vital for dendritic arborization and neuronal morphological complexity (*23*). Neurons from CYFIP1 haploinsufficient animals have smaller and less complex dendritic branching both *in vitro* and *in vivo* (*23*). Another reason could be due to dysregulation of oxytocin (OT) and arginine vasopressin (AVP) in the brain. The dysregulation of the OT system in animals and humans is linked with marked deficits in social behavior and anxiety (*24*). There is a scarcity of OT producing neurons in the paraventricular nucleus of the hypothalamus (PVN) in individuals with PWS (*25*) and in the *Fmr1* KO (knock-out) mice (*24*). OT administration increases trust and diminishes disruptive behavior in individuals with PWS (*26*); in those with FXS OT therapy improves social anxiety (*27*).

3. Treatment

In the treatment of autism, use of intensive early behavioral intervention, such as the Early Start Denver Model (ESDM) has been shown to improve developmental and social outcome in addition to normalization of the EEG abnormalities compared to those treated with community behavioral interventions (*28*). Such intervention is also recommended in young children with FXS both with and without autism or the PWP (*29,30*). A variety of medication use can be helpful for those with FXS both with and without the PWP including; stimulants which can help with attention and appetite; Selective Serotonin Reuptake Inhibitors (SSRIs), such as sertraline for anxiety (*31*); and atypical antipsychotics, such as aripiprazole to stabilize mood, improve autism, aggression and/or tantrums (*6*). However, aripiprazole can cause an increase in weight gain and particularly for those with a CYP2D6 polymorphism that can slow down the metabolism of aripiprazole (*32*).

There are a variety of new-targeted treatments that have been studied in those with FXS. To enhance the GABA deficits in FXS, the GABAB agonist, arbaclofen, showed initial benefit for those with ASD or low sociability (*33*). However, the subsequent phase 3 trial in children and adolescents and adults did

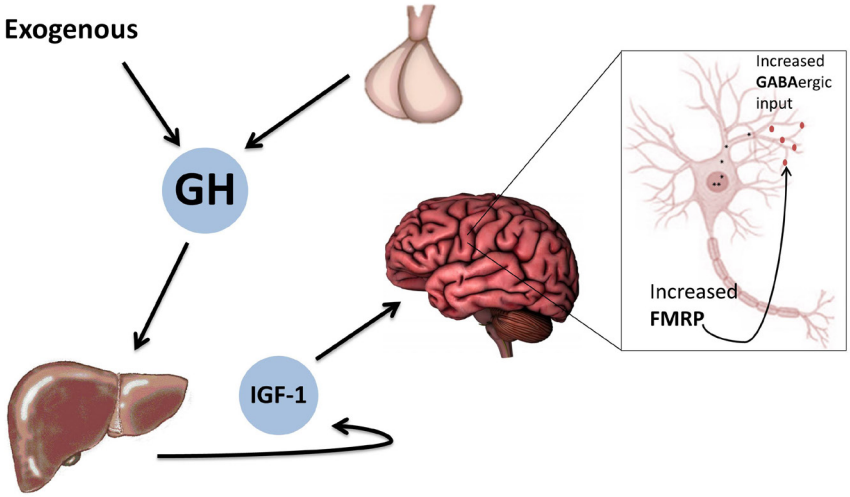

Figure 2. Suggested mechanism of action of GH. GH stimulates the liver to release IGF-1, which activates CREB, and this increases FMRP in neurons. The increase of FMRP leads to increased GABAergic input to some areas of the brain.

not demonstrate efficacy (*34*). The GABAA agonist ganaxolone is being studied in children 6 to 17 years old at the MIND Institute at UC Davis Medical Center and in Belgium utilizing the same protocol (*https://www.clinicaltrials.gov* [NCT]: ID number NCT01725152). Results from this study are expected before of the end of this year. The mGluR5 antagonists developed by Roche and Novartis have not demonstrated efficacy in adult and childhood studies, but further studies are planned for an mGluR5 antagonist. Minocycline has been studied in children with FXS and has demonstrated some efficacy so it is often utilized clinically (*35,36*). Other trials included at multiple centers included metadoxine for improving attention and focus and the IGF-1 analogue developed by Neuren for the treatment of behavioral problems. However, those with the FXS-PWP of are not typically included in such clinical trials because their level of obesity is beyond what is acceptable for inclusion in these trials. Therefore, there are no studies of the treatment in those with FXS-PWP.

In contrast, human growth hormone (GH) has been the panacea for treatment of PWS over the last decade (*37,38*). Most individuals with PWS, but not all, are deficient in GH and studies that were initially focused on improving growth have not only demonstrated this effect, but also improvements in metabolism, body composition, behavior and cognition (*38-44*). However, GH therapy on occasion can be associated with significant side effects, such as the stimulation of adenoid tissue leading to obstructive sleep apnea so it should be used carefully in the treatment of PWS. GH can also promote the growth of some malignant tumors so such a history is a contraindication for GH therapy. Since GH therapy has been so beneficial to those with PWS and because there are remarkable similarities between PWS and the FXS-PWP, it is possible that those with the FXS-PWP will benefit from GH therapy. The observed benefits of the IGF-1 analogue in the KO mouse FXS model support the likelihood that GH therapy should be beneficial in the FXS-PWP. GH stimulates the release of IGF-1 by the liver and this may be the mechanism for the benefit of GH therapy. IGF-1 enhances GABA activity that is deficient in FXS both with and without the PWP (Figure 2)(*45*). In addition, the GABAB receptor-mediated transactivation of IGF-1 receptors leads to cAMP response element-binding protein (CREB) activation which in turn binds to *FMR1* and increases FMRP levels (*46*). Therefore, GH in FXS-PWP could stimulate the release of IGF-1 to enhance the GABA system, and increase the residual expression of *FMR1* particularly in those who are mosaic or partially unmethylated. Further studies are necessary to determine the molecular benefits of IGF-1 analogues and GH in FXS.

In general, metabolic anomalies are suspected in FXS because about 30% are obese (*47*). Metabolic anomalies including increased glucose uptake and excess protein synthesis in the brain have been reported in *Fmr1* KO mice, while in the fly (*dfmr1*), it has been shown that FMRP is required during brain development and may function in neuroblast reactivation by regulating an output of the insulin signaling pathway (*48-52*). Metabolic profiling in the *Fmr1* KO mice also revealed profound consequences in brain metabolism, which in turn lead to alterations in the metabolic response, along with anomalies in other physiological processes and behaviors (*53*). Using Drosophila as a model of FXS it has been shown that the *dfmr1* has elevated levels of drosophila insulin-like peptide 2 (Dilp2) in the insulin-producing cells which result in elevated insulin-signaling *via* the PI3K/Akt/mTOR pathway (*54*). It is also known that increased insulin-signaling leads to defects in the circadian output pathway and in short and long-term memory

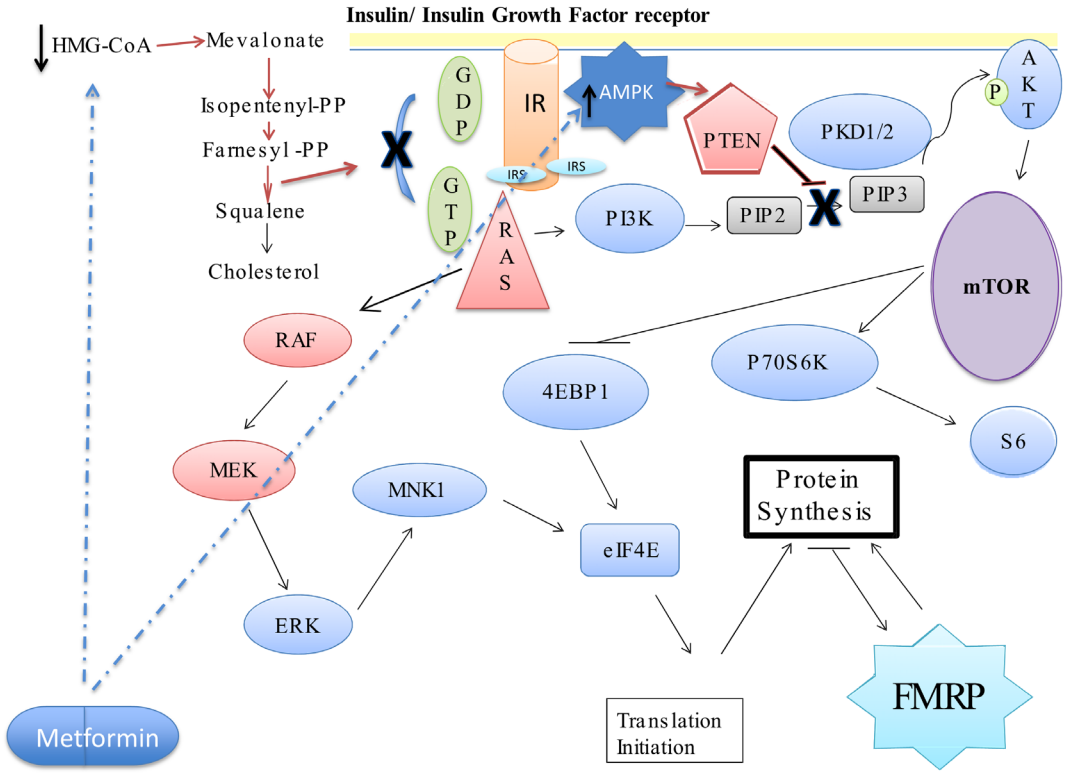

Figure 3. Mechanism of action of metformin. Metformin decreases protein synthesis and insulin signaling (IS) *via* the AMPK/Akt/mTOR pathway, it also inhibits the lipid and sterol biosynthetic pathways

deficits. Interestingly, pharmacological restoration using metformin, rescued memory deficits in the *dfmr1* (54). Metformin is known to decrease body mass index (BMI) and to prevent cognitive deficits in individuals with diabetes (*55-59*). Metformin decreases protein synthesis and insulin-signaling *via* the AMPK/Akt/mTOR pathway, it also inhibits the lipid and sterol biosynthetic pathways (*60-63*) (Figure 3). Therefore, metformin in FXS may decrease insulin-signaling and restore the circadian output pathway and in turn have positive effects on memory and sleep. A pilot, open-label study of response to metformin in 21 children with PWS and six with early morbid obesity (EMO) showed significant improvements in food-related distress, anxiety, and ability to be redirected away from food. Within the PWS group, responders to metformin had higher 2-hour glucose levels on oral glucose tolerance test and higher fasting insulin levels. Additionally, parents of 5/13 individuals with PWS and 5/6 with EMO reported recognition of satiety (*64*). Further studies are necessary to determine the safety and efficacy of metformin in FXS, PWS and FXS-PWP.

4. Future directions for research

Understanding the similarities and differences between PWS and the PWP of FXS will lead to new treatments perhaps for both disorders. Since *CYFIP1* is down-regulated in the PWP and because the clinical phenotypes are so similar across both disorders, it is likely that epigenetic changes or methylation differences may be down-regulating other genes in the 15 q 11-13 region in those with the PWP. Further studies are warranted to determine whether the reduced expression of multiple genes in PWS also occur in FXS-PWP. These genes include, *MKRN3* (*65*), *MAGEL2* (*66*), *MAGED1* (*67*), *NECDIN* (*68,69*) and *SNURF-SNRPRN* (*70*). To understand more about hyperphagia phenotype in FXS-PWP, studies regarding the GABA network in the brain are required. In addition, as chronic hyperghrelinaemia promotes hyperphagia in PWS (*71*), it would be interesting to see ghrelin levels in patients with the FXS-PWP.

A variety of new treatments are currently being studied in PWS and reviewed in Miller *et al*. (2015). Although GH treatment has many beneficial effects, it does not significantly help the hyperphagia. Some of the new medications that are being studied in PWS include Diazoxide, a potent K^+-ATP channel agonist that hyperpolarizes hypothalamic neurons whose activity is impaired by a defective leptin signaling pathway in PWS (NCT02-34071); AZP-573, an unacylated ghrelin analog; Exanatide/Liraglutide, glucagon-like peptide 1 (GLP-1) receptor agonist which can suppress appetite and reduce weight in PWS and obese patients (NCT014448981/ NCT01542242). Clearly the study of those with FXS-

PWP will determine whether they have the potential to benefit from some of these new trials that have occurred in PWS. The future of treatment of both disorders looks bright with the advent of targeted treatments based on the neurobiological studies of both disorders.

Acknowledgements

This work was supported by grants from the National Fragile X Foundation, NICHD grant HD036071, the Department of Defense PR101054, the MIND Institute Intellectual and Developmental Disabilities Research Center U54 HD079125, the Health and Human Services Administration for Developmental Disabilities grant 90DD0596 and by the Seaver Foundation; Dr. Lozano is a Seaver Faculty, Friedman Brain Institute and NIH faculty Scholar.

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(Received October 9, 2016; Revised November 7, 2016; Accepted November 8, 2016)

Original Article

DOI: 10.5582/irdr.2016.01053

Auditory brainstem response and late latency response in individuals with tinnitus having normal hearing

Sreeraj Konadath*, Puttabasappa Manjula

Department of Audiology, All India Institute of Speech and Hearing, Mysuru, Karnataka State, India.

Summary **Tinnitus is a commonly encountered complaint in routine audiology practice. The pathophysiology and exact generation site of tinnitus is not precisely established. Auditory brainstem response (ABR) and late latency response (LLR) findings in individuals with tinnitus show mixed results in the literature. Majority of studies have focused on individuals having tinnitus with peripheral hearing loss. The present study explores ABR and LLR characteristics among tinnitus patients with normal audiometric presentation; with no direct indication of any cochlear lesion. This study aims at characterizing the ABR and LLR findings in individuals with tinnitus having normal audiometric presentation. ABR and LLR waveform characteristics were recorded and compared between participants with tinnitus (Group 1) and those without tinnitus (Group 2). The ABR analysis indicated no significant differences in latency and amplitude between Groups 1 and 2. However, patients with tinnitus showed abnormally reduced absolute amplitudes of peaks I and V. LLR analysis indicated no significant differences in latency and amplitude between Groups 1 and 2 except enhanced amplitude of P1. The reduced amplitude of peaks I and V along with normal absolute latencies of peaks I, III and V indicate that the origin of tinnitus is possibly due to reduced excitation of auditory nerve fibres arising from a peripheral hearing loss beyond 8 kHz. The P1 amplitude enhancement could be attributed to mechanism explaining central gain model; which suggests that central auditory structures recalibrates the mean firing rate, considering the reduced output from sensory structures, generating neural noise perceived as tinnitus.**

Keywords: Auditory brainstem responses, late latency response, tinnitus, central gain mechanism

1. Introduction

Tinnitus is defined as a perceived sound with varied intensity, loudness and pitch in the absence of an external sound (*1*). The pathophysiology and exact generation site of tinnitus is not precisely established. Initially, the origin of tinnitus was attributed to peripheral auditory system (*2,3*). Later, the involvement of central auditory structures was identified (*4-6*). Hazell (1995) proposed that tinnitus has its origin in the cochlear structures and/or within the brainstem as a weak signal that undergoes processes like filtering and amplification before it is perceived at the cortical or sub-cortical level. This neural activity occurs in everyone; but, emotional issues as well as stress can enhance the perception of tinnitus (*7*). Various factors like outer and inner cochlear hair cell lesions, efferent auditory system impairment, cross-talk between the auditory nervous system fibers, ionic imbalances that occur within the cochlea, impaired functioning of cochlear neurotransmitters, various kinds of central auditory processing disorders or auditory neuropathy may lead to the perception of tinnitus (*8*). Thus, it can be assumed that multiple physiological factors across various levels of the auditory nervous system, to different degrees, can result in the development of tinnitus (*9,10*).

About 10-25% of the adult population has prolonged tinnitus (*11-14*). 70-80% of individuals with tinnitus have significant hearing difficulties (*15*). Even though tinnitus is commonly associated with hearing loss (*16*), it also exists among individuals with apparently

Released online in J-STAGE as advance publication September 5, 2016.

**Address correspondence to:*
Mr. Sreeraj Konadath, Department of Audiology, All India Institute of Speech and Hearing, Mysuru 570 006, Karnataka State, India.
E-mail: sreerajkonadath@aiishmysore.in

normal hearing sensitivity (*17*). Hence, there might be a possibility of "hidden hearing loss" (*18*), wherein central auditory structures are involved in tinnitus generation. Even though based on real experiences, the individual with tinnitus believes that the sound is generated within the ear, the neurophysiological standpoint describes the symptom as a perception that happens in the cortical level and is reserved for sensorial modes (*7*). So, in addition to subjective audiological assessments, objective assessment plays a vital role in diagnosis of tinnitus.

Studies have been carried out to explore the phenomenon of tinnitus by means of auditory evoked potentials. Auditory evoked potentials help in understanding synchronous discharge of nerve fibers along the auditory pathway. The ABR are measured by means of scalp electrodes which pick up electrical potentials generated by the synchronous firing of neural populations within the brainstem. Thus, ABR provides an excellent method for assessing auditory function in a clinical set-up by recording the aggregate neural responses objectively and passively. Click evoked ABR has a wide-scale clinical application as a metric for evaluating auditory thresholds and identifying the neuropathologies (*19*). Certain ABR findings like abnormal waveform morphology, fluctuations in peaks III and V, delayed transmission time and increased interaural latency difference of peak V *etc*. are observed in the case of individuals with tinnitus (*20,21*). Thus, ABR helps in probing into the pathophysiology behind the origin of tinnitus.

The long latency responses (LLRs), which are generated from non-primary cortical areas measure the integrity of the auditory system beyond the level of brainstem. Studies have observed alterations in LLRs among individuals with tinnitus, like delayed N1 latency (*22*), and abnormal latency of P2 (*23*). With this background, to explore the role of higher auditory structures in the involvement of tinnitus, the evoked potentials play an integral role. Most of the studies concentrated on the assessment of tinnitus in individuals having elevated thresholds. Even though the prevalence of tinnitus is higher among individuals with hearing loss compared to those with normal hearing sensitivity, tracking its origin among the latter group is more obscure compared to the former one. Involvement of retrocochlear structures need to be examined to explore the tinnitus symptoms in such cases. Hence, this study was aimed at describing the findings of ABR and LLR in individuals with tinnitus having normal audiometric presentation.

2. Materials and Methods

2.1. *Participants*

The participants of the study were categorized into two groups. Group 1 was comprised of 20 individuals (10 males, 10 females) having tinnitus as their primary complaint with normal hearing sensitivity, in the age range from 20 to 48 years (mean ± S.D., 33.15 ± 9.80). Group 2 was comprised of 20 individuals (10 males, 10 females) with normal hearing in the age range from 18 to 22 years (mean ± S.D., 20.50 ± 1.79).

All the participants in Group 1 and Group 2 had their air- and bone- conduction hearing thresholds within 20 dB HL in the frequency range of 250 Hz to 8,000 Hz. The speech identification score in the test ear was greater than 90%. They had 'A' type tympanogram with acoustic reflexes at normal levels. The participants did not have any history of middle ear infections, use of oto-toxic drugs or significant noise exposure. The participants in Group I differed from Group II only in one aspect. The participants of Group I had continuous tinnitus, having a score of greater than 38 (*i.e.*, moderate tinnitus) on Tinnitus Handicap Inventory (THI), whereas, the participants in Group 2 did not complain of tinnitus.

All the participants in Group 1 were individuals with a complaint of tinnitus who reported to the outpatient department of All India Institute of Speech and Hearing. Group 2 participants were selected from the staff and students cluster present at the same institute who had normal hearing and no complaint of tinnitus. Ethical clearance was obtained from the ethics committee of the institute before commencing this research work. Also, written informed consent was obtained from participants of both Groups for their inclusion in the study.

2.2. *Data collection*

As an initial step, a detailed case history was taken from participants of Group 1 to probe into the nature of the problem that the patient was facing, along with details of tinnitus perceived. Following this, a detailed audiological evaluation was carried out to check the air-conduction thresholds between 250 Hz and 8,000 Hz and bone-conduction thresholds from 250 Hz to 4,000 Hz. The modified Hughson-Westlake procedure (*24*) was used to find out behavioral thresholds.

Further, speech reception threshold (SRT) was obtained using a paired-word list developed in the Department of Audiology. The Speech Identification Scores were obtained at 40 dB HL (ref. Speech Recognition Threshold) using Phonemically Balanced Kannada Word Test (*25*). Tympanograms were acquired using 226 Hz probe tone followed by measurement of acoustic reflex thresholds, both ipsilaterally and contralaterally at 500 Hz, 1,000 Hz, 2,000 Hz and 4,000 Hz.

The THI (*26*) questionnaire was administered on participants in Group 1 to find out the details of tinnitus. The ABR and LLR recording was carried out using Biologic Navigator Pro Auditory Evoked Potential equipment (version 7.2.1) to meet the objectives of the

Table 1. Acquisition and Stimulus parameters used for recording ABR and LLR

Parameters	ABR	LLR
Stimulus parameters		
a. Type of stimulus	Clicks	500 Hz tone burst of 500 Hz; Blackman window (2-0-2)
b. Duration of stimulus	100 µsec	Rise/fall: 10 ms; Plateau: 50 ms
c. Polarity	Rarefaction	Alternating
e. Repetition rate	11.1 Hz	1.1/sec
f. Intensity	70 dB nHL	70 dB nHL
Acquisition parameters		
a. Analysis time	15 ms	600 ms
b. Amplification	50,000×	50,000x
c. Filter	30 Hz to 3,000 Hz	1 Hz to 100 Hz
d. Sweeps	1,500	300
e. Mode	Monaural	Monaural
f. Electrode montage	Vertical (Fpz, Cz, M1/M2)	vertical (Fpz, Cz, M1/M2)
g. Electrode impedance		
Absolute	< 5 kΩ	< 5 kΩ
Inter electrode	< 5 kΩ	< 5 kΩ
h. No. of channels	Two	Two

ABR, brainstem response; LLR, late latency response.

study. The stimulus and acquisition parameters used to record ABR and LLR are given in Table 1.

The participants were seated in a comfortable posture to ensure that the artifacts were minimal. The recording sites were cleaned using skin preparation gel. The silver chloride cup electrodes were placed on the test sites. Conduction paste was used to ensure good conductivity between skin and electrodes. After recording the ABR waveform, peaks I, III and V were marked manually by visual inspection by an experienced Audiologist. The latencies and absolute amplitudes of the aforementioned peaks were noted for further analysis. Later, LLR was recorded and the absolute amplitudes and latencies were noted for peaks P1, N1, P2 and N2. The same procedure was followed for recording the waveforms in both Groups.

2.3. *Data analysis*

Data collected from 40 ears (20 ears with tinnitus and 20 without tinnitus) were analyzed further using the statistical package for social sciences (SPSS) software version 21. Shapiro-Wilk's test was administered to check for normality of data. Because the data between group comparisons did not come under normal distribution, a non-parametric test was selected to check for differences between the groups. The variability is accounted for due to heterogeneity in the participants of the study.

3. Results

3.1. *Comparison of absolute latency and amplitude of peak I, III and V between Group 1 and Group 2 for click evoked ABR*

Descriptive statistics to calculate mean and standard deviation was carried out for Group 1 and Group 2.

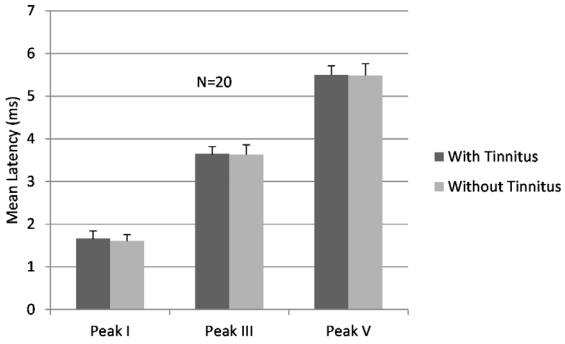

Figure 1. Mean and S.D. of latency of I, III and V peaks in Group 1 (with tinnitus) and Group 2 (without tinnitus).

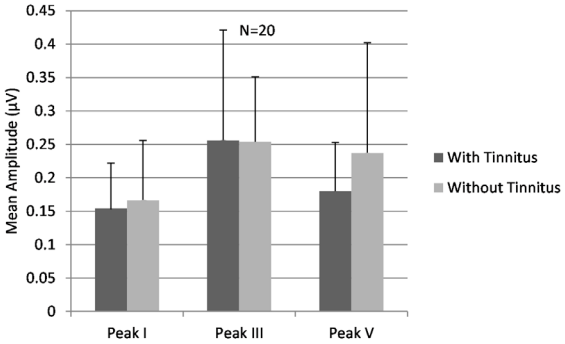

Figure 2. Mean and S.D. of amplitude of I, III and V peaks in Group 1 (with tinnitus) and Group 2 (without tinnitus).

At 11.1 repetitions per second, the mean latency and amplitude for I peak for the participants was 1.67 ms (S.D.-0.171) and 0.15 µV (S.D.-0.068) respectively for Group 1; whereas it was 1.61 ms (S.D.-0.144) and 0.166 µV (S.D.-0.089) for Group 2. The mean latency and amplitude of III peak was 3.65 ms (S.D.-0.168) and 0.256 µV (S.D.-0.165) respectively for Group 1; whereas it was 3.63ms (S.D.-0.228) and 0.254 µV (S.D.-0.097) for Group 2. The mean latency and amplitude for V peak,

Table 2. Z values and level of significance for latency and amplitude of ABR and LLR

	ABR Latency			*LLR Latency*			
	I peak	III peak	V peak	P1	N1	P2	N2
Group 1 *vs*. Group 2	1.128, $p > 0.05$	0.950, $p > 0.05$	0.448, $p > 0.05$	0.323, $p > 0.05$	0.217, $p > 0.05$	1.354, $p > 0.05$	0.604, $p > 0.05$
	ABR Amplitude			*LLR Amplitude*			
	I peak	III peak	V peak	P1	N1	P2	N2
Group 1 *vs*. Group 2	0.068, $p > 0.05$	0.257, $p > 0.05$	0.691, $p > 0.05$	2.27, $p < 0.05$	0.338, $p > 0.05$	0.243, $p > 0.05$	1.429, $p > 0.05$

ABR, brainstem response; LLR, late latency response.

was 5.50 ms (S.D.-0.213) and 0.180 µV (S.D.-0.0728) respectively for Group 1, whereas it was 5.48 ms (S.D.-0.280) and 0.223 µV (S.D.-0.165) for Group 2. The mean latencies (with S.D.) and amplitudes of peaks I, III and V for the Group 1 and Group 2 is shown in Figure 1 and Figure 2 respectively.

Further, Mann Whitney U test was performed to look for any significant difference between the two groups in terms of latency and amplitude for the I, III and V peaks. Z and *p* values obtained in the Mann Whitney U test are given in Table 2. Results in Table 2 indicate that only P1 amplitude showed a statistically significant ($p < 0.05$) difference between the groups among all the other parameters studied.

3.2. *Comparison of absolute latency and amplitude of peaks P1, N1, P2 and N2 of LLR between Group 1 and Group 2*

Descriptive statistics was carried out to find out the mean and standard deviation in LLR peaks for Group 1 and Group 2.The mean Latency and amplitude for the P1 peak for the participants is 47.33 ms (S.D.-11.29) and 2.80 µV (S.D.-1.681) respectively for Group 1, whereas it was 49.79ms (S.D.-14.041) and 1.543 µV (S.D.-1.017) for Group 2. Peak N1 mean Latency and amplitude for the participants was 88.91 ms (S.D.-10.435) and -3.534 µV (S.D.-1.827) respectively for Group 1, whereas it was 89.56ms (S.D.-14.368) and -3.573 µV (S.D.-2.449) for Group 2. For P2 peak, mean Latency and amplitude for the participants was 158.304 ms (S.D.-38.723) and 3.449 µV (S.D.-1.774) respectively for Group 1, whereas it was 175.341ms (S.D.-20.550) and 4.309 µV (S.D.-3.265) for Group 2. Further, for N2 peak, mean Latency and amplitude for the participants was 262.726 ms (S.D.-39.444) and -1.264 µV (S.D.-1.1864) respectively for Group 1, whereas it was 251.860 ms (S.D.-33.441) and -1.957 µV (S.D.-0.948) for Group 2.

Mean latencies (with S.D.) and amplitudes of P1, N1, P2 and N2 peaks for Group 1 and Group 2 are shown in Figure 3 and Figure 4 respectively.

Mann Whitney U test was performed to look for any significant differences between the groups in terms of

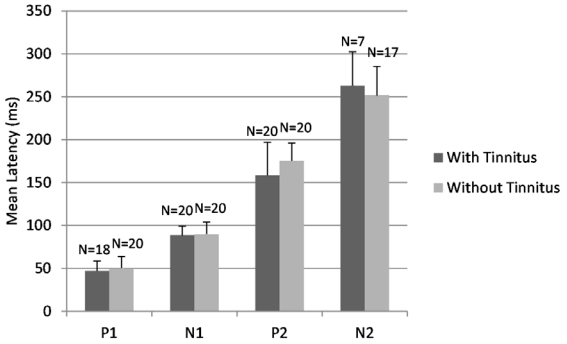

Figure 3. Mean and S.D. of latency of P1, N1, P2 and N2 peak in Group 1 (with tinnitus) and Group 2 (without tinnitus).

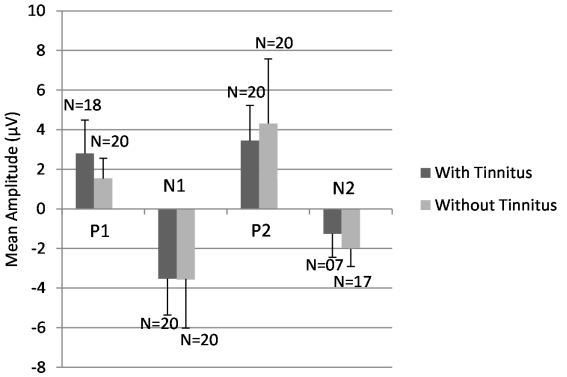

Figure 4. Mean and S.D. of amplitude of P1, N1, P2 and N2 peak in Group 1 (with tinnitus) and Group 2 (without tinnitus).

latency and amplitude for peaks P1, N1, P2 and N2. Z and *p* values obtained in the Mann Whitney *U* test are given in Table 2.

The representative Waveforms of ABR and LLR for both groups are given in Figure 5 and 6 respectively. From Figure 5 it can be noted that there was no significant change in the ABR latency and Amplitude between the Groups. However, Figure 6 shows an increase in P1 amplitude in Group 1 LLR waveform.

4. Discussion

The aim of the present study was to characterize

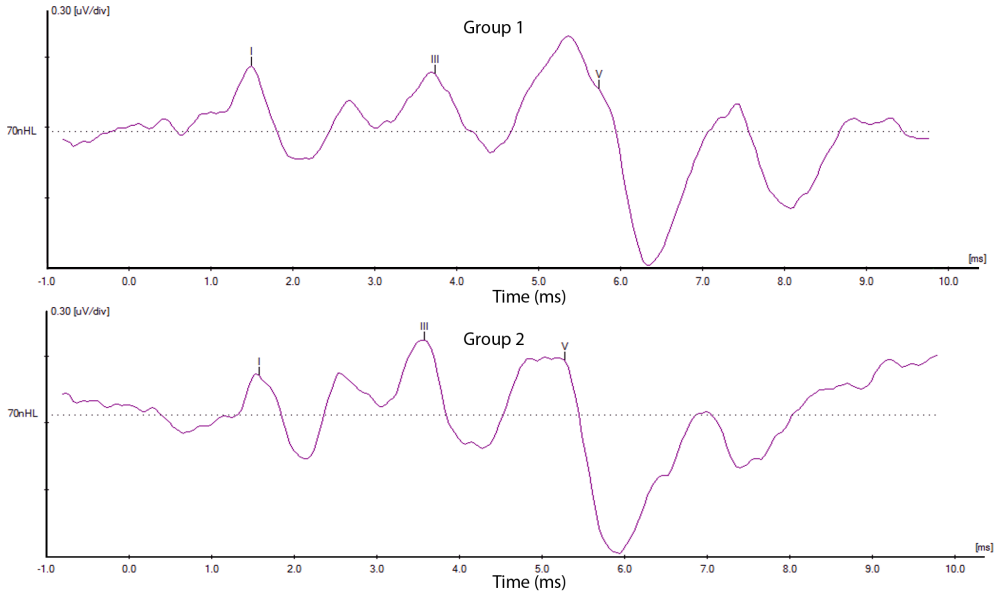

Figure 5. Representative waveform of ABR for Group 1 and Group 2. ABR, brainstem response.

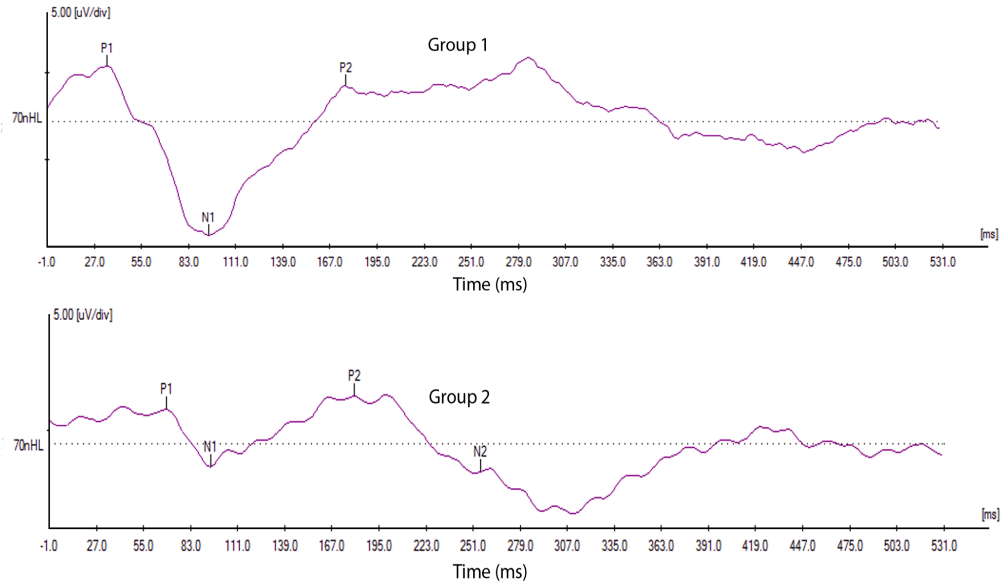

Figure 6. Representative waveform of LLR for Group 1 and Group 2. LLR, late latency response.

the findings of ABR and LLR in individuals with and without tinnitus having normal audiometric presentation. This would further target determining changes in the auditory structures that have occurred due to pathological changes of which tinnitus is a consequence.

The results from ABR indicated no significant differences in latency and amplitude between Group 1 and Group 2. However, participants with tinnitus (Group 1) showed abnormally reduced absolute amplitudes of peaks I and V. There are some possible explanations for the reduction in amplitude of peak I in tinnitus patients compared to individuals without tinnitus. The peak I of ABR is generated from the auditory nerve (*27,28*). Reduced amplitude of peak I could have resulted from a lesser contribution of responsiveness from auditory nerve fibers, or dys-synchrony in discharge of the auditory nerve fibers, or both (*18*). A similar, but detailed explanation is that, even if inner hair cells and auditory nerve fibers are intact, the excitability of the fibers might be reduced *via* lateral olivocochlear efferents which terminate on their endings. This perhaps may lead to the reduction in amplitude of Peak I (*29*). Another possibility is that; there might be damage to the higher-threshold auditory nerve fibers; but not to the lower-threshold fibers, which determine the behavioral threshold. This conclusion is from an animal study in which, after recovering from a temporary threshold shift,

acoustically over-exposed mice presented with a normal set of inner hair cells; but had degeneration of auditory nerve fibers (*30*). Further, the lesser amplitude of peaks Peak I and V could be due to peripheral hearing loss at frequency regions in the cochlea beyond 8 kHz (*31*). The findings of this study support the fact that peripheral loss (*3,32*) (not observed in routine audiological evaluation), occurring due to hair cell damage could be a probable reason for reduced output from nerve fibers at the brainstem level. When absolute latencies are taken into account, no statistically significant difference was found between the two groups. This is similar to the findings by Barnea, Attias, Gold and Shahar (1990) (*33*), wherein, absolute latencies of peaks I, III and V were within normal limits for individuals with normal hearing and tinnitus. Tinnitus with a normal conventional audiogram does not indicate an appreciable lesion at the level of the brainstem. The reduced amplitude of peaks I and V along with normal absolute latencies of peaks I, III and V in the present study indicate that the underlying factor for the origin of tinnitus is possibly due to reduced excitation of auditory nerve fibers. The findings also can be due to existence of peripheral hearing loss beyond 8 kHz, which was not assessed in this study.

The results of LLR indicated that there was no significant difference in the latency and amplitude between Group 1 and Group 2, except for enhanced amplitude of the P1 peak in Group 2. The P1 component of LLR is generated from the pedunculopontine tegmental nucleus, which is a cholinergic sub-division of reticular formation that receives auditory input (*34*). The increased amplitude of the P1 peak in Group 2 could be attributed to a central gain adaptation mechanism which, when confronted with decreased peripheral input, boosts the neural gains to increase spontaneous activity to a point where it is perceived as sound (*35*). The involvement of central auditory structures in the generation and perception of tinnitus is evident in the literature (*36-38*).

Peripheral damage at the level of the cochlea leads to reduced auditory input. Output from central auditory neurons is modulated in response to incoming alterations in signals from the periphery (*39*). The reduced output from the periphery leads to sensory deprivation, which in turn leads to altered neural activity in various areas of the brain (*40*). Evidence suggests that this kind of altered neural activity is seen at the level of the inferior colliculus and auditory cortex (*41-43*). The central gain model suggests that the central auditory structures recalibrate the mean firing rate considering the reduced output from the sensory structures, generating neural noise perceived as tinnitus (*44*).

5. Conclusion

The present study tried to explore the sensorineural correlates of tinnitus among tinnitus patients with normal audiometric thresholds. The electrophysiological investigations using ABR and LLR revealed considerable differences in findings between individuals with normal hearing with and without tinnitus. The abnormally reduced amplitudes of peaks I and V in ABR could be attributed to reduced excitation of auditory nerve fibers. The findings also can be attributed to existence of peripheral hearing loss beyond 8 kHz, which needs to be explored. The increased amplitude of the P1 component of LLR in individuals with tinnitus could be attributed to a central gain adaptation mechanism which, when confronted with decreased peripheral input, boosts neural gains to increase spontaneous activity to a point where it is perceived as sound.

Acknowledgements

Authors acknowledge Dr. S. R. Savithri, Director, All India Institute of Speech and Hearing, Mysuru and HOD Audiology for permitting to publish this research work.

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(Received June 29, 2016; Revised August 12, 2016; Accepted August 15, 2016)

Brief Report

DOI: 10.5582/irdr.2016.01064

Targeted sequencing approach to identify genetic mutations in Nasu-Hakola disease

Jun-ichi Satoh*, Motoaki Yanaizu, Youhei Tosaki, Kenji Sakai, Yoshihiro Kino

Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Tokyo, Japan.

Summary **Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder characterized by sclerosing leukoencephalopathy and multifocal bone cysts, caused by a loss-of-function mutation of either *TYROBP (DAP12)* or *TREM2*. TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Premortem molecular diagnosis of NHD requires genetic analysis of both *TYROBP* and *TREM2*, in which 20 distinct NHD-causing mutations have been reported. Due to genetic heterogeneity, it is often difficult to identify the exact mutation responsible for NHD. Recently, the revolution of the next-generation sequencing (NGS) technology has greatly advanced the field of genome research. A targeted sequencing approach allows us to investigate a selected set of disease-causing genes and mutations in a number of samples within several days. By targeted sequencing using the TruSight One Sequencing Panel, we resequenced genetic mutations of seven NHD cases with known molecular diagnosis and two control subjects. We identified homozygous variants of *TYROBP* or *TREM2* in all NHD cases, composed of a frameshift mutation of c.141delG in exon 3 of *TYROBP* in four cases, a missense mutation of c.2T>C in exon 1 of *TYROBP* in two cases, or a splicing mutation of c.482+2T>C in intron 3 of *TREM2* in one case. The results of targeted resequencing corresponded to those of Sanger sequencing. In contrast, causative variants were not detected in control subjects. These results indicate that targeted sequencing is a useful approach to precisely identify genetic mutations responsible for NHD in a comprehensive manner.**

Keywords: DAP12, Nasu-Hakola disease, targeted sequencing, TREM2, TYROBP

1. Introduction

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL; OMIM 221770), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts (*1,2*). Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide. Clinically, patients show pathological bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, followed by progressive dementia and death until the fifth decade of life (*3*). Pathologically, the brains of NHD patients exhibit extensive demyelination, accumulation of axonal spheroids, microglia activation and intense astrogliosis predominantly in the white matter of frontal and temporal lobes and the basal ganglia (*4*).

NHD is caused by various homozygous loss-of-function mutations located in one of two genes, TYRO protein tyrosine kinase-binding protein (*TYROBP*), alternatively named DNAX-activation protein 12 (*DAP12*) on chromosome 19q13.1 or triggering receptor expressed on myeloid cells 2 (*TREM2*) on chromosome 6p21.1 (*5,6*). TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia

Released online in J-STAGE as advance publication October 4, 2016.

**Address correspondence to:*
Dr. Jun-ichi Satoh, Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo, Japan.
E-mail: satoj@my-pharm.ac.jp

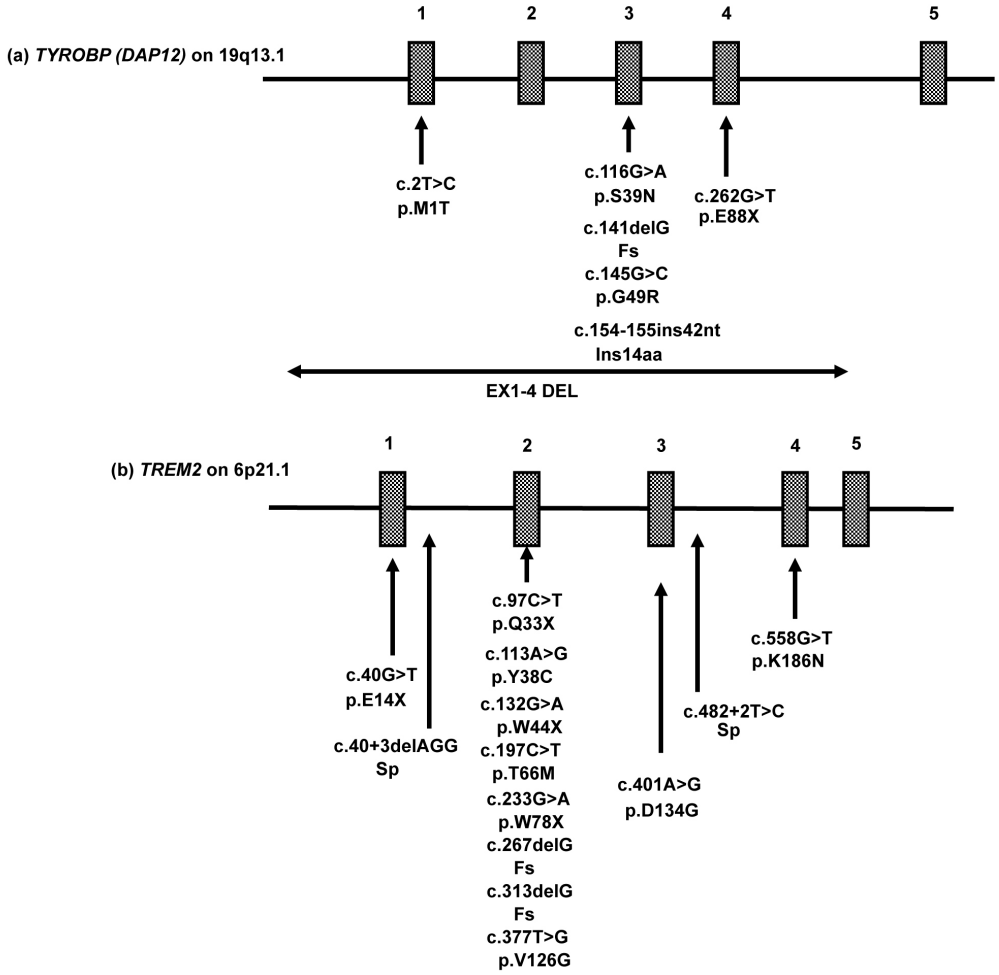

Figure 1. Twenty different mutations causative of NHD. NHD-causing mutations in **(a)** *TYROBP (DAP12)* or **(b)** *TREM2* are shown with genetic and protein definitions and the position of exons.

(*7*). Premortem diagnosis of NHD requires genetic analysis of both *TYROBP* and *TREM2*. Up to the present, a panel of 20 different NHD-causing mutations was reported (Figure 1) (*8,9*). Due to genetic heterogeneity, it is often difficult to identify the exact mutation responsible for NHD. Furthermore, homozygous or compound heterozygous mutations, comprised of c.40+3delAGG, p.Q33X, p.Y38C, p.T66M, p.D86V and p.W198X in *TREM2*, cause frontotemporal dementia (FTD)-like syndrome without bone involvement (*10,11*). Importantly, accumulating evidence indicates that heterogeneous variants of p.R47H and p.R62H in *TREM2*, confer a substantial risk for development of late-onset Alzheimer's disease (LOAD) (*11,12*).

Recently, the revolution of the next-generation sequencing (NGS) technology has had a great impact on the field of genome research. Targeted sequencing with focused gene panels, which are designed to include genomic regions causative of genetic diseases, allows us to investigate rapidly and efficiently a selected set of candidate genes in many samples (*13*). By using the TruSight One Sequencing Panel, we resequenced genetic mutations of seven NHD cases with known molecular diagnosis.

2. Materials and Methods

2.1. *Ethics*

The Human Research Ethics Committee (HREC) of the Meiji Pharmaceutical University (MPU) approved this study (No. 1904, 2015), which follows the Ethical Guidelines for Analytical Research on the Human Genome/Genes, Japan. Written informed consent was taken from all participants.

2.2. *Targeted resequencing*

All patients showed clinical characteristics of NHD, such as multiple bone cysts and diffuse leukoencephalopathy. Before targeted sequencing, the causative mutations were characterized by Sanger sequencing of the *TYROBP* or *TREM2* gene. Fifty ng of genomic DNA extracted from peripheral blood mononuclear cells (PBMC) was processed for DNA library preparation by using MiSeq Reagent Kit V3 (Illumina, San Diego,

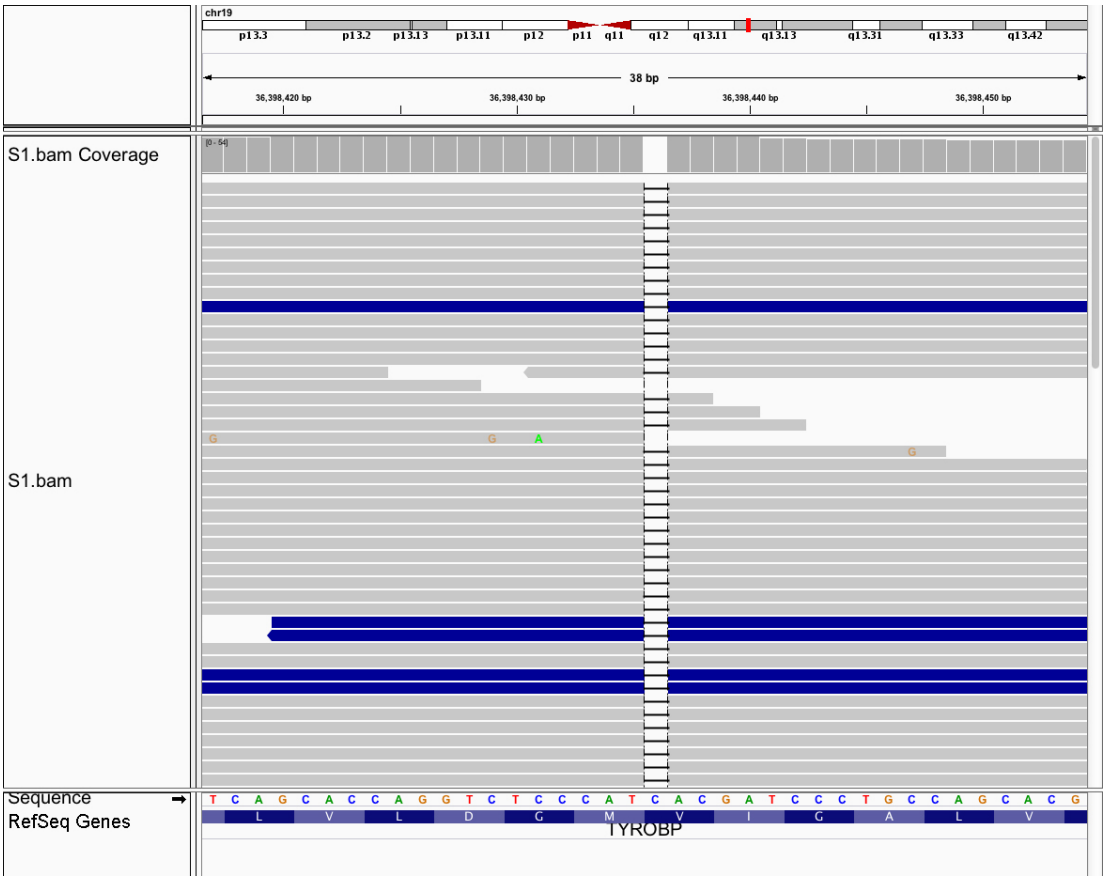

Figure 2. A frameshift mutation of c.141delG in exon 3 of *TYROBP*. By targeted resequencing, we identified a frameshift mutation of c.141delG in exon 3 of *TYROBP*, causing premature termination at amino acid residue 52 in four NHD cases. The genetic mutation was visualized by importing the sequence alignment data into IGV.

CA, USA) and target enrichment by the TruSight One Sequencing Panel that contains coding regions of 4,813 genes associated with known clinical phenotypes (Illumina). The genes were selected from the Human Gene Mutation Database (HGMD; *http://www.hgmd.cf.ac.uk*), the Online Mendelian Inheritance in Man database (OMIM; *http://omim.org/search/advanced/entry*), the GeneTests database (*http://www.genetests.org*), the Illumina TruSight Exome content set, and other available sequencing panels. The full list of the genes is available online (*http://www.illumina.com/content/dam/illumina-marketing/documents/products/gene_lists/gene_list_trusight_one.zip*). The concentration of target DNA was quantified by Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The prepared library was loaded on the Flowcell of MiSeq (Illumina) for sequencing.

2.3. *Processing of sequencing data*

The sequencing data, composed of 150 bp paired-end reads, were processed for MiSeq Reporter (Illumina), which is endowed with Burrows-Wheeler Aligner (BWA) for alignment on a reference sequence of hg19 and Genome Analysis Toolkit (GATK) for variant calling. Then, the Variant Call format (VCF) file was processed for analyzing on VariantStudio (Illumina), which contains annotation databases, such as the ClinVar database (ClinVar; *http://www.ncbi.nlm.nih.gov/clinvar*), OMIM, the dbSNP database (dbSNP; *http://www.ncbi.nlm.nih.gov/SNP*), the Polymorphism Phenotyping v2 database (Polyphen-2; *http://genetics.bwh.harvard.edu/pph2*), and the Sorting Intolerant from Tolerant database (SIFT; *http://sift.jcvi.org*). The genetic mutations were visualized by importing a BAM format file into Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA, USA).

3. Results and Discussion

Variant calling data enclosed in a VCF file were filtered by VariantStudio under the condition of homozygous mutations, quality value > 1000, read depth > 30, and only variants with ClinVar annotation. Pathogenic and probably pathogenic mutations with ClinVar significance were selected. Benign mutations on PolyPhen-2 and tolerated mutations on SIFT were excluded. Finally, we were able to narrow down the set of disease causing-mutations in all NHD cases, composed of a frameshift mutation of c.141delG in exon 3 of *TYROBP* (*14*), causing premature termination at amino acid residue 52 in four cases (Figure 2), a missense mutation of c.2T>C

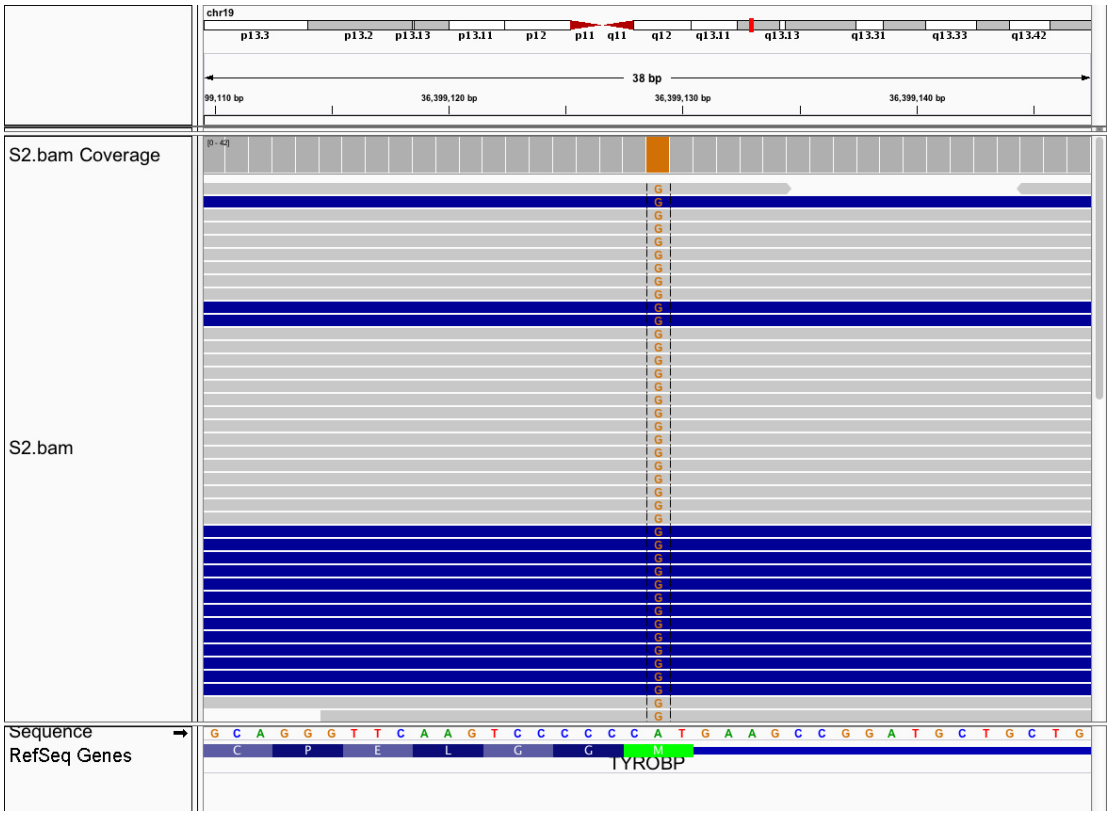

Figure 3. A missense mutation of c.2T>C in exon 1 of *TYROBP*. By targeted resequencing, we identified a missense mutation of c.2T>C at the initiation codon in exon 1 of *TYROBP* in two NHD cases. The genetic mutation was visualized by importing the sequence alignment data into IGV.

at the initiation codon in exon 1 of *TYROBP* (*14*) in two cases (Figure 3), or a splicing mutation of c.482+2T>C at the splice-donor consensus site in intron 3 of *TREM2* (*15*) in one case (Figure 4). The entire process was completed within a couple of days. The results of targeted resequencing corresponded completely to those of Sanger sequencing. Causative variants were not detected in control subjects.

In the present study, by targeted sequencing on a commercially available focused gene panel, we resequenced *TYROBP* or *TREM2* mutations in seven NHD cases with known molecular diagnosis. We identified genetic variants in all seven NHD cases, which were previously validated by Sanger sequencing, indicating that this approach is highly reliable and reproducible. Thus, targeted sequencing provides a rapid, convenient, high throughput, and comprehensive platform for molecular diagnosis of NHD in a clinical setting. Furthermore, targeted sequencing is capable of detecting novel unpredicted variants located in focused genes, including noncausative but disease-modifying ones. In contrast, Sanger sequencing of individual genes and exons is often laborious with low throughput, although it does not require expensive NGS machines. With respect to the disadvantage, the results of targeted sequencing are derived exclusively from a limited set of focused genes. Furthermore, this approach could not detect noncoding or deep intronic mutations and copy number variations. However, targeted sequencing produces a much smaller dataset size, compared with that of whole genome or whole exome sequencing, making interpretation of the data more concise (*16*).

A recent study sequenced genomic DNA of 79 patients with sporadic inclusion body myositis (sIBM) on a panel of 38 target genes, and identified 27 rare coding missense variants with those including disease-causing mutations in the valosin containing protein (*VCP*) gene, suggesting that targeted sequencing is a clinically meaningful approach for genetic evaluation of sIBM (*17*). More recently, targeted sequencing with the TruSight One sequencing panel, causative mutations were found in six of 17 families of pediatric patients with various genetic diseases, such as Sotos syndrome, Joubert syndrome, and neurofibromatosis type 1A (*16*). In different studies, targeted sequencing with the TruSight One Sequencing Panel showed somatic mosaicism of a disease-causing mutation in a patient with CDKL5-related encephalopathy (*18*), and served as a prenatal diagnostic tool for LAMA2-related muscular dystrophy (*19*). Furthermore, targeted sequencing with the TruSight One Sequencing Panel identified three distinct rare homozygous and compound heterozygous variants causative of nephronophthisis-related ciliopathy (NPHP-RC), an autosomal recessive cystic kidney disease, in three patients (*20*).

In conclusion, targeted sequencing is a useful

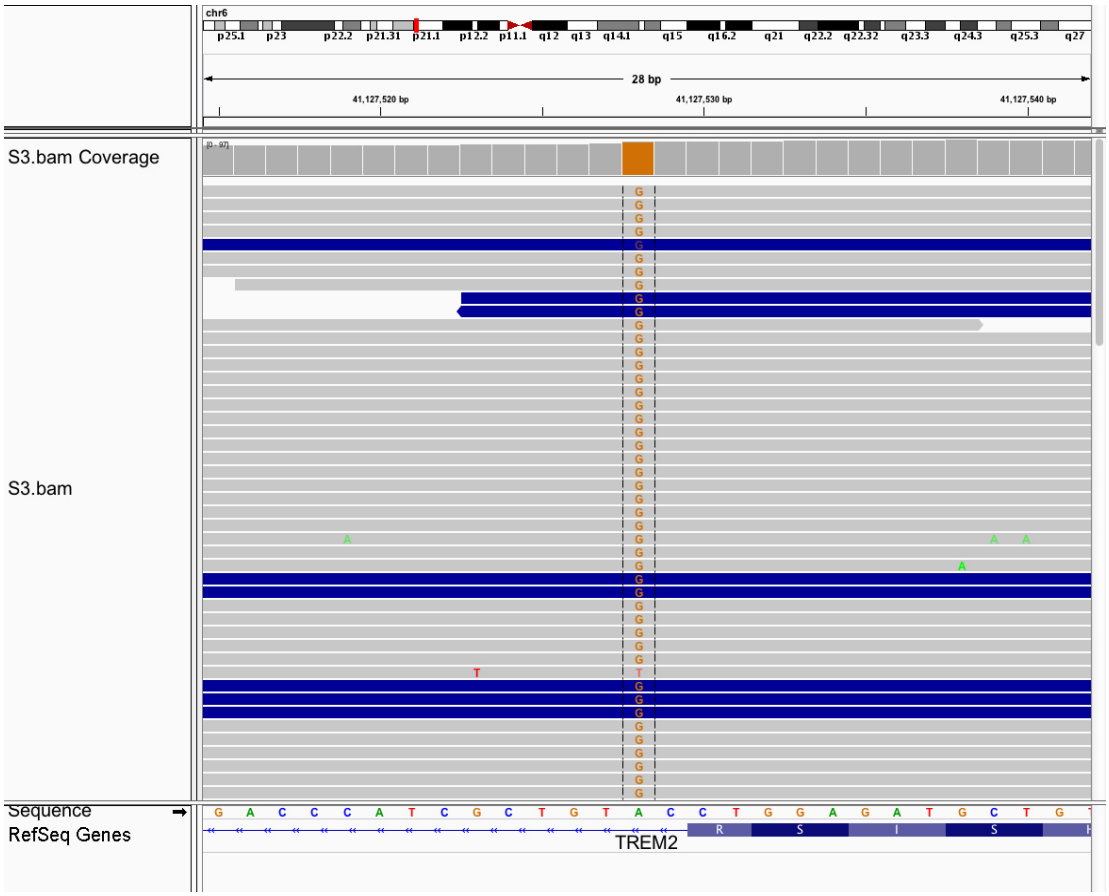

Figure 4. A splicing mutation of c.482+2T>C in intron 3 of *TREM2*. By targeted resequencing, we identified a splicing mutation of c.482+2T>C at the splice-donor consensus site in intron 3 of *TREM2* in one NHD case. The genetic mutation was visualized by importing the sequence alignment data into IGV.

approach for the exact molecular diagnosis of rare Mendelian diseases, including NHD, by investigating genetic mutations in a comprehensive manner.

Acknowledgements

This work was supported by grants from the Research on Intractable Diseases, entitled “Clinicopathological and genetic studies of Nasu-Hakola disease” (H21-Nanchi-Ippan-201; H22-Nanchi-Ippan-136), the Ministry of Health, Labour and Welfare of Japan, and the JSPS KAKENHI (C25430054 and 16K07043) and the Dementia Drug Development Research Center (DRC) project (S1511016), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. We thank Ms. Shiho Saito for technical assistance.

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(Received August 10, 2016; Revised September 1, 2016; Accepted September 2, 2016))

Brief Report

DOI: 10.5582/irdr.2016.01086

Expression of gp91phox and p22phox, catalytic subunits of NADPH oxidase, on microglia in Nasu-Hakola disease brains

Jun-ichi Satoh[1,*], Yoshihiro Kino[1], Motoaki Yanaizu[1], Youhei Tosaki[1], Kenji Sakai[1], Tusyoshi Ishida[2], Yuko Saito[3]

[1] *Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Tokyo, Japan;*
[2] *Department of Pathology and Laboratory Medicine, Kohnodai Hospital, NCGM, Chiba, Japan;*
[3] *Department of Laboratory Medicine, National Center Hospital, NCNP, Tokyo, Japan.*

Summary **The superoxide-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex of phagocytes (phox) plays a key role in production of reactive oxygen species (ROS) by microglia. The catalytic subunits of the NADPH oxidase are composed of p22phox and gp91phox. Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder caused by a loss-of-function mutation of either *TYROBP* (*DAP12*) or *TREM2*. Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes. However, a pathological role of the gp91phox-p22phox complex in generation of leukoencephalopathy in NHD remains unknown. We clarified the expression of gp91phox and p22phox in the white matter of the frontal cortex derived from five NHD and eight control subjects. We identified the expression of p22phox and gp91phox immunoreactivity almost exclusively on microglia. Microglia overexpressed gp91phox in NHD brains and p22phox in myotonic dystrophy (MD) brains, when compared with non-neurological control (NC) brains. These results suggest that the enhanced expression of gp91phox by microglia might contribute to overproduction of ROS highly toxic to myelinating oligodendrocytes, resulting in oligodendrocyte cell death that induces leukoencephalopathy in NHD brains.**

Keywords: gp91phox, leukoencephalopathy, microglia, Nasu-Hakola disease, p22phox

1. Introduction

The superoxide-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) complex of phagocytes (phox) plays a key role in the elimination of invading pathogens by phagocytic cells (*1*). The core catalytic enzymes called flavocytochrome b558 are composed of a membrane-associated heterodimeric complex, consisting of p22phox (α subunit) and gp91phox (Nox2, β subunit) (*2*). Upon stimulation of phagocytes, regulatory subunits, including p40phox, p47phox, p67phox, and Rac1/2, are activated by posttranslational modification, conformational change, and protein-protein interaction, and translocates from the cytosol to the membrane, resulting in activation of the gp91phox-p22phox complex (*2*). Following the assembly of the oxidase complex, the flavocytochrome b558 transports electrons from intracellular NADPH to extracellular or phagosomal oxygen, leading to production of superoxide anion. Superoxide anion is short lived and reacts with various molecules to form reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals, and hypochlorous acid, and peroxynitrite (*1*). An excessive amount of ROS causes oxidative stress and cell death by inducing oxidative modification of lipids, proteins, and nucleic acids, whereas a low level of ROS play a key role in redox-dependent intracellular

Released online in J-STAGE as advance publication November 7, 2016.

**Address correspondence to:*
Dr. Jun-ichi Satoh, Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo, Japan.
E-mail: satoj@my-pharm.ac.jp

signaling that amplifies the proinflammatory response (*3,4*).

Microglia, the resident myeloid cells in the central nervous system (CNS), have a capacity to constantly scavenge invading pathogens, apoptotic debris, unwanted synapses, and aggregated proteins by sensing them with a panel of pattern recognition receptors (PRRs) (*5*). Microglia serve as a major source of ROS through the expression of the phagocytic Nox in the lesions of cerebral ischemia, traumatic brain injury, multiple sclerosis (MS), and Alzheimer's disease (*6-9*). In these diseases, the microglial gp91phox-p22phox complex is promptly activated in response to neurotoxic stimuli, followed by ROS-mediated neuronal damage (*3*).

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL; OMIM 221770), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of either *TYROBP*(*DAP12*) or *TREM2* (*10*). TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide. Clinically, the patients with NHD show recurrent bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, and progressive dementia and death until the fifth decade of life (*11*). Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia (*12*). However, a pathological role of the gp91phox-p22phox complex with relevance to leukoencephalopathy in NHD remains largely unknown. In the present study, we have attempted to clarify the expression of gp91phox and p22phox in the white matter lesions of NHD brains by immunohistochemistry.

2. Materials and Methods

2.1. *Human brain tissues*

The brain autopsies were performed at the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Japan, Kohnodai Hospital, National Center for Global Health and Medicine (NCGM), Japan, and affiliated hospitals of Research Resource Network (RRN), Japan. The comprehensive examination by established neuropathologists (YS and TI) validated the pathological diagnosis. In all cases, written informed consent was obtained. The Ethics Committee of the NCNP for the Human Brain Research, the Ethics Committee of the NCGM on the Research Use of Human Samples, and the Human Research Ethics Committee (HREC) of the Meiji Pharmaceutical University (MPU) approved the present study.

For immunohistochemical studies, serial sections containing the white matter of the frontal cortex were prepared from four subjects who died of non-neurological causes (NC), composed of a 63-year-old man who died of prostate cancer and acute myocardial infarction (NC1), a 67-year-old man who died of dissecting aortic aneurysm (NC2), a 57-year-old man who died of alcoholic liver cirrhosis (NC3), and a 61-year-old man who died of rheumatoid arthritis with interstitial pneumonia (NC4), four neuropsychiatric disease controls affected with myotonic dystrophy (MD), composed of a 68-year-old man (MD1), a 61-year-old man (MD2), a 60-year-old man (MD3), and a 53-year-old woman (MD4), and five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5). The homozygous mutation of a single base deletion of 141G (141delG) in exon 3 of DAP12 was identified in NHD1, NHD2, and NHD5, while the genetic analysis was not performed in NHD3 or NHD4, as described previously (*13*).

2.2. *Immunohistochemistry*

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 110℃ for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Concord, CA, USA). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. They were then incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4℃ overnight with rabbit polyclonal anti-p22phox antibody (sc-20781, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with mouse monoclonal anti-gp91phox antibody (ab139371, Abcam, Cambridge, UK). After washing with PBS, tissue sections were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with diaminobenzidine tetrahydrochloride (DAB) substrate (Vector, Burlingame, CA, USA). They were processed for a counterstain with hematoxylin. Negative controls underwent all the steps except for exposure to primary antibody. In limited experiments, double immunolabeling of mouse anti-gp91phox antibody and rabbit antibodies against p22phox, Iba1 (Wako Pure Chemical, Tokyo, Japan; a marker of microglia/macrophages), GFAP (Dako, Tokyo, Japan;

a marker of astrocytes), or NeuN (Abcam; a marker of neurons), was performed, followed by incubation with HRP-conjugated or alkaline phosphatase-conjugated anti-mouse or anti-rabbit secondary antibody and exposure to DAB substrate and Warp Red chromogen (Biocare Medical).

2.3. *Quantification of gp91phox and p22phox immunoreactivity*

To quantify immunolabeled areas, the images derived from three fields of the white matter were captured at a 200 X magnification on the Olympus BX51 universal microscope. They were then processed for quantification by using ImageJ software (National Institute of Health, Bethesda, MD, USA). The gp91phox- or p22phox-immunolabeled area was calibrated by the Iba1-immunolabeled area of the identical field. The difference in the average of immunopositive areas between NHD and the controls was evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test.

3. Results and Discussion

By immunohistochemistry, we identified the intense expression of gp91phox and p22phox immunoreactivity chiefly on microglia/macrophages in the white matter of the frontal cortex derived from NC, MD and NHD subjects (Figure 1, panels a-d and Figure 2, panels a-d). By double immunolabeling, the expression pattern of gp91phox overlapped with that of p22phox (Figure 3, panel a) and Iba1-positive microglia expressed gp91phox (Figure 3, panel b), whereas myelinating oligodendrocytes, GFAP-positive astrocytes and NeuN-positive neurons did not express gp91phox (Figure 3, panels c, d), suggesting that oligodendrocytes, astrocytes and neurons do not express discernible levels of the gp91phox-p22phox complex in the human brain. By quantitative analysis, the immunolabeled area of gp91phox-positive microglia was increased significantly in NHD brains, when compared with NC brains but not with MD brains (Figure 4, panel a; $p = 0.0338$). In contrast, the immunolabeled area of p22phox-positive microglia was not significantly different between NC and NHD brains, whereas it was increased significantly in MD brains, when compared with NC brains (Figure 4, panel b; $p = 0.0096$).

The enhanced expression of the gp91phox-p22phox complex plays a key role in microglial production of superoxide anion and ROS (*3,7*). We previously found accumulation of numerous Iba1-positive microglia in NHD brains and showed that mRNA levels of microglia/macrophage marker genes, such as CD68, CD163, and MSR1, were elevated in NHS brains (*12,14*). The present study indicated that microglia overexpressed gp91phox in NHD brains and p22phox in MD brains, when compared with NC

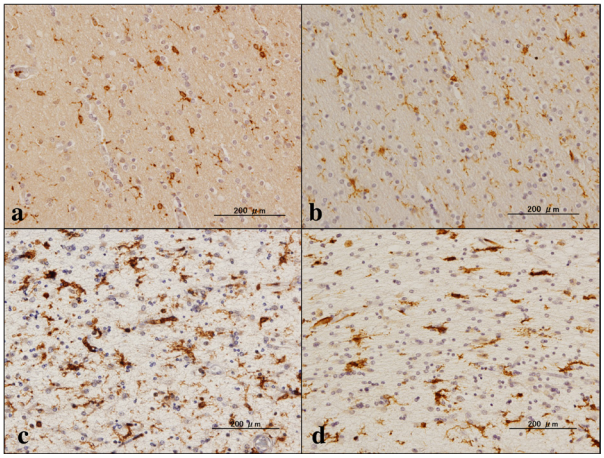

Figure 2. Expression of p22phox and Iba1. (a) NC, p22phox, **(b)** NC, the same area of **(a)**, Iba1, **(c)** NHD, p22phox, and **(d)** NHD, the same area of **(c)**, Iba1.

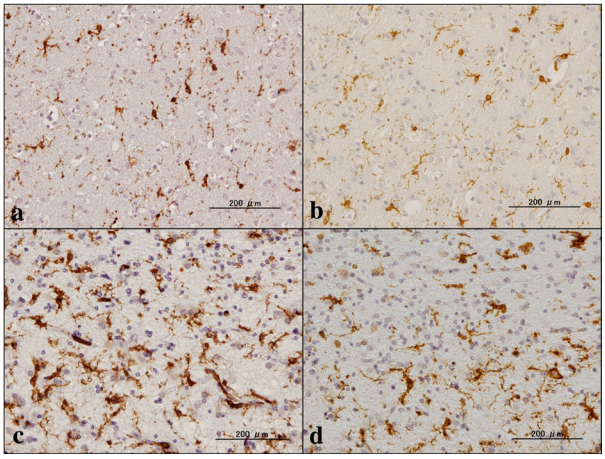

Figure 1. Expression of gp91phox and Iba1. (a) NC, gp91phox, **(b)** NC, the same area of **(a)**, Iba1, **(c)** NHD, gp91phox, and **(d)** NHD, the same area of **(c)**, Iba1.

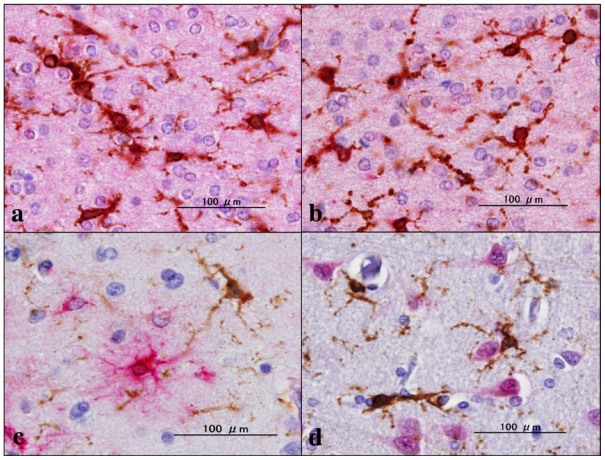

Figure 3. Double labeling of gp91Phox and cell type-specific markers. (a) MD, gp91phox (brown), p22phox (red), **(b)** MD, gp91phox (brown), Iba1 (red), **(c)** MD, g91phox (brown), GFAP (red), and **(d)** gp91phox (brown), NeuN (red).

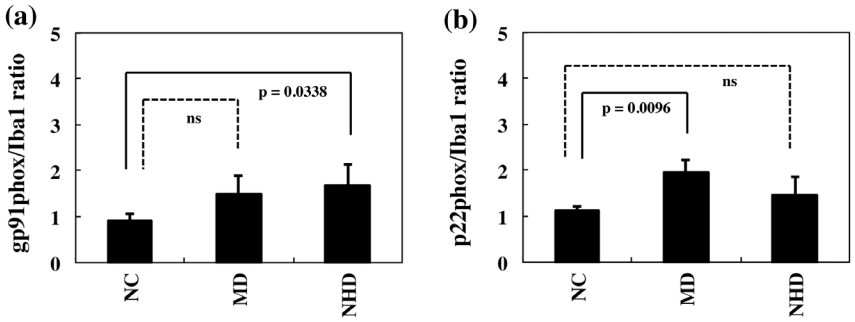

Figure 4. Quantitative analysis of gp91phox and p22phox expression on microglia in NC, MD, and NHD brains. (a) The ratio of immunolabeled area of gp91phox/Iba1, and **(b)** The ratio of immunolabeled area of p22phox/Iba1.

brains. The elevation of p22phox expression levels in MD brains is attributable to a high prevalence of white matter damage in this disease (*15*). More importantly, our observations raise a possible scenario that the enhanced expression of gp91phox in NHD brains contributes to overproduction of ROS highly toxic to myelinating oligodendrocytes (*16,17*), resulting in oligodendrocyte cell death, followed by development of leukoencephalopathy. Supporting this, previous studies showed that the expression of gp91phox and p22phox was upregulated in activated microglia accumulating in initial lesions of MS, an inflammatory demyelinating disease in the CNS, suggesting a role of oxidative tissue damage in progression of demyelination (*8,18*).

Notably, a population of activated microglia expressed gp91 in the penumbra of transient cerebral ischemia (*6*). The expression of gp91 was upregulated in ameboid microglia, which generate superoxide anion and peroxynitrite, distributed in the pericontusional area following traumatic brain injury (*7*). Microglia play a key role in lipopolysaccharide (LPS)-induced dopaminergic cell death in a manner dependent on the expression of gp91phox (*19*). All of these observations suggest that microglia contain a functionally active NADPH oxidase capable of generating ROS. Microglia, promptly activated by proinflammatory cytokines, disease-associated proteins, and environmental toxins, express high levels of gp91phox and produce a large amount of ROS that cause oxidative stress leading to neuronal and myelin damage (*3,16,17*).

In conclusion, the enhanced expression of gp91phox on microglia, a central component of the NADPH oxidase, might be involved in development of leukoencephalopathy in NHD brains *via* myelin damage caused by superoxide anion-inducible ROS.

Acknowledgements

The authors thank Drs. Kenji Jinnai, Nobutaka Arai, Kiyotaka Nakamagoe, Nobutaka Motohashi, and Saburo Yagishita for providing us brain samples. This work was supported by grants from the Research on Intractable Diseases, entitled "Clinicopathological and genetic studies of Nasu-Hakola disease" (H21-Nanchi-Ippan-201; H22-Nanchi-Ippan-136), the Ministry of Health, Labour and Welfare of Japan, and grants from the JSPS KAKENHI (C25430054 and 16K07043) and the Dementia Drug Development Research Center (DRC) project (S1511016), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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(Received October 24, 2016; Accepted November 3, 2016)

Brief Report

DOI: 10.5582/irdr.2016.01085

Metachromatic leukodystrophy: Biochemical characterization of two (p.307Glu→Lys, p.318Trp→Cys) arylsulfatase A mutations

Adem Özkan, Hatice Asuman Özkara*

Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey.

Summary **Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by Arylsulfatase A (ASA) deficiency. The hallmark of the disease is central and peripheral neurodegeneration. More than 200 mutations have been identified in *ARSA* gene so far. Some of these mutations were characterized. The aim of this study is to reinforce genotype-phenotype correlation and to understand the effect of mutations on the enzyme by biochemical characterization. Two missense mutations (c.919G→A, p.307Glu→Lys and c.954G→T, p.318Trp→Cys in exon 5) were constructed on WT-ASA cDNA and were confirmed by DNA sequence analysis. Plasmid DNA carrying mutant or normal ASA cDNA was transferred to Chinese Hamster Ovary (CHO) cells through transient transfection. ASA protein was produced by CHO cells. Hexosaminidase beta-subunit gene was cotransfected into the CHO cells as a control gene of transfection efficiency. 48 hours after transfection, cells were collected and homogenized. ASA and hexosaminidase activities were measured in supernatant. ASA enzyme activity is decreased 100% according to the control by the effect of both mutations. The mutations are located in the higly conserved region of the protein. In this study, we showed that both mutations result in null ASA activity in CHO cells making the protein nonfunctional. We confirmed that p.307Glu→Lys and p.318Trp→Cys mutations cause late infantile form of MLD disease.**

Keywords: Missense mutations, *in vitro* mutagenesis, transfection, CHO cells, genotype-phenotype correlation

1. Introduction

Metachromatic leukodystrophy (MLD) is an autosomal recessive sphingolipid storage disease that occurs as a result of deficiency of lysosomal Arylsulfatase A (ASA) or its activator protein. Its frequency is estimated to be 1 in 69,890 newborns in Turkey (*1*) and 1 in 40,000 newborns in Northern Sweden (*2*).

ASA catalyses the desulfation of sphingolipid sulfatide. Sulfatide is found in high concentrations in myelin sheat of the nervous system. As a result of ASA deficiency, sulfatide is accumulated in the nervous system and affects the oligodendrocytes and results in neurodegeneration. Sulfatide is also stored in various organs such as liver and kidney. According to the starting age MLD shows three clinical phenotypes: late infantile, juvenile and adult. Clinically, the most severe type is late infantile type. It starts around 2-year-old and ends in early childhood with death. Juvenile and adult types start between 4-16-year-old and any age after puberty. ASA can be 5-15% of reference value in some patients with pseudodeficiency which can lead to misdiagnosis. DNA-based methods for pseudodeficiency allele and mutation detection and activator protein detection can be useful for further diagnosis of MLD types (*3*).

To date 200 mutations have been described in the *ARSA* gene (*4*). Most of them are missense mutations. A few of these mutations occur only with high frequency. There is a genotype-phenotype correlation in MLD. Patients with late infantile phenotype usually have homozygous mutations. Mutations result in very low level of enzyme activity. However, patients with juvenile

Released online in J-STAGE as advance publication November 7, 2016.

**Address correspondence to:*
Dr. Hatice Asuman Özkara, Hacettepe University Faculty of Medicine, Department of Medical Biochemistry 06100 Ankara, Turkey.
E-mail: ozkara@hacettepe.edu.tr

phenotype usually have one severe allele and one mild allele combinations and have some residual enzyme activity. Adult patients are frequently homozygous for alleles expressing residual enzyme activities. Severity of the disease correlates inversely with the residual enzyme activity (*3*).

In this study we constructed two missense (in exon 5 c.919G→A, p.307Glu→Lys and c.954G→T, p.318Trp→Cys) mutations by site-directed mutagenesis on wild-type *ARSA* gene, transiently transfected to the CHO cells, and characterized biochemically.

2. Materials and Methods

2.1. *Materials*

Cell culture media were obtained from Gibco (Germany). Taq DNA polymerase, oligonucleotides and restriction enzymes were purchased from Sigma Chemical Co. (Germany). DH5-alpha cells were purchased from Invitrogene (Germany), Quickchange site-directed mutagenesis kit was purchased from Qiagen (Germany). Wild-type ASA plasmid was kindly supplied by Prof. Dr. Volkmar Gieselmann (Bonn University). Other reagents were from Sigma and Merck.

2.2. *In vitro mutagenesis, amplification of ARSA genes and DNA sequencing*

In vitro mutagenesis was performed according to the protocol of QuickChange site-directed mutagenesis kit on the wild-type *ARSA* gene. The sequences of the oligonucleotides used for the introduction of the mutations were: c.919G→A, p.307Glu→Lys, 5' GA AAGGGAACGACCTACAAGGGCGGTGTCCGAG AG 3' and c.954G→T, p.318Trp→Cys, 5' CTGCCTTG GCCTTCTGTCCAGGTCATATCGCTC 3'. Mutations were confirmed by DNA sequencing.

2.3. *Cell culture and transfection*

Chinese hamster ovary (CHO) cells were grown in Dulbecco's Modified Eagles Medium (DMEM) with 1% glutamine and 10% fetal calf serum (FCS) at 37°C in 5% CO_2. Four µg of vector was transfected to the CHO cells (40% confluent) using Superfect Transfection Reagent (Qiagen). After 48 h of transfection the medium was discarded, the cells were washed 3×, scraped and centrifuged. Cell pellet dissolved in 100 µL Tris-HCl pH 7,8 and were mixed with protease inhibitor mix and immediately lysed by freezing and thawing 3X in liquid nitrogen. Then supernatants were used for protein measurement by bicinconinic acid method.

2.4. *Enzyme analysis*

Hexosaminidase and Arylsulfatase A activities were measured according to the protocol described before (*5,6*) by using fluorometric substrate 4MUG and spectrophotometric substrate p-nitrocathecol sulfate. As a control, β-hexosaminidase activity was assayed and used to correct variations in transfection efficiencies.

3. Results and Discussion

Metachromatic leukodystrophy is common in Turkey among patient with sphingolipid storage disease. Minimum calculated incidence is 1.43/100,000 live births in Turkey (*1*). Worldwide incidence of MLD is 1/40,000 to 1/160,000 (*7*). Previously we reported two ASA mutations causing late infantile MLD in two different Turkish patients (*8*): *(a)* a c.919G→A transition in exon 5 causing a p.307Glu→Lys and *(b)* a c.954G→T transition in exon 5 causing a p.318Trp→Cys. The patients were homozygotes for the mutations. Confirmation of the mutations' effect by *in vitro* mutagenesis and reinforcement genotype-phenotype correlation are important for using those mutations in prenatal diagnosis. It is also important for understanding the functional domains of ASA protein and underlying mechanism of the disease. Here we analyzed the effect of these mutations on the function of the protein in CHO cells. Two missense mutations (*8*) were created on ASA cDNA constructs by site-directed mutagenesis, and their DNA sequence confirmed using the automatic sequence analyzer from Applied Biosystem. CHO cells were transiently transfected with each of the cDNAs, collected after 48 h, mixed with protease inhibitors, lysed and then subjected to enzyme and protein analysis. Enzyme activities were measured in supernatants of two independent transfection experiments and the results were expressed as nmol/hr/mg pr. ASA activity was 2 folds of mock transfected cells in WT-ASA cDNA transfected CHO cells. In mutant ASA cDNA transfected CHO cells ASA activity was almost equal to the activity measured in mock transfected cells. On the other hand, lysates of both mutant transfected CHO cells contained only background ASA activity (the same activity with the mock transfected). The human Hex activity was found to be 1.8-2 times higher in transfected cells than in mock transfected cells and activities were found to be in the same range in all transfected cells. Activity measurements obtained from two independent transfection experiments were calculated as mean values, subtracted from mock transfected cells' value, and expressed as % activity of the normal control. ASA activity was found deficient in both mutant protein containing samples according to the control (Table 1). We found that 307Glu→Lys and 318Trp→Cys missense mutations impairs enzyme activity. On the other hand, in wild-type transfected CHO cells two-fold ASA activity was observed.

Secondary structure of ASA is very well-defined

Table 1. Verification of ASA activities in transiently transfected CHO cells expressing two mutant *ARSA* gene as observed in Turkish late-infantile Metachromatic Leukodystrophy patients

ARSA gene expressed	Clinical phenotype	ASA activity* (nmol/hr/mg protein)	ASA activity** (% of wild type)
Wild-type	Positive control	215 ($n = 2$)	100
c.919G→A, p.307Glu→Lys	Late infantile	0 ($n = 2$)	0
c.954G→T, p.318Trp→Cys	Late infantile	0 ($n = 2$)	0

*Mean of two independent transfection experiments. **100% of ASA activity equals the activity found in wild type. Each enzyme activity value subtracted from mock transfected activity. Enzyme activities are calculated as % activity of wild-type activity.

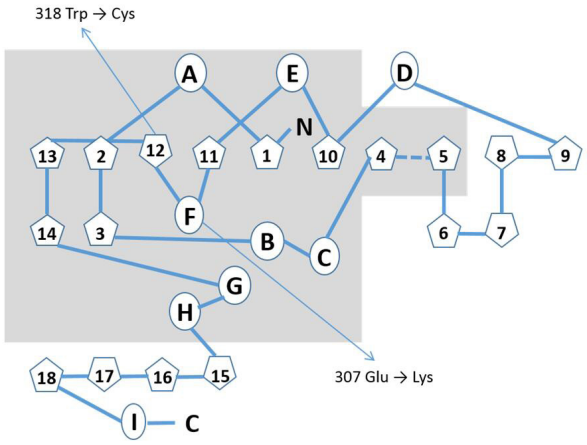

Figure 1. Simplified schematic represantation of the secondary structure of Arylsulfatase A modified from Lukatela G. *et al*., 1998 (*9*). ⬯ indicates alpha helices (A to I) and ⬠ indicates beta-structures (1 to 18). Grey area shows the higly conserved structural elements among human sulfatases. The locations of two Turkish mutations are indicated with arrows.

(*9*). It has a mixture of alpha-helix and beta-plated structure (26% α-helix and 3_{10} helix, 16% β-sheet, 46% β and 3_{10} turn and 12% primary structure). Center of the enzyme contains two significant β-sheets: minor and major. The minor includes 4 anti-parallel β-strands and the major includes 10 different beta strands. The major β-pleated sheet is located between A, D and E helices on one side and B, C, G and H helices on the other side. The short helix F connects anti parallel β11 and β12 (Figure 1). Major and minor β sheets linked by hydrogen bonding and a disulfide bond between Cys300-Cys414 (*9,10*). 919 G→A mutation in exon 5 of ASA gene leads to a substitution of a basic amino acid lysine into an acidic amino acid glutamate (p.307Glu→Lys) in the protein. Glu307 is a member of F helix (Figure 1) and it is located in a highly conserved region of human sulfatases. F helix connects the anti parallel beta sheets. The substitution of a basic amino acid (Lys) into an acidic amino acid (Glu) may result in changes in interaction of beta-sheets in the active center, cause deficient enzyme activity both in patients (*8*) and *in vitro* by probably disrupting the alpha-helix structure of F helix (Figure 1). ASA activity was found 0% of wild transfected in mutant-protein expressing CHO cells. Different mutations have been identified at the adjacent amino acids involved in alpha-helical structure in the literature. All of those mutations are caused late infantile type of MLD. Enzyme activity was found completely deficient in transiently transfected COS-1 cells carrying p.308Gly→Val substitution which is found in European and Japanese patients (*11*). On the other hand, enzyme activity was found 13% of control in *in vitro* mutagenesis study of p.309Gly→Ser mutation. Protein found to have entered to the lysosome but unstabile (*12*).

c.954 G→T mutation in ASA gene causes p.318Trp →Cys substitution. Instead of a hydrophobic amino acid tryptophan, SH group containing uncharged polar amino acid cysteine is located in the protein. 318Trp is the member of primary structure between beta-sheet 12 and anti-parallel beta-sheet 13 (Figure 1). ASA protein has 15 cysteine amino acids. 12 of them make disulfide bridges. One disulfide bond combines 2 beta-sheets, 2 stabilize the hairpin structure of beta6, and beta7, 3 makes the node structure of 20 amino acids at the C-terminal. This 6 cysteine clusters are special and specific for ASA, the rest 3 are single. Cys38 and Cys294 are located at the intermediate surface of homodimer, Cys69 is converted to formylglycine after synthesis of the protein and Fgly69 is the active site amino acid of the protein. p.318Trp→Cys substitution creates a new Cys residue in the secondary structure of the protein. Deficient enzyme activity both in patient's samples and transfected CHO cells, show that p.318Trp→Cys substitution makes important structural changes. This effect can be a new disulfide bond with one of the free Cys residues or active site amino acid Cys prior to posttranslational modification to formyl glycine. The clinical phenotype of the patient is late infantile (*8*). Defect in the posttranslational modification of the Cys69 leads to deficiency of all sulfatases. Sulfate binding amino acid is FGly69, but Lys123, Ser150, His229 and Cys69 are the contributing amino acid residues for sulfate binding at the active center of the enzyme (*9,10*).

As a conclusion, p.307Glu→Lys and p.318Trp →Cys substitutions in the ASA protein affect enzyme activity and cause late-infantile type MLD. Biochemical characterization of the disease causing mutations is important for correlating genotype and phenotype, for understanding molecular basis of the disease, for prenatal diagnostic testing and for developing new therapeutic strategies like chaperone therapy.

Acknowledgements

Authors thank Prof. Dr. Volkmar Gieselmann for his gift of WT ASA plasmid. This study is supported by Hacettepe University Scientific Research Project Coordination Unit Grant number 727.

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(Received October 22, 2016; Revised November 2, 2016; Accepted November 3, 2016)

Case Report

DOI: 10.5582/irdr.2016.01067

Can acetazolamide be used to treat diseases involving increased bone mineral density?

Juan David González-Rodríguez[1,*], María Isabel Luis-Yanes[2], Esther Inglés-Torres[1], Pedro Arango-Sancho[2], José Eugenio Cabrera-Sevilla[1], María Rosario Duque-Fernández[2], Salvador Gil-Sánchez[1], Víctor Manuel García-Nieto[2]

[1] *Paediatric Nephrology Unit, Department of Pediatrics, Santa Lucía General University Hospital, Cartagena, Spain;*
[2] *Paediatric Nephrology Unit, Department of Pediatrics, Nuestra Señora de Candelaria University Hospital, Tenerife, Spain.*

Summary **Sclerosing bone dysplasias are a series of clinically and genetically heterogeneous diseases characterized by functional failure of the osteoclasts in bone resorption, leading to an excessive amount of bone mineral density (BMD) which could have serious clinical consequences. We treated three children affected with seriously high levels of BMD with acetazolamide, with the intention of inducing metabolic acidosis, thus increasing bone resorption and reducing BMD. All our patients tolerated and followed the treatment well and the clinical response was satisfactory in all cases.**

Keywords: Acetazolamide, osteopetrosis, carbonic anhydrase inhibitor, craniometaphyseal dysplasia, osteochondrodysplasia, sclerosing bone dysplasia

1. Introduction

Sclerosing bone dysplasias are a series of clinically and genetically heterogeneous diseases characterized by functional failure of the osteoclasts in bone resorption, giving rise to anomalies in bone formation and modelling, leading to an excessive amount of bone mineral density (BMD). This defect makes the bones brittle and can cause bone marrow failure, delayed eruption of permanent teeth, nerve entrapment syndrome and growth deficiencies (*1*).

Osteopetrosis is a rare disease, affecting 1:20,000 live births in its dominant form and 1:250,000 in its recessive form. It is characterized by the presence of pathological fractures. Although osteopetrosis comprises a heterogeneous group of conditions, encompassing a range of molecular lesions and clinical signs, all of its forms share the hallmark of osteoclast dysfunction (*2,3*).

Released online in J-STAGE as advance publication November 5, 2016.

**Address correspondence to:*
Dr. Juan David González Rodríguez, Paediatric Nephrology Unit, Department of Pediatrics, Santa Lucía General University Hospital, C/ Mezquita, s/n, Paraje Los Arcos, 30202, Santa Lucía – Cartagena (Murcia), Spain.
E-mail: juandavid.gonzalez@um.es

Craniometaphyseal dysplasia is a form of osteochondrodysplasia characterized by hyperostosis and sclerosis of the base of the skull, cranial vault and facial bones, as well as metaphyseal widening of the long bones. Cranial sclerosis may cause mandibular asymmetry and compression of the cranial nerves, which may eventually lead to hearing loss and facial paralysis (*4*). Most cases display a pattern of dominant autosomal pedigree (1:20,000) with mutations in the ANKH gene, which codifies a protein that regulates intracellular to extracellular movement of pyrophosphate (PP), although there is also a more severe recessive autosomal form (1:250,000), the gene for which has not yet been identified. Accordingly, its diagnosis is essentially clinical and based on radiology findings (*5*).

Although there has been some success with the use of calcitriol and interferon gamma in treating osteopetrosis, bone marrow transplant is the only cure for more serious forms of the disease (*6*).

Acetazolamide is a carbonic anhydrase (CA) inhibitor that suppresses the activity of carbonic anhydrase type IV, which is associated with the membranes. Acetazolamide interferes with the tubular resorption of bicarbonate (HCO_{3^-}), inducing bicarbonate diuresis and metabolic acidosis (*7*).

However, bone marrow transplant involves considerable risks, requiring heavy doses of

immunosuppressants and involving the possibility of graft *vs* host disease. As there is no treatment for craniometaphyseal dysplasia (CMD) or osteopetrosis, we treated three children affected with seriously high levels of BMD with acetazolamide, after approval of off-label use, with the intention of inducing metabolic acidosis, thus increasing bone resorption and reducing BMD.

2. Clinical cases

Case 1. Three-year-old male referred due to his unusual phenotype and the fortuitous detection of cranial sclerosis during radiography performed after a mild head injury. No personal or perinatal antecedents of interest were seen. Patient had normal psychomotor development, and was performing well at school. Parents young and healthy, not consanguineous. Brother aged six years, healthy. No family antecedents of interest.

Physical examination showed normal weight (89th percentile) and height (96th percentile). Prominent forehead with normal superciliary arches, orbital hypertelorism, large ears and a small nose, with flat root and bridge and progressive lateral widening, anteverted nostrils and short columella (Figure 1). Wide philtrum, large mouth with normal palate and teeth, mouth breathing were seen. Mammary hypertelorism. No evident abnormalities of spinal column. Extremities normal, single transverse palmar crease, articulations normal, articular mobility conserved, no cramping. The feet showed clinodactyly of the 5th toe and 1/3 soft-tissue syndactyly of the 2nd and 3rd toes. Neurological examination showed only unstable gait; the rest, including the cranial nerves, tested within normal range.

Complementary testing returned normal blood count, acid basic balance and elementary biochemistry and erythrocyte sedimentation rate (ESR) values. Karyotype 46XY. Blood and urine test results are given in Table 1.

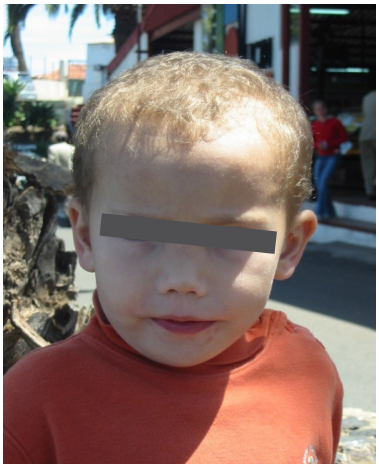

Figure 1. Patient's craniometaphyseal dysplasia on diagnosis, age 3 years: orbital hypertelorism, flat root and bridge of nose, anteverted nostrils and macrocephaly.

Radiology showed a marked sclerosis at the base of the skull and frontal region (Figure 2). The long bones of the lower limbs showed evidence of sclerosis of the diaphysis, the metaphysis being relatively radiolucent. A cranial computed tomography showed, in addition to sclerosis of the base of the skull, thickening of the bone in the nasal pyramid and of the lamina cribrosa (Figure 3). Neurophysiology testing showed discreet impairment of bilateral sight and mild bilateral cochlear sensorineural hypoacusis. Bone densitometry taken at lumbar level showed a significant increase in BMD (0.718 g/cm^2; z-BMD + 6.83).

Physical examination of the parents returned normal results. Molecular tests for dominant autosomal craniometaphyseal dysplasia returned negative, so the child was diagnosed with autosomal recessive craniometaphyseal dysplasia and began treatment with acetazolamide, maintained over seven years to the present day. In the third year of therapy, the auditory evoked potentials had normalized, although at the following check-up the patient once again presented with mild bilateral sensorineural hypoacusis as well as a mild demyelinating lesion of both optic nerves, not affecting his day-to-day activity. In the fourth year, ultrasonography detected calyceal microlithiasis (left side), and so the patient began treatment with oral potassium citrate; and the lesions were observed to disappear over subsequent check-ups.

Case 2. Female aged 11.5 years, from Ecuador, with a clinical diagnosis of osteopetrosis and compatible radiology findings. The patient has a history of multiple pathological fractures since she was five years old (wrist, coccyx, hip, forearm (twice) and fingers), as well as recurring bone pain requiring regular painkillers; she also tires during moderate physical exercise. Patient had normal psychomotor development, and school performance. Menarche at age 10. Parents young and healthy, consanguineous. Sister aged 7 years, healthy. No family antecedents of interest, except for 2nd degree nephrourolithiasis.

Physical examination: Weight in 42nd percentile, height in 4th percentile, head circumference in 68th percentile, blood pressure 110/60 mmHg (p50-90), peculiar phenotype with bulging forehead, large ears and narrow thorax. No further abnormalities were found, except limited abduction of the left hip and a level II/VI protomesosystolic murmur in the mesocardium, which normalized at later check-ups.

Complementary testing returned normal blood count, ESR, acid basic balance, elementary biochemistry, immunoglobulin, proteinogram, complement test and urine values. Test results given in Table 1.

Dual-energy X-Ray absorptiometry (DXA) revealed a BMD of 2.578 g/cm^2 at lumbar level (Z score + 6.25); no auditory or visual deficiencies. Normal abdominal ultrasound. Normal baseline and stress spirometry

Table 1. Clinical, analytical and radiology variables before and after treatment

Items	Case 1. Craniometaphyseal dysplasia				Case 2. Female, Dominant Osteopetrosis			Case 3. Male, Dominant Osteopetrosis		
	Baseline	Year 1	Year 3	Year 7	Baseline	Year 1	Year 6	Baseline	Year 1	Year 6
Z Score BMD	+6.83 (170%)	+3.9 (142%)	+1.21 (114%)	+0.7	+6.25 (300%)	+7.91 (277%)	T score + 20.5 (305%)	+7.55	+5.37 (295%)	+6.5 (221%)
pH/HCO_3-/SBE (mmol/L)	7.35/ 22/-3	7.29/18 /-7.7	7.32/22/ -9.3	7.28/24/ -3.2	7.38/ 23.5/-1	7.34/21.4/ -2.7	7.34/23.5/ -1.4	7.39/24/ -0.1	7.33/22.5/ -0.4	7.29/20.9/ -4.6
Cit/Cr urine (mmol/mol)	98.5	70.4	123.2	218.4	-	42.4	322.4	-	239.2	578.6
Calcium/IPh (mmol/L)	2.4/1.58	2.4/1.87	2.2/1.49	2.5/1.65	2.4/1.58	2.3/1.78	2.2/1.58	2.2/1.74	2.2/1.74	2.2/1.74
Ca/Cr urine (mol/mol)	116.7	183.9	95.5	84.9	0.18	14.1	31.8	0.18	28.3	53.0
Ca/Cit urine (mol/mol)	1.22	2.63	0.75	0.40	-	0.06	0.10	-	0.12	0.06
iPTH (pmol/L)	3.17	1.19	2.07	2.08	5.79	5.71	3.18	5.79	5.71	3.82
β Crosslaps (mmol/L)	-	-	-	-	-	0.21	0.16	-	0.26	0.50
DP/Cr urine (nmol/mmol)	86.29	52.03	27.32	37.2	-	-	-	-	-	-
ALP (µkat/L)	7.78	4.86	-	5.06	6.08	3.0	2.37	4.18	3.72	4.51
Osteocalcin (mmol/L)	10.26	3.59	1.84	7.86	-	5.71	4.83	-	11.03	14.59
Renal ultrasound	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
BAEP	Mild bilateral sn hypoacusis	Mild bilateral sn hypoacusis	Normal	Mild bilateral sn hypoacusis	-	-	-	-	-	-
Acetazolamide	No	9.9 mg/ kg/day 425 mg/ day/1.73 m^2	8 mg/ kg/day 375 mg/ day/1.73 m^2	7.2 mg/ kg/day 366 mg/ day/1.73 m^2	No	5 mg/ kg/day 300 mg/ day/1.73 m^2	12.3 mg/ kg/day 816 mg/ day/ 1.73 m^2	No	7 mg/ kg/day 375 mg/ day/1.73 m^2	8.6 mg/ kg/day 534 mg/ day/1.73 m^2
Height/GR	p93	p87/ -1.81 SD	p83/ +1.83 SD	P67/ -2.26 SD	p4 /-0.14 SD	p6/ 1.16 SD	P3/ 0 SD	p4/ 0.13 SD	p3/ -2.43 SD	P12/ +0.10 SD

ALP: Alkaline phosphatase; BAEP: Brainstem auditory evoked potentials; BMD: Bone mineral density in lumbar region; Ca/Cit: Calcium/Citrate ratio; Ca/Cr: Calcium/Creatinine ratio; Cit/Cr: Citrate/Creatinine ratio; DP/Cr: Deoxypyridinoline/Creatinine ratio; GR: Growth rate; IPh: Inorganic phosphorus; iPTH: Intact parathyroid hormone; p: Percentile; SBE: Standard base excess; SD: Standard deviation; sn: sensorineural.

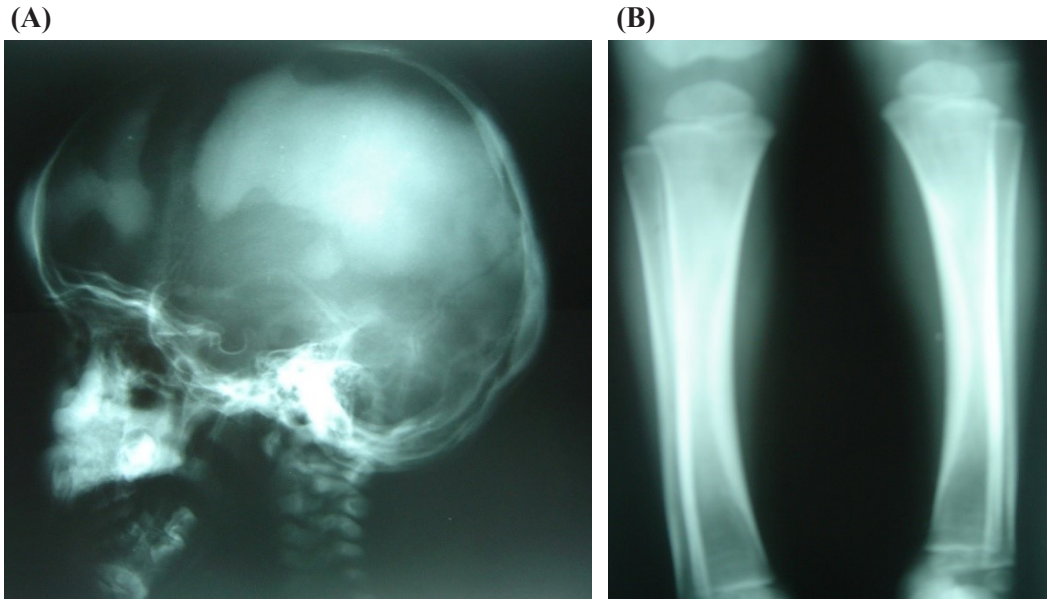

Figure 2. Patient's craniometaphyseal dysplasia. (A), Cranial radiography on diagnosis: Irregular cranial sclerosis. **(B)**, Radiography of lower limb on diagnosis: Metaphyseal widening with areas of hyperdensity and hypodense zones in diaphysis

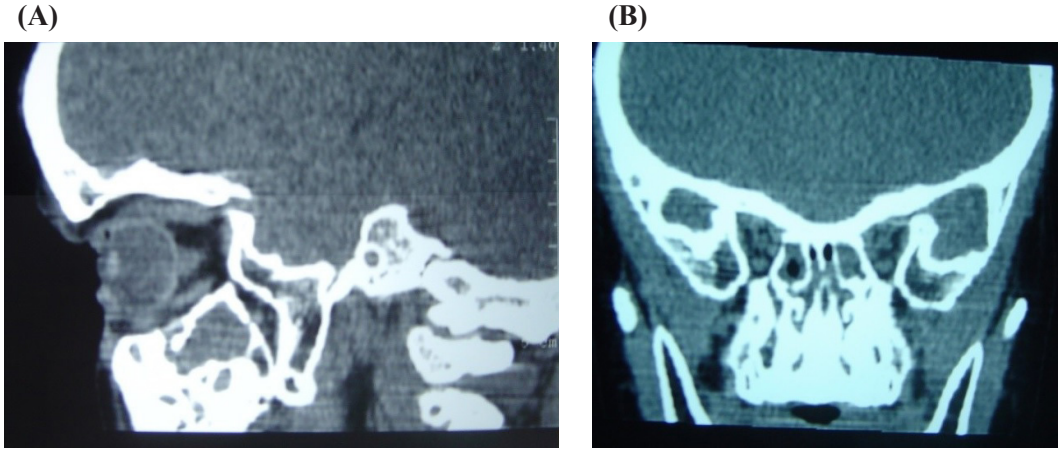

Figure 3. Patient's craniometaphyseal dysplasia. Cranial computed tomography on diagnosis: Notable abnormalities in bone density and thickness of cranial vault, differentiated abnormalities in bone layers. Bone thickening around nasal pyramid and lamina cribrosa. **(A)**, Sagittal plane; **(B)**, Coronal plane.

normal. No electrocardiographic (ECG) abnormalities, although echocardiography showed findings compatible with dilated cardiomyopathy, with ventricular function preserved. This was treated with Enalapril and in subsequent check-ups the values had returned to normal. Habitual toxicity tests were conducted, as well as extended blood testing (including the Trypanosoma cruzi serology) and ultrasound testing of the family, all returning normal values. A molecular study of gene ClCN7 was also conducted, revealing a heterozygous p.Leu213Phe (c.637C>T) mutation, confirming the diagnosis of dominant osteopetrosis.

After the good results obtained in treating the patient's CMD, we began treatment with acetazolamide. The patient's BMD levels stabilized and she reacted extremely well to therapy over six years of follow-up, to the present day, reducing her reliance on analgesics. She had suffered no new fractures, although in this past year she has had two fractures of her fingers, associated with increased BMD and requiring a higher acetazolamide dose. Patient followed and tolerated therapy well, presenting no adverse effects, except for the expected mild metabolic acidosis and hypocitraturia (Table 1).

Case 3. Male aged 9.5 years, brother of Patient 2, with radiology findings compatible with osteopetrosis and a history of suffering fractures from mild knocks since age six years (right wrist (twice), hip), frequent bone pain and tiring when performing vigorous physical activity. Normal psychomotor development, performing well at school.

Physical examination: Weight in 25th percentile, height in 4th percentile, head circumference in 40th percentile, blood pressure 90/50 mmHg (< p50), peculiar phenotype with bulging forehead, large ears and narrow thorax. The rest of the physical examination likewise evidenced limited abduction of the left hip and a level I-II/VI systolic murmur in the mesocardium, resolving over time.

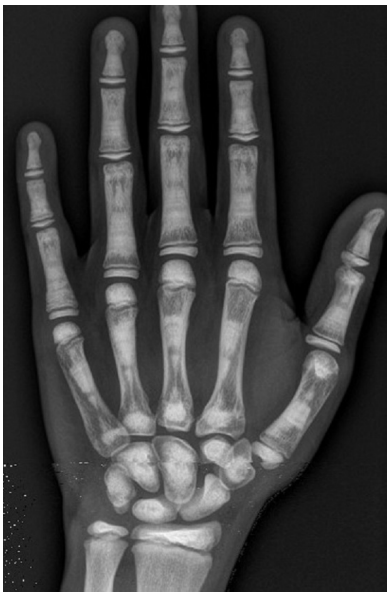

Figure 4. Osteopetrosis. Mottled sclerotic pattern in bones of left hand of male patient.

Complementary testing returned normal blood count, ESR, acid basic balance, elementary biochemistry, immunoglobulin, proteinogram, complement test and urine values. Test values given in Table 1.

DXA showed BMD of 1.969 g/cm^2 at lumbar level (Z score + 7.55); without auditory or visual deficiencies. Normal abdominal ultrasound, ECG and spirometry. Echocardiography showed a dilated cardiomyopathy, like his sister, with negative serology and toxicology results. Treatment commenced with enalapril, and values returned to normal over time.

Molecular analysis of the ClCN7 gene also revealed a heterozygous p.Leu213Phe (c.637C>T) mutation, which was also confirmed in the mother of the patients who, having remained asymptomatic to date, under DXA had a BMD at lumbar level in the normal range (T score 1.71).

Like his sister, the patient commenced treatment with acetazolamide. His BMD and bone pain decreased after the same follow-up period. His incidence of fractures has decreased, although in the past year he broke a bone in a toe, related to increased BMD, requiring a higher dose of the drug.

Patient followed and tolerated therapy well, presenting mild compensated metabolic acidosis and hypocitraturia, as well as reduced growth rate (Table 1). Because of this, his acetazolamide dose was reduced in the third year of therapy, after his BMD Z score had been reduced to + 3.73 (243%) (Figure 4).

3. Discussion

Bone is a metabolically-dynamic, constantly-changing tissue. Bone formation is a complex process, requiring the sequential intervention of a large number of local and systemic factors, and the simultaneous and balanced participation of two types of cells: osteoclasts, giant TRAP-rich multinucleate cells which derive from myeloid processes and break down organic bone matrix; and osteoblasts, which derive from pluripotent mesenchymal cells, synthesize bone matrix and determine the eventual activation of the osteoclasts *via* several mediators, RANKL/RANK activating interactions and osteoprotegerin-blocking inhibitors. Mature osteoclasts adhere to the bone surface using their ruffled border, breaking down the bone matrix by acidifying the bone surface and secreting proteolytic enzymes such as Cathepsin K. Dysfunctional acidification of the resorption lacunae of the osteoclasts, caused by type II intracellular CA deficiency, proton pump deficiency and/or abnormalities in the chloride channels, is associated with several forms of osteopetrosis (*2,8*). On the other hand, the dominant autosomal form of CMD is associated with mutations in ANHK, leading to increased mineralization due to decreased transport of intracellular PP into the extracellular matrix (*7*).

Moreover, studies have shown that the skeleton contains a massive reserve of alkaline mineral (hydroxyapatite) and that, during periods of metabolic acidosis, bone acts as a buffer, helping to stabilize extracellular pH, reducing mineralization and stimulating the osteoclasts. This reduces bone calcium deposits and increases its elimination in the urine, although this mechanism has not been reproduced in all studies on humans, which show a reduction in the number of osteoblasts and osteoclasts when the body suffers from chronic metabolic acidosis (*9,10*).

In our cases, we decided on treatment with acetazolamide in order to induce mild metabolic acidosis, principally by inhibiting membrane-bound CA in the brush border of the proximal tubule, thus favoring bone resorption *via* the well-known buffer effect of the bone (*11,12*). Previous publications have described attempts to treat these diseases with calcitriol, calcium restriction, steroids or parathyroid hormone, all with the intention of increasing bone resorption, but with no conclusive findings in the majority of cases; likewise interferon therapy prior to hematopoietic stem cell transplantation in severe forms of the disease has been used (*2*). There have also been attempts made with calcitonin, which inhibits osteoclast activity, reducing bone formation by negative feedback, as osteoblast differentiation is influenced by proteins secreted during bone resorption (*8*). RANKL and the activation of alternative osteoclast acidification mechanisms, including the Na^+/H^+ antiporter, have also been proposed as potential therapeutic targets (*2*). In our group, we had previously trailed treatment with ammonium chloride, also attempting to generate acidosis and thereby bone resorption, but it was not tolerated well by patients (results not published).

All our patients treated with acetazolamide tolerated and followed the treatment well, in spite of it being necessary to increase the dose-probably due to its action decreasing during chronic acidosis. The proper dosage was determined by clinical outcome, patient tolerance without side effects and the findings in DXA and laboratory testing desired (metabolic acidosis). The clinical response was satisfactory in all cases, with improvements in visual and auditory disorders in the case with CMD, and a notable reduction in fractures and pain in patients with osteopetrosis. Once treatment commenced, all patients showed mild metabolic acidosis and hypocitraturia, with an increased urine calcium/citrate ratio. The patient with CMD began treatment with potassium citrate to treat an episode of lithiasis, resolving spontaneously with no increase in BMD.

Hypocitraturia in the three patients would seem to suggest intracellular acidosis (*13*). This contrasts with previous basic investigation work and those performed on adult women, which show the existence of osteoclast membrane-bound CA, as in the proximal renal tubule, which would act like a metabolon with intracellular CA, reducing bone resorption and increasing intracellular pH and osteoclast apoptosis after treatment with a CA inhibitor (*14-16*). Nevertheless, not all cases show the same results when acetazolamide is administered specifically, and in some, decreased osteoblast activity is also observed (*14,16*).

In our patients, the effect on BMD determined by DXA, was a reduction in the Z score in the male patient with CMD and the male patient with osteopetrosis, as reflected in Table 1. In spite of inducing acidosis and a good clinical evolution, the same effect was not achieved in the female patient with osteopetrosis, although, as the BMD was not adjusted to her bone size, due to a lack of standardization in this regard, and as BMD measured by DXA is influenced by the size of the bone, the increased Z score attained in the first year of treatment could be falsely magnified by growth (*17*). Nevertheless, the

subsequent reduction in BMD was not as noteworthy as in the male patients, which could also be affected by not being adjusted to pubertal stage and/or body composition, and by the influence of estrogen on RANKL (*8*).

With regard to bone remodelling biochemical markers, Cases 1 and 2 showed a tendency towards reduction of both formation parameters (Alkaline phosphatase and osteocalcin) and resorption parameters (β Crosslap and urine Deoxypyridinoline/ Creatinine ratio), compatible with the findings of Domrongkitchaiporn *et al*. in acidosis, and those of Shinohara *et al*. after administering acetazolamide (*10,16*). On the other hand, the male patient with osteopetrosis (Case 3) showed increased absolute values for both parameters in the third year after treatment commenced. This could be influenced by pubertal development, or the wide variation and analytical interference of these markers (*18*); these markers showed a reduction in the last check-up over the previous.

Also worthy of note was the absence of symptoms and bone anomalies in the mother of the two patients with osteopetrosis, even though she is a carrier of the same heterozygous mutation. This situation has already been described in the literature with the variable penetrance of the disease and its modulation by modifying factors, seen only in 66% of patients with mutations in the ClCN7 gene (*19*).

Finally, and within the complex equilibrium of bone formation, clearly displaced in favor of appositional growth during childhood, puberty and adolescence, we believe that the predominant beneficial effect of acetazolamide in our patients may be due to a reduction in bone formation and resorption secondary to metabolic acidosis induced by inhibiting membrane-bound CA in the proximal renal tubule, because otherwise there should also be osteoblast dysfunction in sclerosing dysplasias caused by primary defects in osteoclast resorption (*1-3*).

In any case, in view of the lack of effective treatment for most sclerosing bone dysplasias, acetazolamide is presented as a new form of therapy that may improve patients' prognosis and quality of life, circumventing the need for surgery. However, analytical and ultrasound controls are necessary once treatment has commenced, due to the risk of metabolic alteration and kidney stones. Nevertheless, further studies will be required at both the clinical and experimental level, to extract more solid conclusions and determine the exact action of treatment on bone metabolism and even assess the association with other treatments.

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(Received August 30, 2016; Revised October 12, 2016; Accepted October 17, 2016)

Case Report

DOI: 10.5582/irdr.2016.01059

Infective abdominal aortitis due to *Campylobacter fetus* bacteremia: A case report and review of literature

Abhinav Agrawal[1,*], Rutuja R Sikachi[2]

[1] *Division of Pulmonary, Critical Care & Sleep Medicine, Department of Medicine, Northwell Health – Hofstra Northwell School of Medicine, New York, USA;*
[2] *Department of Anesthesiology, Lilavati Hospital & Research Center, Mumbai, India.*

Summary **Infectious aortitis (IA) is a rare but life-threatening condition, and most commonly affects the abdominal aorta or thoracic aorta. Various microorganisms have been associated with infectious thoracic aortitis, most commonly *Staphylococcus*, *Enterococcus*, *Streptococcus*, and *Salmonella species*. *Campylobacter fetus* (*C. fetus*) has been seen as a cause of infective aortitis only in a few case reports. We report a rare case of infective aortitis of the abdominal aorta caused due to *C. fetus* bacteremia. While *C. fetus* infections usually occur in patients with immunosuppression, such as malignancy, or those with diabetes mellitus, but our patient was not immunocompromised. Furthermore, the IA occurred in the absence of an aortic aneurysm, unlike its usual presentation. Thus, it is extremely important to establish an early diagnosis of IA and find out the causative organism for appropriate medical treatment, because this condition is potentially life threatening.**

Keywords: Aortitis, *campylobacter fetus*, aortic aneurysm, abdominal aorta

1. Introduction

Infectious aortitis (IA) is a rare but life-threatening condition, and most commonly affects the thoracic or the abdominal aorta (*1*). IA usually affects patients with atherosclerotic and/or aneurysmal disease and/or infective endocarditis (*1*). The epidemiology of aortitis, especially IA, as a distinct entity is poorly studied. The most common microorganisms causing IA are *Staphylococcus*, *Enterococcus*, *Streptococcus*, and *Salmonella species* (*2*). Cases of aortitis due to Mycobacterium tuberculosis have been observed in developing countries (*3*). *Campylobacter fetus* (*C. fetus*), a gram-negative bacilli has mostly been associated with endovascular and valve infections (*4*). We present a rare case of infectious aortitis associated with *C. fetus* bacteremia.

Released online in J-STAGE as advance publication October 24, 2016.

**Address correspondence to:*
Dr. Abhinav Agrawal, Division of Pulmonary, Critical Care & Sleep Medicine, Department of Medicine, Northwell Health – Hofstra Northwell School of Medicine, New York, USA.
E-mail: Abhinav72@gmail.com

2. Case Report

A 66-year-old male with a medical history of hypertension, hyperlipidemia, gastroesophageal reflux disease (GERD), hypothyroidism and gout presented to the emergency room with a one-week history of back pain accompanied by fever and chills. The patient was initially suspected to have prostatitis as an outpatient and had failed treatment with oral levofloxacin. He denied having upper respiratory symptoms, difficulty breathing, diarrhea, recent intake of uncooked meat or intravenous drug use. Hematological workup revealed mild leukocytosis with white count of 13,000 and neutrophilia of 82%. There was elevation of acute inflammatory markers including erythrocyte sedimentation rate (ESR) of 90 and C-reactive protein (CRP) of 170. His chest X-ray and urinalysis were normal. Computed tomography (CT) scan of the abdomen and pelvis revealed focal wall thickening and fluid attenuation with inflammatory changes within and around the distal infrarenal abdominal aorta to proximal aortic bifurcation involving 7 cm in length, consistent with focal aortitis (Figure 1 and Figure 2)

He was admitted to the hospital, blood cultures were drawn and intravenous (IV) meropenem and

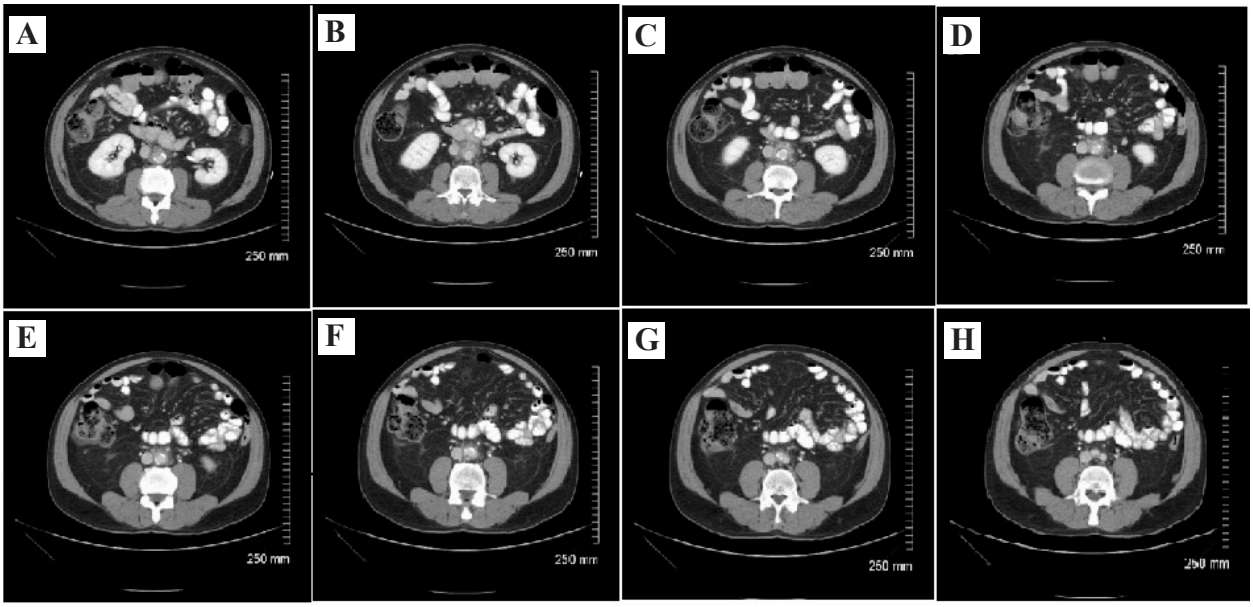

Figure 1. Contrast material enhanced sequential axial images of the abdomen *via* CT of the abdomen (A-H). There is nodular circumferential soft tissue thickening of the infra-renal aortic wall and the peri-aortic region with hazy peri-aortic inflammatory changes. The extent of the inflammatory changes involve the infra-renal aorta up to the more proximal aspect of the aortic bifurcation. Additionally, atherosclerotic changes of the infra-renal abdominal aorta are noted, consisting of calcified and non-calcified plaques.

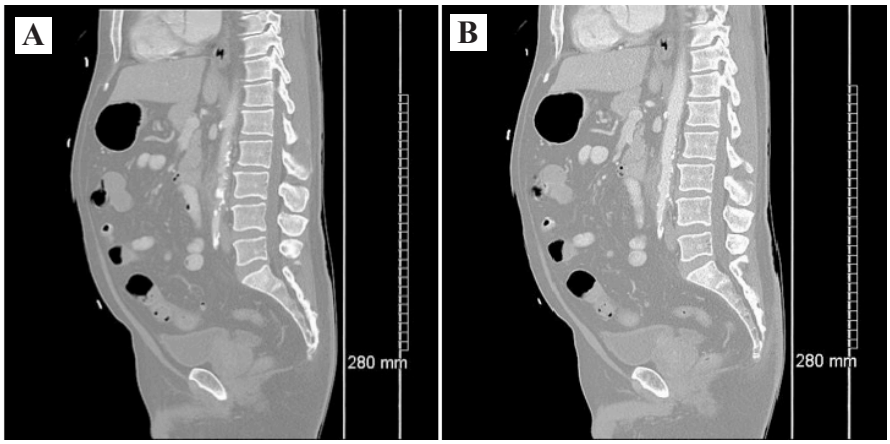

Figure 2. Contrast material enhanced sagittally reconstructed images of the abdomen and pelvis (A and B). There is evidence of diffuse circumferential soft tissue wall thickening of the abdominal aorta with surrounding peri-aortic inflammatory hazy changes. The superior extent of the inflammatory changes begin approximately at the proximal aspect of the infra-renal aorta and extend inferiorly just beyond the aortic bifurcation. Additionally, atherosclerotic changes of the infra-renal abdominal aorta are noted, consisting of calcified and non-calcified plaques.

ciprofloxacin were started empirically. Blood cultures returned positive for gram-negative rods eventually identified as *C. fetus*. While hospitalized, the patient received meropenem for 12 days, and ciprofloxacin for 9 days, which were eventually switched to gentamicin with final identification of *C. fetus*. Repeat blood cultures remained negative. A cardiac echocardiogram done did not reveal valvular vegetations or other abnormalities and repeat CT of the abdomen & pelvis showed stable focal aortitis with no signs of aortic aneurysmal dilatation or dissection. The patient's back pain significantly improved over time and a peripheral intravenous central catheter was placed anticipating long-term antibiotic therapy. His discharge medications included IV meropenem and IV gentamicin in addition to his home medications - aspirin, amlodipine, olmesartan, levothyroxine, atorvastatin, colchicine, pantoprazole and rosuvastatin. The patient's repeat blood cultures and CT of the abdomen remained stable.

3. Discussion

IA also known by bacterial, microbial, or cryptogenic aortitis, or mycotic or infected aneurysm, is a rare

entity in the antibiotic era. It can affect the thoracic as well as the abdominal aorta. IA is frequently related to infectious endocarditis, occurring in 86% cases in the pre-antibiotic era (*1*). Infectious aortitis has mostly been described in the thoracic aorta in the literature rather than the abdominal aorta. There are only a few cases in the literature describing IA in the absence of an aneurysm or rupture. If it is left untreated, an infected nonaneurysmal aorta (typically with atherosclerosis) will likely progress to a mycotic aneurysm (*2*).

The most common pathogens associated with infective thoracic aortitis are Gram-positive bacteria, such as *Staphylococcus*, *Enterococcus* and *Streptococcus species*, causing 60% of the infections. On the other hand, infective abdominal aortitis is most frequently caused by Gram-negative bacilli, most commonly the Salmonella species (*1*). Campylobacter has been reported to cause infective abdominal aortitis in a few case reports (*3-7*). Patients with underlying chronic debilitating illness, *e.g.*, diabetes, thalassemias, malignancy, cardiac conditions, or cirrhosis, are at increased risk for *C. fetus* bloodstream infections (*8*). The mechanisms of infections include *i*) bacteremic seeding on an existing atherosclerotic plaque; *ii*) septic emboli from infective endocarditis; *iii*) contiguous infective focus extending to the abdominal wall; and *iv*) direct bacterial inoculation at the time of trauma (penetrating injury). The virulence of *C. fetus* is thought to be mainly related to its production of a high-molecular-weight surface protein, which acts as a microcapsule with high affinity for the vascular endothelium (*9*). Blaser et al reported that *C. fetus* strains were serum resistant, which explains the high affinity of the organism to endothelial tissues (*10*).

Patients may present with fevers, chills, abdominal pain, and back pain. The patient may be asymptomatic, if he has no aneurysm formation. Leukocytosis and neutrophilia is seen in patients. ESR and CRP will also be elevated. Pentraxin-3 (PTX-3) is another promising potential biomarker for IA. Blood cultures can help isolate the microorganism, like in our case. Due to its association with infective endocarditis (IE), an echocardiogram is recommended to rule out IE. CT with contrast is the initial imaging method of choice (*11*). Magnetic resonance imaging (MRI) with gadolinium contrast enhancement or positron emission tomography (PET) scan can also be used. Invasive aortography is reserved for cases in which diagnosis of acute aortic syndrome cannot be excluded by noninvasive methods and carries the risk of rupture of the fragile aortic wall. The possible complications include aneurysm formation, if the patient does not have an already existent aneurysm. Complications of the aneurysm include rupture and bleeding, aortic thrombosis with distal embolisation, aortic dissection and aortic insufficiency. Acute coronary syndromes can occur in patients with involvement of coronaries (*11*).

As soon as the diagnosis is suspected, broad-spectrum intravenous antibiotics should be initiated. A longer course should be considered for immunosuppressed patients and if biochemical parameters of inflammation do not return to normal. Resection of the infected aorta can be performed with the intent of confirming the diagnosis, controlling sepsis, controlling hemorrhage (if rupture occurred), and reconstruction of the arterial vasculature. While surgery is recommended for most cases of infective aortitis, our patient was treated only with intravenous antibiotics because of the absence of aneurysm and clinical improvement with appropriate antibiotic therapy. It is important to identify the organism and initiate appropriate antibiotic therapy. *C. fetus* has been known to infect prosthetic joints and heart valves. In a case series of 21 patients with *C. fetus* bacteremia, all isolates were susceptible to amoxicillin, amoxicillin-clavulanate, imipenem, and gentamicin. Based on their study results, the authors suggest that imipenem should be preferred as first-line therapy for severe *C. fetus* infection, such as meningitis or endovascular infections (*12*). Gentamicin has low MICs for *C. fetus* isolates, and no gentamicin-resistant *C. fetus* isolates were seen in a report from Quebec (*13,14*). Based on microbiological data and clinical experience with treatment of *C. fetus* bacteremia, the use of a regimen that includes gentamicin has been advocated (*15*). Our patient responded well to gentamycin therapy. Endovascular techniques need further studies and longer follow-up periods in order to better define their role in IA management.

Factors apparently associated with a poor prognosis are advanced age, diagnosis delay, gram-negative bacilli infection, immunosuppression, thoracic location, and complication occurrence (such as rupture, embolization, or septic shock). Some authors recommend close medical follow-up that includes old and new biomarkers, serial blood cultures and imaging techniques (*16*) while others do not perform such approaches, and recommend only clinical surveillance (*17*).

Our patient presented with infective aortitis in the absence of formation of an aneurysm. He was found to have *C. fetus* bacteremia along with the aortitis in the absence of any underlying chronic debilitating illnesses. The patient showed an elevated leukocyte count and elevated ESR and CRP. The patient was diagnosed on the basis of the CT scan which showed focal aortitis in the absence of an aneurysm formation. Gentamycin has low MIC for treatment of *C. fetus* and our patient responded well to gentamycin therapy. Serial follow up CT scans showed resolution and no aneurysm formation was observed.

In conclusion, we report a rare case of infective aortitis of the abdominal aorta caused due to *C. fetus* bacteremia. It is extremely important to establish an early diagnosis of IA and find out the causative organism to institute antibiotic therapy and prevent fatal complications.

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(Received July 19, 2016; Revised September 30, 2016; Accepted October 5, 2016)

Case Report

DOI: 10.5582/irdr.2016.01063

Presentation of idiopathic retroperitoneal fibrosis at a young age: A rare case report

Priyanka Minocha[1,*], Ankur Setia[2]

[1] *Department of Pediatrics, SMS Medical College and Hospital, Jaipur, Rajasthan, India;*
[2] *Department of Gastroenterology, Fortis Escort Hospital, Jaipur, Rajasthan, India.*

Summary **Abdominal pain is a very common symptom in all age groups but retroperitoneal fibrosis is a rare differential diagnosis suspected in young patients presenting with nonspecific abdominal pain and symptoms of obstructive uropathy. Presented here is a case of a 16-year-old boy who presented with symptoms of persistent abdominal pain and a previous history of swelling in the left leg. A computed tomography (CT) scan suggested retroperitoneal fibrosis and an exploratory laparotomy and histopathological examination were performed for definitive diagnosis. This case report is intended to promote awareness of retroperitoneal fibrosis in young patients among health care providers.**

Keywords: Retroperitoneal fibrosis, children, abdominal pain, obstructive uropathy

1. Introduction

Retroperitoneal fibrosis is a rare disorder characterized by the presence of a chronic mass of fibroinflammatory tissue, which usually starts by first entrapping the aorta and then gradually entrapping surrounding structures including the ureter, vena cava, psoas muscle, and other retroperitoneal tissues. The peak age for the incidence of retroperitoneal fibrosis is usually in the 60 s or 70 s. The average age at onset is 60 years, with a male-to-female ratio of 1.9:1 (*1*). Childhood presentation is extremely rare. The most common manifestations include abdominal pain, abdominal distension, lumbago, weight loss, and pitting edema in the lower extremities (*1*).

2. Case Report

A 16-year-old male presented with persistent abdominal pain and vomiting that began 15 days earlier. The patient also had a history of swelling in the left leg from 4 years prior. Findings from a systemic examination were unremarkable. There was no localised tenderness in the abdomen, no organomegaly, and no lymphadenopathy.

Laboratory results in the form of the complete blood count revealed microcytic hypochromic anemia (hemoglobin: 9 gm/dL) for the patient's age and sex while total leucocyte counts and platelet counts were within normal limits. A routine urine examination indicated a mild degree of proteinuria with 4-6 pus cells and 3-5 red blood cells per high power field. Blood urea nitrogen (BUN) was 128 mg/dL and creatinine was 1.8 mg/dL. Other biochemical results were within normal limits. Inflammatory markers in the serum were elevated, including C-reactive protein (CRP) at 1.2 mg/dL (normal: < 0.6 mg/dL) and an erythrocyte sedimentation rate (ESR) of 95 mm/h (normal: 0-20 mm/h), but immunoglobulin G4 (IgG4) was normal at 105 mg/dL (normal: 1-291 mg/dL). The patient tested negative for antinuclear antibodies (< 1.0 IU).

Ultrasonography of the abdomen showed right renal enlargement with grade 3 hydronephrosis and a small left kidney. No evidence of stones was noted. Computed tomography (CT) (Figure 1) suggested an irregularly shaped soft-tissue mass 10.5 × 9.1 × 8.9 cm in size in the presacral region. The mass was located more to the left and it extended along the left lateral pelvic wall, protruding in the left sacral foramina at levels S1-2 and S2-3. Iliac vessels on both sides abutted

Released online in J-STAGE as advance publication October 24, 2016.

**Address correspondence to:*
Dr. Priyanka Minocha, Department of Pediatrics, SMS Medical College and Hospital, Jaipur, Rajasthan 302004, India.
E-mail: dr.priyankaminocha@gmail.com

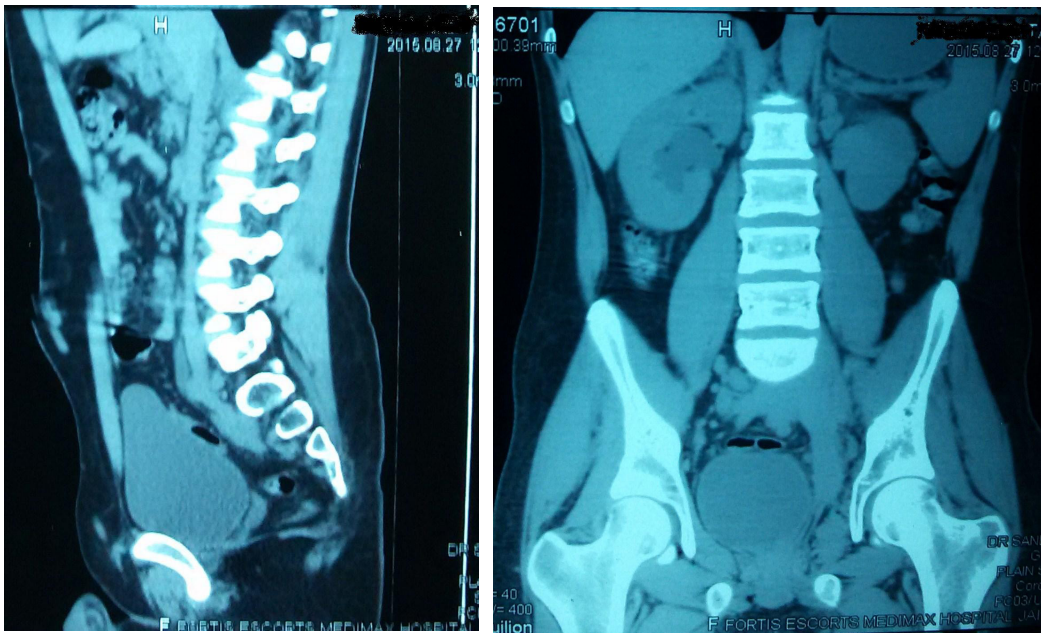

Figure 1. Computed tomography scan of the abdomen showing a retroperitoneal mass encasing the ureter and iliac vessels on both sides. The left kidney is 7.6 cm smaller in length and heterogeneously hyperdense.

or were entrapped within the mass (L > R). Both ureters also entrapped by the mass. The left kidney was small, 7.6 cm in length and heterogeneously hyperdense with loss of corticomedullary differentiation. Prominent vascular collaterals were found in the subcutaneous plane of the anterior abdominal wall and the bilateral inguinal region and pelvis.

Given the risk of bleeding from prominent vascular collaterals present in the subcutaneous plane of the anterior abdominal wall and the inaccessibility of a deep-seated mass, a biopsy was performed via an exploratory laparotomy rather than a laparoscopic procedure. There was a hard, irregular, and poorly circumscribed mass at the base of the bladder that encased both ureters and iliac vessels. The left ureter had a thick wall and narrow lumen. A biopsy sample was submitted for histopathology examination. A DJ stent was placed on both sides to relieve obstruction. Biopsy of the mass revealed several irregular greyish white pieces of soft tissue 0.6 × 0.4 cm in size. Microscopically, the mass consisted of mainly fibroadipose tissue with few congested blood vessels and chronic infiltration by non-specific inflammatory cells. Evidence of tuberculosis or malignancy was not evident.

Corticosteroids were started to treat retroperitoneal fibrosis, and the patient responded well. One month after the start of treatment, the patient became clinically asymptomatic and his creatinine level returned to normal (0.7 mg/dL). Another CT scan is planned for follow up.

3. Discussion

Cases of retroperitoneal fibrosis have previously been reported, but the current case is unusual. This case has been for 2 reasons: to promote awareness regarding the overlooked features of retroperitoneal fibrosis and to prompt consideration of retroperitoneal fibrosis in young patients as well.

Retroperitoneal fibrosis was first described by Albarran, a French urologist, as ureter obstruction by retroperitoneal tissue (*2*), but Ormond (*3*) defined it as an established clinical disease. The cause of retroperitoneal fibrosis is not readily understood. About three-fourths of cases are idiopathic while other cases may be related to retroperitoneal injury, drug therapy with methysergide, β-blockers and haloperidol, or genetic factors. The incidence of the idiopathic form has been reported to be 0.1 per 100,000 person-years, with a prevalence of 1.4 per 100,000 (*4*). This disease usually occurs in elderly patients, but it has also been reported in children. Males are affected more often than females (*1*).

Most frequently, the patient presents with nonspecific symptoms like dull abdominal pain and features of chronic renal failure, hypertension, pain, a backache, weight loss, and an abdominal mass (*5*). In the current case, the patient presented with features of a urinary tract obstruction and renal failure. However, the patient also had a past history of swelling in the left leg. At the time, ultrasonography of the perforators was performed, but the results were normal and the patient was advised to wear stockings. That swelling might be part of the disease process, but this disease entity is very rare, so health care providers seldom consider retroperitoneal fibrosis. When a patient presents with nonspecific symptoms or symptoms of obstructive uropathy and elevated ESR and CRP, retroperitoneal fibrosis should be considered. Biopsy is the gold standard for diagnosis but

a CT scan and magnetic resonance imaging (MRI) scan should also done to rule out secondary causes (*6*).

Recently, the concept of a correlation between IgG4 and idiopathic retroperitoneal fibrosis has been proposed. Zen *et al*. (*7*) studied 17 patients with retroperitoneal fibrosis and immunohistochemistry revealed numerous IgG4-positive plasma cell infiltrates in 10 cases (IgG4-related) but only a few positive cells in 7 cases (non-IgG4-related). Serologically, serum IgG and IgG4 levels were significantly higher in cases of IgG4-related retroperitoneal fibrosis. The current patient had normal serum IgG4 levels, and the patient's condition may have fallen into the category of non-IgG4-related retroperitoneal fibrosis.

Secondary retroperitoneal fibrosis is treated by treating the underlying cause while idiopathic cases of retroperitoneal fibrosis are treated by excising the mass with ureterolysis and administering corticosteroid therapy (*8*). Immunosuppression with azathioprine and mycophenolate mofetil and hormonal treatment with tamoxifen are also cited as treatment modalities for retroperitoneal fibrosis (*9-11*).

Surgical treatment includes temporary decompression by percutaneous nephrostomy or ureteral stenting for obstructive uropathy, and definitive treatment includes open or laparoscopic ureterolysis, anterior transposition or omental wrapping of the involved ureter, and more recently, ureterolysis and omental wrapping with Gore-Tex, excision of the ureter and reanastomosis, fashioning of a posterior preperitoneal flap, and renal autotransplantation (*12,13*).

The prognosis for retroperitoneal fibrosis usually depends on the underlying cause and the degree of renal impairment at the time of presentation. Usually, the idiopathic form has a good prognosis (*14*) while the malignant form has a poor prognosis. Even if the idiopathic form has a good prognosis, lifelong follow up is required to monitor recurrence and complications.

In conclusion, this case report is intended to promote awareness of retroperitoneal fibrosis in young patients among health care providers. Definitive diagnosis should be based on a CT scan or MRI findings along with a biopsy.

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(Received August 5, 2016; Revised September 13, 2016; Accepted September 27, 2016)

Case Report

DOI: 10.5582/irdr.2016.01058

Dent's disease complicated by nephrotic syndrome: A case report

Guohua He[1,2,*], Hongwen Zhang[1,*], Shanshan Cao[1,3], Huijie Xiao[1], Yong Yao[1,]**

[1] *Pediatrics, Peking University First Hospital, Beijing, China;*
[2] *Pediatrics, Foshan Maternal and Children's Hospital, Foshan, China;*
[3] *Health Center, Beijing Entry-Exit Inspection and Quarantine Bureau, Beijing, China.*

Summary **Dent's disease is an X-linked recessive proximal tubular disorder that mostly affects male patients in childhood or early adult life. The condition is caused by mutations in the *CLCN5* (Dent disease 1) or *OCRL* (Dent disease 2) genes located on chromosome Xp11.22 and Xq25, respectively. In most male patients, proteinuria is subnephrotic but may reach nephrotic levels. Here, we report the first case of Dent's disease complicated by nephrotic syndrome. Dent's disease should be considered in the differential diagnosis of nephrotic syndrome, and especially in male patients with early onset of nephrotic syndrome. A urinary α1-microglobulin/albumin ratio > 1 may provide the first clue to a tubulopathy.**

Keywords: Dent's disease, proteinuria, nephrotic syndrome, *OCRL* gene

1. Introduction

Dent's disease is an X-linked recessive proximal tubulopathy that presents with hypercalciuria, low-molecular-weight proteinuria (LMWP), nephrolithiasis, nephrocalcinosis, and progressive renal failure (*1,2*). Dent's disease is divided into two types depending on the phenotype: Dent's disease 1 (OMIM #300009), which is caused by mutations in the *CLCN5* gene located on chromosome Xp 11.22, and Dent's disease 2 (OMIM#300555), which is caused by mutations in the *OCRL* gene located on chromosome Xq25 (*3*). Dent's disease mainly affects males, whereas female carriers may display a milder phenotype (*4,5*). Patients are usually diagnosed in childhood or in their early adult years. LMWP is the most consistent feature, occurring in 99% of affected male patients. Proteinuria is usually subnephrotic but may reach nephrotic levels (*2,6,7*). To the extent known, there are no previous reports on Dent's disease complicated by nephrotic syndrome.

Reported here is a case involving a 4-year-old boy. Consistent with the diagnosis of nephrotic syndrome, renal pathology indicated minimal change disease (MCD), and mutation analysis indicated a c.2435T>C (p.L812P) mutation in the *OCRL* gene. The boy was found to have Dent's disease complicated by nephrotic syndrome.

2. Case Report

A 4-year-old Chinese boy who had intermittent proteinuria (urine protein of 1.91 g/24 h, 172 mg/Kg) and edema for 13 months presented with hypoalbuminemia of 15.3 g/L and hypercholesterolemia of 7.80 mmol/L. The boy was initially treated for nephrotic syndrome at a local hospital; the boy received a full dose of corticosteroids for 8 weeks, 3 doses of intravenous methylprednisolone (MP), and then 8 doses (one dose per month) of cyclophosphamide (CTX). The boy then received oral prednisone and mycophenolate mofetil (MMF) for 4 months. The boy's proteinuria appeared to subside but did not completely resolve (urine protein of 0.45-1.11 g/24 h). The boy was referred to this Hospital on August 1, 2015. An examination at admission indicated a height of 90 cm and weight of 11 Kg, both of which were below the 1st percentile. A 24-h urine collection at this Hospital revealed urine protein of 0.48 g/24 h (44 mg/Kg). Urinalysis revealed urine α1-microglobulin of 307 mg/L and urine albumin of 103 mg/L. The boy's urine α1-microglobulin/albumin ratio was > 1. Urine protein electrophoresis was performed, and the pattern revealed a low-molecular-weight protein fraction

Released online in J-STAGE as advance publication September 12, 2016.

*These authors contributed equally to this works.
**Address correspondence to:
Dr. Yong Yao, Pediatrics, Peking University First Hospital, No.1 Xi An Men Da Jie, Beijing 100034, China.
E-mail: yaoyong3238@126.com

Table 1. Clinical data from different times the current patient was hospitalized

Hospitalization	Status of NS	Alb (g/L)	Cholesterol (mmol/L)	UPE (mg/kg/24h)	α1 MG (mg/L)	MicroAlb (mg/L)	α1 MG/ albumin	Low-molecular-weight protein fraction (%)	Treatment
1st	remission	40.0	5.06	0.48	307	103	2.98	64.4	Pre + MMF
2nd	relapse	17.3	16.13	4.33	208	3,106	0.07	10.1	Pre
3rd	remission	30.5	8.79	0.90	208	138	1.51	61.7	Pre
4th	relapse	27.5	10.96	2.01	462	1,452	0.32	29.8	Pre
5th	remission	38.1	6.13	0.68	366	234	1.56	63.1	Pre + CsA

Alb: albumin; CsA: cyclosporine; MG: microglobulin; MMF: mycophenolate mofetil; Pre: prednisone; UPE: urinary protein excretion; α1MG: α1-microglobulinuria.

of 64.4%. Hypercalciuria (0.18 mmol/Kg/24h, ratio of urine calcium to creatine = 0.32) was also noted. Further investigation revealed normal creatine (49.2 umol/L) and an estimated glomerular filtration rate (eGFR) of 100 mL/min/1.73 m^2. A chemistry panel revealed a serum albumin level of 44 mg/L and a cholesterol level of 5.06 mmol/L. The boy's autoimmune profile, including C3 and C4 complements, was normal. Serum phosphate was 1.66 mmol/L (1.45-2.1 mmol/L), alkaline phosphatase was 147 U/L (< 750 U/L), and serum calcium was 2.42 mmol/L (2.11-2.52 mmol/L).

Renal ultrasonography revealed no evidence of abnormalities. Renal biopsy indicated normal glomeruli with minimal proliferation of mesenteric cells and matrix, vacuolar degeneration of the glomerular basement membrane, and vacuolar and granular degeneration of tubular epithelium. Immunofluorescence was negative. Electron microscopy (EM) revealed segmental fusion of epithelial foot processes but no obvious abnormalities in tubules. The results of EM coincided with glomeruli minimal change. The boy was tentatively diagnosed with minimal-change disease (MCD). A genetic test indicated a heterozygote mutation of the *OCRL* gene c.2435T>C (p.L812P), confirming the diagnosis of Dent's disease 2. The boy's mother was subsequently identified as a carrier for the same *OCRL* gene mutation. The child's Gesell adaptive score was normal except for a slight delay in language development. An ophthalmological examination revealed congenital cataracts (zonular).

Hydrochlorothiazide (1 mg/Kg daily) was given orally as primary treatment as soon as the diagnosis of Dent's disease 2 was confirmed. Potassium citrate was also given orally, and immunosuppressive agents were gradually tapered off. Over a follow-up of 9 months, the patient had no recurrence of edema, urine protein of 0.42-0.51 g/24h, and urine calcium of 0.89-1.35 mmol/24 h (0.08-0.12 mmol/Kg/24h); all of the parameters had improved markedly.

Nine months after diagnosis (08/10, 2015), the boy had recurrence of edema, oliguria, and an increase in urine protein from 1+ to 3+ after an upper respiratory infection. A 24-h urine collection revealed urine protein of 4.33 g/24h. A chemistry panel revealed serum albumin of 17.3 g/L and cholesterol of 16.13 mmol/L. The boy's urine α1-microglobulin/albumin ratio was < 1 (urine α1-microglobulin of 208 mg/L and urine albumin of 3,106 mg/L). The pattern of urine protein electrophoresis revealed a low-molecular-weight protein fraction of 9.1%, an albumin fraction of 69.0%, and a high-molecular-weight protein fraction of 21.9%. The boy's urine calcium/creatine ratio and 24-h urine calcium were normal. At this stage, relapse of nephrotic syndrome was considered, so the boy was started on 60 mg/m^2 of prednisone. Two weeks later, urine protein decreased to 1.83 g/24h and serum albumin increased to 30 g/L. After four weeks of a full dose of prednisone, the drug was gradually tapered off. A chemistry panel revealed serum albumin of 30.5-44.2 g/L. A 24-h urine collection revealed urine protein of 0.9-1.1 g/24h, urine calcium of 1.53-1.85 mmol/24h. Urinalysis revealed urine α1-microglobulin of 208-321 mg/L and urine albumin of 183-219 mg/L.

Three months after the first relapse (21/01, 2016), the boy was admitted for revaluation while taking 25 mg of prednisone (1.78 mg/Kg) every other day. The boy had no obvious edema. A 24-h collection of urine revealed urine protein of 2.01 g/24h. A chemistry panel revealed serum albumin of 27.5 g/L and cholesterol of 10.96 mmol/L. The boy's urine α1-microglobulin/ albumin ratio was < 1 (urine α1-microglobulin of 462 mg/L and urine albumin of 1452 mg/L). The pattern of urine protein electrophoresis revealed a low-molecular-weight protein fraction of 29.8%, an albumin fraction of 64.6%, and a high-molecular-weight protein fraction of 5.6%. A 2nd relapse of nephrotic syndrome was considered although there was no obvious edema. The dose of oral prednisone was increased to 1 mg/Kg daily and cyclosporine was added at a total daily dose of 3.3 mg/Kg (a half dose 2 times per day). Two weeks after the adjustment, urine protein decreased to 0.98 g/24h. The dose of prednisone was gradually reduced, and the boy's course was satisfactory at a 6-month follow-up; his urine protein maintained stable and renal function was within the normal range (Table 1).

3. Discussion

Dent's disease is an X-linked recessive proximal tubular disorder that mostly affects male patients in childhood or early adult life. In most male patients, proteinuria is subnephrotic but may reach nephrotic levels (*8,9*). The current report describes the first case of Dent's disease

complicated by nephrotic syndrome.

A 4-year-old boy presenting with intermittent edema, persistent proteinuria at a nephrotic level, transient hypoalbuminemia, and hyperlipidemia responded somewhat to glucocorticoids (GC) and other immunosuppressive agents. However, his urine protein did not return to normal. Further investigation revealed hypercalciuria, hypercholesterolemia, retardation of growth, and congenital cataracts. Renal biopsy led to a tentative diagnosis of MCD, and genetic testing revealed a c.2435T>C (p.L812P) mutation in the *OCRL* gene. Dent's disease was diagnosed based on the presence of LMWP, hypercalciuria, retardation of growth, and congenital cataracts, and this diagnosis was confirmed based on a mutation in the *OCRL* gene (*3*). Nephrotic syndrome was diagnosed based on the presence of edema, severe proteinuria (172 mg/Kg), hypoalbuminemia (15.3 g/L), and hypercholesterolemia of 7.8 mmol/L, and this diagnosis was confirmed based on pathologic evidence of MCD under light microscopy and fusion of epithelial cell foot processes on EM. Thus, the current patient had Dent's disease complicated by nephrotic syndrome.

Dent's disease and nephrotic syndrome are two distinct diseases. A look at the pathogenesis of proteinuria in these two diseases indicates that proteinuria originates due to two different processes. In idiopathic nephrotic syndrome (INS), proteinuria is mainly albuminemia, so hypoalbuminemia is commonly found in INS with substantial albumin loss *via* urine. Non-specific histological abnormalities of the kidney, including minimal changes, focal and segmental glomerular sclerosis (FSGS), and diffuse mesangial proliferation, are present in INS. Glomeruli had a fusion of epithelial cell foot processes on EM and no significant deposits of immunoglobulins or complements according to immunofluorescence. In Dent's disease, however, proteinuria is mainly LMWP. LMWP is a result of tubular dysfunction that prevents effective reabsorption of low-molecular-weight proteins, including α1 and β2 microglobulins, retinol-binding protein (RBP), Clara cell protein, and vitamin D binding protein, that are filtered through the glomerular basement membrane (*10-12*). However, albuminuria will be produced when there are defects in the glomerular basement membrane. In some cases, Dent's disease manifests as proteinuria in the nephrotic range, which is a combination of albuminuria and LMWP. This indicates that both of the aforementioned processes can occur in the same patient. That said, none of the reported patients had hypoalbuminemia, indicating that LMWP is the primary cause of proteinuria while albuminuria is only slight cause of proteinuria in Dent's disease. In other words, Dent's disease and nephrotic syndrome were two independent diseases in the current case.

The current treatment for Dent's disease is mainly supportive, including hydrochlorothiazide (HCTZ) and potassium citrate in order to prevent nephrolithiasis (*13-15*). In contrast, the treatment for nephrotic syndrome is mainly immunosuppressive agents, with glucocorticoids (GC) being the agent of choice. In cases of Dent's disease complicated by nephrotic syndrome, both of those treatments should be used. The dose of GC should be reduced as the disease subsides, while HCTZ and potassium citrate should be maintained. The question then is how to evaluate the efficacy of treatment. How can the dose of GC be reduced while urine protein remains positive?

α1-Microglobulin has long been considered an effective biomarker of tubular dysfunction (*16,17*). A study found a correlation between α1-microglobulin and albuminuria in diabetic children (*17*), which could indicate tubular dysfunction is present in diabetes. In Dent's disease, urinary α1-microglobulin increases significantly due to the pathological changes occurring mainly in tubules. In the current case, the ratio of urine α1-microglobulin to albumin proved to be a useful indicator of LMWP. The patient's 24-h urine protein level was monitored and the patient's urine α1-microglobulin/albumin ratio was calculated. When nephrotic syndrome was adequately controlled, the patient's 24-h urine protein level tended to be lower, but LMWP caused by Dent's disease resulted in urine protein remaining positive. The patient's urine α1-microglobulin/albumin ratio was monitored when nephrotic syndrome relapsed. The patient had severe albuminuria and an α1-microglobulin/albumin ratio far below 1. Urine protein was monitored when the recurrence of nephrotic syndrome was under control. The patient's urine protein decreased back to its usual level and the patient's urine α1-microglobulin/albumin ratio increased from far below 1 to more than 1. At this point, nephrotic syndrome was apparently under control even though urine protein was still positive (Table 1). The protein in urine was presumably due mainly to the LMWP caused by Dent's disease. When nephrotic syndrome was consistently controlled, the α1-microglobulin/albumin ratio remained more than 1.

In conclusion, reported here is the first case of Dent's disease complicated by nephrotic syndrome, which is significant since Dent's disease and nephrotic syndrome are two independent diseases. A urine α1-microglobulin/albumin ratio > 1 was successfully used as a parameter to differentiate LMWP caused by Dent's disease from albuminuria caused mainly by relapsing nephrotic syndrome, suggesting that this parameter could be used as a diagnostic marker of tubular proteinuria.

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(Received July 18, 2016; Revised August 29, 2016; Accepted August 31, 2016)

Case Report

DOI: 10.5582/irdr.2016.01055

Systemic Kikuchi-Fujimoto disease bordering lupus lymphadenitis: A fresh look?

Aram Behdadnia[1], Seyyed Farshad Allameh[2], Mehrnaz Asadi Gharabaghi[3], Seyed Reza Najafizadeh[1], Ahmad Tahamoli Roudsari[1], Alireza Ghajar[1,4], Morsaleh Ganji[1,4], Mohsen Afarideh[1,4,*]

[1] *Lupus and Rheumatology department, Vali-Asr Hospital, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran;*
[2] *Department of General Internal Medicine, Imam Khomeini Hospital, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran;*
[3] *Department of Pulmonary Medicine, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran;*
[4] *Endocrinology and Metabolism Research Center (EMRC), Vali-Asr Hospital, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.*

Summary **A 31-year old woman with persistent fever for 6 weeks and unresponsive to antibiotic therapy came for rheumatologic investigation. After computed tomography (CT) studies of her neck, thorax and abdomen revealed bilateral cervical, axillary and retroperitoneal lymph node enlargements, histopathologic evaluation of the resected nodes showed features of histiocytic necrotizing lymphadenopathy suggestive of Kikuchi-Fujimoto's lymphadenopathy. Kikuchi-Fujimoto Disease (KFD) involving the retroperitoneal nodes is extremely unusual and even more challenging to diagnose when there are no early signs of extranodal involvement or abdominopelvic pain. We present a case of systemic KFD involving the cervical, axillary and retroperitoneal lymph nodes and emphasize the clinical interest to properly differentiate between the benign condition of KFD that requires no more than minimal to low dosage steroid therapy and the potentially life-threatening lupus lymphadenitis that mandates intensive immunosuppressive treatment.**

Keywords: Kikuchi-Fujimoto disease, necrotizing lymphadenitis, systemic lupus erythematosus, lupus lymphadenitis

1. Introduction

Kikuchi-Fujimoto's necrotizing lymphadenitis is a predominantly benign disease characterized by regional lymphadenopathy, low-grade fever and night sweats often involving the cervical lymph nodes in young people with less frequent involvement of lymph nodes elsewhere (*1*). From 1972 with the first reports of Kikuchi-Fujimoto disease (KFD) in Japan (*2*), the knowledge of KFD has been confined to case reports and series. This lack of evidence surrounding the pathophysiology of KFD has caused two major questions: *i*) does KFD belong to an independent category of autoimmune diseases or *ii*) does KFD develop as a bystander autoimmune-like phenomenon in many autoimmune diseases (*e.g.* systemic lupus erythematosus, SLE)? Credentials of the earlier suggestion have been put into question by two major findings: *i*) the association of SLE with KFD is well documented in the literature and the importance of lymph node biopsy is also well established; *ii*) in addition to SLE, KFD occurs in many other autoimmune diseases (*3,4*). Here we delineate a 31-year old woman with a 5-year history of SLE. In this case, two points may lead us into the diagnosis of KFD: first, the distinct clinicopathologic features of KFD as reported from the biopsy of resected nodes; and second, a demographic profile of the patient that fits well with the epidemiological background of KFD.

Released online in J-STAGE as advance publication October 24, 2016.

**Address correspondence to:*
Dr. Mohsen Afarideh, Lupus and Rheumatology department, Vali-Asr Hospital, Tehran University of Medical Sciences, P.O. Box 13145-784, Tehran, Iran.
E-mail: mhafarideh@gmail.com

2. Case Report

A 31 year old G3P1 Ab2 (7 months, 4 months) woman

was admitted into our unit (Lupus and Rheumatology department, Vali-Asr Hospital) due to persisting complaints of 6-week unremitting low-grade fever and chills. The fever was persistent in nature with no defining pattern and aggravating factors, however it was relieved to some extent with acetaminophen intake and was associated with, nausea, vomiting, malaise, arthralgia, nocturnal diaphoresis and a weight loss of 15 lb. during the past 40 days. From the age of 19 with a diagnosis of rheumatoid arthritis she has been on chloroquine 5 mg/kg/day and 5 years ago, she was diagnosed with SLE based on the findings of the American College of Rheumatology (ACR) criteria: presence of photosensitivity, malar rash, large symmetric joint arthritis involvement and positive antinuclear antibody (ANA). She denied chest pain, shortness of breath, dizziness, diarrhea, urinary symptoms, vaginal discharge, rash, headache, myalgia or rigor. She denied sick contacts, recent travel, exposure to pets/animals, bug bites, new medications, and herbal supplements. Her drug history included prednisone 5 mg/day, hydroxychloroquine 200-400 mg/day and folinic acid. Prior to admission, the patient was evaluated at a community urgent care center for possible infectious etiologies and was started on IV broad-spectrum antibiotic therapy; however, her condition remained unresponsive to this treatment and she was subsequently presented to our department. Her past medical was remarkable for minor β-thalassemia and cataract surgery. On physical examination, the patient was pale, she was febrile (101.48 F), tachycardic at 100, and had a blood pressure of 100/60. Physical exam showed diffuse, rubbery and slightly tender enlargement of bilateral posterior cervical lymph nodes and bilateral axillary lymph nodes with the largest node measuring to 1.5 cm. Enlarged lymph nodes were noted to be firm, discrete and mobile. There was no joint tenderness, warmness, erythema, swelling, limited range of motion or stiffness. Examination of the gastrointestinal, respiratory, cardiovascular and nervous systems was normal with no hepatosplenomegaly.

2.1. *Investigations*

Lab Investigations revealed hemoglobin 9.3 g/dL, WBC count 11,960 cells/mm (neutrophils-51% , lymphocytes-36% with no atypical lymphocytes) with platelet count of 276,000 cells/cu.mm, erythrocyte sedimentation rate (ESR) –30 mm/hr and C-reactive protein (CRP) –6.7 mg/dL; alanine aminotransferase (ALAT) 15 IU/L (normal up to 31); aspartate aminotransferase (ASAT) 24 IU/L (normal up to 31); alkaline phosphatase (ALP) 94 IU/L (64-306); serum lactate dehydrogenase (LDH) 493 U/L (normal 225-500); serum Iron 57 µg/dL (37-158), ferritin 110 ng/mL (10-126 ng/mL), total iron-binding capacity (TIBC) 289 µg/dL (210-440); serum 25-hydroxyvitamin D level 30.56 (desirable > 30); ANA positive with a titer of 1:80; complement C3 and C4 were decreased [C3 32.2 (reference: 90-180), C4 3.1 (reference: 10-40)] ; low CH50 at 30.5; the anti-double stranded DNA (anti-dsDNA) positive at 141.2 IU/mL. Additional tests including for HIV (ELlSA antibody), tuberculosis (PPD and Chest X-ray, a positive PPD skin test was defined as ≥ 10 mm intradermal induration), brucellosis (Wright, Coombs W.), typhoid (Widal TO and TH serology) and repeated blood cultures were negative. Urinalysis was normal except for a sterile pyuria (U/A: WBC, 16-18; RBC, 1-2; few epithelial cells; specific gravity, 1,005; negative nitrate; negative proteinuria) with no evidence of infection (Zero colony count); renal parameters were within normal ranges as were her thyroid function tests.

Double-contrast Spiral computed tomography (CT) of the abdominopelvic region revealed enlarged retroperitoneal lymph nodes in lateral aspect of the aorta at the level of the renal hilum, with no evidence of hepato-splenomegaly, ascites and bowel thickening. IV contrast CT study of the neck and thorax was noticeable for cervical lymphadenopathy located bilaterally in levels 1, 2 and 3 and left-sided in levels 4 and 5 and bilateral axillary enlarged nodes, with no mediastinal mass lesion, nodule, effusion, thickening or lymphadenopathy being detected. Maximum short axis diameters for enlarged lymph nodes were 8 mm and 15 mm for cervical and axillary lymph nodes, respectively.

2.2. *Outcome, treatment and follow-up*

With the initial diagnosis of lupus flare-up, the patient had dramatic clinical improvement to an increased daily dose of prednisolone to 10 mg and the constellation of her symptoms concerning cervical and axillary lymphadenopathies promptly resolved. Following the core needle biopsy of the cervical enlarged lymph nodes, the patient was discharged from the hospital. In the ensuing pathology report, histology of the resected nodes indicated the nodal architecture was preserved, as highlighted by CD20 stain of follicular germinal centers which were bcl2 negative. The parenchyma contained areas of karyorrhectic necrosis, with no neutrophilic reaction surrounded by plasmacytoid monocytes and lymphoid cells. The biopsy report confirmed these findings were consistent with the diagnosis of histiocytic necrotizing lymphadenitis (Kikuchi Fujimoto disease). Three months later, the patient emerged in our unit complaining of daily spells of abdominal pain and nausea/vomiting. In that time, she was diagnosed with lupus necrotizing pancreatitis and started on pulsed methylprednisolone and endoxan. Induction treatment by pulsed methyl prednisolone was received in daily doses of 1 g IV for 3 days, followed by monthly injections of 1 g IV endoxan for 7 months. Oral prednisone was initiated on the fourth day of treatment, at 1 mg/kg per day, for no more than 3

months and was tapered off in 4 to 6 months thereafter. She was in total remission with cellcept which was started on the seventh month after the manifestation of necrotizing pancreatitis, at a daily dosage of 2 mg/kg (1 mg/kg BID), and continued for 1.5 years.

3. Discussion

KFD is characterized as a rare, benign and self-remitting condition that is likely to be caused by a strong immunological response to a viral infection (*5*). However, the evidence linking KFD to a specific viral infection such as Epstein-Barr virus (EBV) and Herpes virus 6 are scarce (*6,7*) and numerous other inciting infectious and autoimmune triggers have been described, including the role of SLE in the pathogenesis of KFD. Affected women of Asian background in their early twenties to their mid-thirties typify the classic picture of KFD that is most commonly characterized by acute/ subacute febrile illness associated with lymph node enlargement of mainly posterior cervical lymph nodes (*2*). Our patient manifested bilateral cervical and axillary lymphadenopathy as well as engorged retroperitoneal lymph nodes. Generalized lymphadenopathy can present occasionally, but involvement of mediastinal or retroperitoneal nodes by KFD is extremely uncommon (*8*). Other accompanying symptoms in KFD include malaise, anorexia, myalgias and arthralgias (*2*).

Though the diagnosis of KFD is made prior to, simultaneous to or after the diagnosis of SLE (*9*), review of the literature in published cases from 1991 onwards indicates that SLE predominantly predates or concurs with KFD. However, it is not clear whether those cases were genuine KFD or were indeed lupus lymphadenitis mimicking KFD because lupus lymphadenitis is clinically indistinguishable from KFD in the absence of hematoxylin bodies and abundant plasma cells. For this reason, the important diagnostic challenge is to differentiate KFD from the more common entity of lupus lymphadenitis. Considering the potentially life-threatening nature of lupus lymphadenitis versus the self-limiting character of KFD, lupus lymphadenitis should always be suspected in patients with SLE who present with necrotizing lymphadenitis. To this aim, careful examination of lymph node histopathology in correlation with clinical features is the most reliable way to differentiate KFD from lupus lymphadenitis and other serious entities that it may mimic, such as fulminant EBV-infection and T-cell lymphoma. For instance, The CD20 and CD3 staining results in our case do not support a diagnosis of B- or T-cell lymphoma.

Depending on the stage of disease, KFD is demonstrated by cortical and paracortical necrotizing nodules, apoptotic debris, proliferation of CD68 histiocytes and plasmacytoid monocytes, immunoblasts, abundant CD8 T-cells, and an absence/paucity of neutrophils (*7,10*). In contrast, lupus lymphadenitis is diagnosed in patients who meet the validated revised ACR criteria for SLE together with typical biopsy findings of necrotic and thrombosed blood vessels, presence of necrotizing neutrophilic infiltrates as is seen in drug-induced lymphadenopathy and the pathognomonic feature, hematoxylin bodies (*7*). However, it is suggested that there may be a significant overlap between pathomorphological features of KFD and lupus lymphadenitis. Therefore, it is imperative for pathologists to be aware of the possibility that lupus lymphadenitis may mimic KFD even on histopathology, and pathologists should inform the clinician of this possibility. The pathologist should also become alert to the possible diagnosis of lupus lymphadenitis if extensive necrosis is seen (as in our case; Table 1). It is commonly accepted that hematoxylin bodies which resemble condensed complexes of DNA and anti-dsDNA-antibodies discriminate between KFD and SLE as ANA, antiphospholipid antibody (APLA), Anti-dsDNA, rheumatoid factor (RF) are all usually negative in KFD; although positive ANA (like in our case) has also been reported in patients with KFD (*11*). Nevertheless, a high level of Anti-dsDNA in our case favors the diagnosis of lupus lymphadenitis. Meanwhile, presence of abundant cytotoxic T-cells on histopathology (*12*), which was a finding in our case, is consistent with diagnosis of KFD. However, it has also been suggested that patients with KFD tend to have higher incidence rates of cutaneous manifestations (*13*) which was not present in our patient.

Despite the characteristic histopathologic diversity, clinical features of SLE and KFD can be very similar; fever, lymphadenopathy, fatigue, and joint pain can be seen in both diseases. Hence, there is an ongoing debate on whether KFD is indeed an atypical manifestation of lupus lymphadenitis. Cramer *et al*. in their report suggested that KFD may be a histopathologic alternative form of lupus lymphadenitis representing a 'forme fruste' rather than being an independent disease entity (*14*). Reported estimates from lupus lymphadenitis range between 12% to 58% of patients with SLE (*15*), far more common than that of KFD. Perhaps, one of the reasons for such rarity of reported cases with KFD is that lymph node biopsy is seldom performed in patients with SLE to establish the diagnosis of either KFD or lupus lymphadenitis. We believe the concurrent presence of positive ANA/Anti-ds DNA/ low C3, C4, which support a diagnosis of SLE and pathological evidence suggestive of KFD in our case may imply that lupus lymphadenitis and KFD could in fact belong to the same entity.

The course of KFD is benign and self-limiting in the overwhelming majority of patients, most often resolving within several weeks to 6 months after the initial diagnosis (*15,16*). However and based on the natural history, a thorough prediction of the severity of outcomes can be challenging in some cases. The majority of patients with KFD have a normal complete

Table 1. Anti-HBV response of TCM and related active compounds in clinical trials

Items	Message
1	The association between SLE and KFD is well documented in the literature and the importance of lymph node biopsy in guiding the definitive diagnosis is also well established.
2	Characteristic morphology and histopathology of the resected lymph nodes should thoroughly distinguish KFD (particularly the type with necrotizing pathology) from lupus lymphadenitis since these two entities have different management protocols.
3	Regular follow-up visits are recommended in more severe and systemic manifestations of KFD from three critical aspects: First, to preclude relapses of coexisting SLE in those already diagnosed with SLE; second, to follow the recurrences of KFD which are generally not worrisome; and third to watch for possible development of lupus lymphadenitis which is of significant clinical urgency for careful management.
4	Laboratory measures of ANA, APLA, Anti-dsDNA and RF may not be accurate and reliable indicators to differentiate correctly between lupus lymphadenitis and KFD. Instead, histopathologic features such as abundant specimen cytotoxic T-cells are more sensitive and specific to detect KFD from lupus lymphadenitis. Extensive necrosis seen on lymph node biopsy may suggest a possible diagnosis of lupus lymphadenitis as the more likely diagnosis.
5	Systemic involvement of KFD with retroperitoneal lymphadenopathies may be associated with unusual presentations of flare-up (*e.g.*, lupus pancreatitis in our case) that require active, intensive and lengthy management.
6	Low serum complement levels (C3, C4 and CH50) in patients with KFD potentially indicate a more severe, systemic and chronic stage of the disease. These patients should be actively investigated and monitored for possible late onset of flare-ups.

ANA: antinuclear antibody; Anti-dsDNA: anti-double stranded DNA; APLA: antiphospholipid antibodies; KFD: Kikuchi-Fujimoto disease; RF, rheumatoid factor; SLE: systemic lupus erythematosus.

blood count with elevated LDH, although anemia of chronic disease (as seen in our case) is found in those with a more severe disease (*12*). Furthermore, presence of night sweats, nausea, vomiting, weight loss, diarrhea accompanying fever and lymphadenopathy (as in our patient), which represents the systemic form of KFD, is more prominent in patients with extranodal involvement and could be associated with a more severe course of disease. This should trigger further assessment for aggressive forms of KFD, which tend to be fatal in some cases and only respond to early and intensive immunosuppressive therapy. Bearing in mind, making a diagnosis of KFD in patients with SLE might mislead clinicians to delay the possible diagnosis of lupus lymphadenitis, thereby delaying the appropriate treatment as may have been the case in our patient with enlarged retroperitoneal lymph nodes and the subsequent development of lupus pancreatitis.

4. Conclusion

Fortunately, even with systemic involvement, KFD is a self-limiting condition and it is imperative to recognize this condition in a patient who fits the epidemiological background, with typical clinical features, to avoid a burdensome investigative work up and adding unnecessary anxiety to the patient. Regular follow-up visits are particularly required in patients with KFD in a bid to detect possible SLE relapses/onsets. Nevertheless, even without the occurrence of SLE, these patients should still be followed in proper intervals since recurrences of KFD itself can also continue to occur for many years (*16*).

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(Received July 8, 2016; Revised September 25, 2016; Accepted September 28, 2016)

Case Report

DOI: 10.5582/irdr.2016.01061

A rare case of kissing gastric ulcers caused by trauma

Yan Zhang[1], Yafei Zhang[2], Shiyang Ma[3,*], Haitao Shi[3], Xiaolan Lu[3]

[1] *Department of Cardiology, Weinan Central Hospital, Weinan, Shaanxi, China;*
[2] *Department of General Surgery, Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi, China;*
[3] *Department of Gastroenterology, Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi, China.*

Summary **We present a rare case of a 32-year-old female with gastric ulcers caused by pressure from trauma. The patient was diagnosed with stress-related acute gastritis by a local hospital and she was discharged 1 week later after her symptoms improved. She was given oral proton pump inhibitors (PPI). However, epigastric pain intensified, so the woman was seen at this Hospital 3 days later. Gastric endoscopy revealed kissing ulcers in the lower body of the stomach. A point worth mentioning is that the kissing gastric ulcers were caused by trauma due to impact with the steering wheel.**

Keywords: Kissing gastric ulcers, pressure ulcer, trauma

1. Introduction

A pressure ulcer is a lesion in the skin and/or underlying tissue, usually over bony prominences, caused by pressure and/or shear (*1*). Pressure is not a common cause of peptic ulcers. Pressure ulcers in the stomach are rare, as evident in the fact that there are only 4 reported cases of those ulcers (*2-5*). The causes and mechanisms of pressure ulcers differ; some are due to the use of non-steroidal anti-inflammatory drugs (NSAIDs) and some are due to trauma. In the case reported here, kissing gastric ulcers developed after blunt abdominal trauma.

2. Case report

A 32-year-old woman abruptly applied the brakes while driving and impacted the steering wheel, and she was brought to the Emergency Department of a local hospital for abdominal pain. The woman had no history of alcohol or drug abuse, was not using NASIDs, and she was negative for a *Helicobacter pylori* infection. Right after the accident, the woman had severe and persistent epigastric pain radiating to her back, and this pain was alleviated by bending at the waist. The woman indicated that she had nausea and vomiting but no fever or diarrhea. Her vital signs were stable and a physical examination indicated marked tenderness with focalized guarding in the epigastric region. Abdominal computed tomography (CT) was normal and laboratory results were all within normal limits.

The woman was diagnosed with stress-related acute gastritis by the hospital and she was discharged 1 week later after her symptoms improved. She was given oral proton pump inhibitors (PPI). However, epigastric pain intensified, and the woman was seen at this Hospital 3 days later. Gastric endoscopy revealed kissing ulcers in the lower body of the stomach (Figure 1). Endoscopic ultrasound (EUS) was performed and the ulcers had penetrated into the deep mucosal layer (Figure 2). An easily digestible diet was recommended and oral PPI were continued for 6-8 weeks. The patient had no further complaints of abdominal pain a month later during follow-up. Biopsy samples obtained from the margin and base of the ulcers ruled out an *H. pylori* infection.

3. Discussion

Peptic ulcers are highly prevalent among patients in Gastroenterology. The most common causes of gastric ulcers are an *H. pylori* infection and use of aspirin or non-steroidal anti-inflammatory drugs. Other causes include acute pancreatitis, smoking, acute stress, congenital anomalies, a gastric motility disorder,

Released online in J-STAGE as advance publication September 12, 2016.

**Address correspondence to:*
Dr. Shiyang Ma, Department of Gastroenterology, Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, 157 West 5th Road, Xi'an 710004, China.
E-mail: shiyangma@163.com

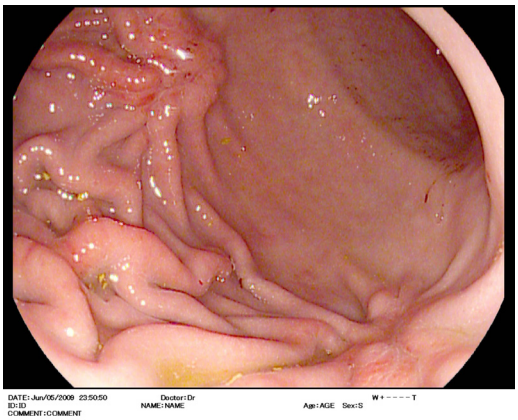

Figure 1. An endoscopic examination revealed kissing ulcers in the body of the stomach.

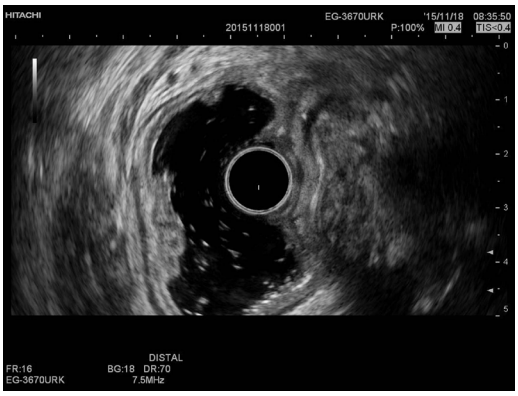

Figure 2. EUS revealed that the ulcer had invaded submucosa layer.

foreign bodies, and trauma. In the current case, ulcers were caused by trauma.

Given the abrupt development of the gastric ulcers, common causes were ruled out. Research has shown that stress causes peptic ulcers much like those associated with *H. pylori* infection or non-steroidal anti-inflammatory drugs (*6*). Endoscopic studies have revealed that between 74-100% of critically ill patients have stress-related mucosal damage within 24 h of admission (*7*). Although multiple factors were presumably responsible for causing gastric ulcers in the current patient, stress was not one such factor.

In terms of morphology, peptic ulcers are more common in the anterior wall of the duodenal bulb, gastric angle, and gastric antrum. Kissing gastric ulcers in both the anterior and posterior gastric wall might be the result of acute gastric ischemia caused by external force. A reduction in mucosal blood flow resulted in the sequence of events that led to formation of an acute gastric mucosal lesion. To the extent known, mucosal damage associated with acute shear is also a cause of gastric ulcers. When the patient is in a stationary state, shear may occur between the fixed skin and the moving subcutaneous tissue, and the results of this shear may be more evident near the bony prominences (*8-10*). This may explain why the ulcers were in front of the spine in the current patient. A series of changes will occur after shear damage, such as neuroendocrine changes and activation of immune cells. Pathological biopsy will reveal accumulation of a large number of inflammatory cells such as neutrophils, monocytes/macrophages, and lymphocytes in the microcirculation, and inflammatory mediators that are subsequently released will aggravate the injury to gastric mucosa (*1*).

In the current case, the patient was fasting on the day of the accident. Therefore, a bold conjecture may be advanced: food in the stomach may be protective since it may absorb or buffer pressure to some extent. An appropriate treatment for peptic ulcers may be fasting with parenteral nutrition, use of PPI, use drugs that protect the mucosa, or even gastrointestinal decompression.

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(Received July 28, 2016; Revised August 19, 2016; Accepted August 31, 2016)

Commentary

DOI: 10.5582/irdr.2016.01025

Orphan drug development in China – Turning challenges into opportunities

Xiaowei Jin*, Li Chen

Hua Medicine, Shanghai, China.

Summary **Of over 7,000 known rare diseases, only 5% currently have an available treatment option worldwide. Moreover, the vast majority of rare disease patients in China have no access to treatment due to limited availability and the lack of appropriate infrastructure in China's healthcare system. Despite increased interest in orphan drug development, drug companies in China with active programs on drugs to treat rare diseases are still limited. Hence, there is a huge unmet need in China, with over 10 million patients suffering from rare diseases. Nonetheless, this has created unprecedented opportunities for the Chinese drug development market. Life science innovation in China has recently received a healthy boost from the 13th National Five-Year Plan and from on-going reform of the China Food and Drug Administration (CFDA). Rare diseases are now recognized as a national priority with increasing governmental support, creating tremendous opportunities for both domestic and multinational drug companies. China is anticipated to play an increasingly important role in the global fight against rare diseases. To ensure future success, Chinese drug companies should leverage the valuable knowledge assembled over the past three decades by Western countries in the area of orphan drug development.**

Keywords: Orphan drug development, rare disease in China, challenges and opportunities

Recent successes in development of orphan drugs to treat rare diseases are in stark contrast to the challenge of decreased productivity faced by the global pharmaceutical industry with traditional research and development (R&D) models for more common diseases. Following this trend, several large multinational pharmaceutical companies such as Pfizer and GlaxoSmithKline have established in-house business units specializing in rare diseases (*1,2*). Many others have been actively working to acquire or partner with orphan drug companies, *e.g.*, Sanofi acquired the leading orphan drug company Genzyme in 2011 (*2*); Biogen has partnered with Ionis to target spinal muscular atrophy (SMA) and with Applied Genetics to develop gene therapies for X-linked retinoschisis (XLRS) (*3*). Furthermore, several large companies specializing in rare diseases such as Shire and Alexion have seen tremendous growth over the past few years (*2*). Rare diseases are now such an attractive sector that orphan drugs have become the global drug industry's leading area of specialization (Table 1).

Despite recent success in the orphan drug sector, effective therapies are only available for less than 5% of over 7,000 rare diseases, many of which are life-threatening and debilitating. The situation is even grimmer in China and many developing nations, where the vast majority of patients with a rare disease currently have no access to appropriate care due to low awareness of the diseases and limited access to specialists, diagnostic testing, and treatment. As a result, less than one-third of the > 500 orphan drugs that have been approved in the United States of America (USA) and other Western countries are available in China and developing nations. In China, the main type of indication for currently available orphan therapies is rare cancer, accounting for almost half of all orphan drugs (*4*). Since the majority of orphan drugs in China are not covered by health insurance, this further limits accessibility because of the high cost of most orphan

Released online in J-STAGE as advance publication October 5, 2016.

**Address correspondence to:*
Dr. Xiaowei Jin, Hua Medicine, 257 Ai Di Sheng Road, Zhangjiang Hi-Tech Park, Pudong, Shanghai, China.
E-mail: xwjin@huamedicine.com

Table 1. Several major acquisitions and partnership deals in the orphan drug industry

Buyer	Seller	Type of deal, year of transaction	Estimated value of the deal	Major rare disease assets acquired
Sanofi	Genzyme	acquisition, 2011	$20.1 billion	Gaucher's disease treatment Cerezyme and the Fabry disease drug Fabrazyme
Shire	Baxalta	acquisition, 2016	$32 billion	several approved orphan drugs for hemophilia and Gaucher's disease, along with a rich pipeline in rare diseases
Shire	Dyax	acquisition, 2015	$5.9 billion	several late-stage assets in HAE + early-stage pipeline
Shire	NPS Pharma	acquisition, 2015	$5.2 billion	Gattex for shore bowel syndrome, Natpara for hypoparathyroidism
Alexion	Synageva BioPharma	acquisition, 2015	$8.4 billion	Kanuma for LAL deficiency + a pipeline of rare disease programs
Alexion	Enobia	acquisition, 2011	$1.1 billion	Strensiq for hypophosphatasia
BioMarin	Prosensa	acquisition, 2014	$840 million	Disapersen for DMD
BioMarin	Zacharin	acquisition, 2013	upfront $10 million + milestone up to $134 million	small molecules for lysosomal storage disorders (preclinical stage) including MPS III, Tay Sachs and Sandhoff; proprietary SensiPro® platform
BioMarin	ZyStor	acquisition, 2010	upfront $22 million + milestone up to $93 million	ERT for the treatment of lysosomal storage disorders such as Pompe's disease
Pfizer	FoldRx Pharmaceutical	acquisition, 2010	undisclosed amount	Tafamidis for familial amyloid polyneuropathy
Roche	Trophos	acquisition, 2015	$0.5 billion	SMA
Biogen	Ionis (formerly ISIS)	partnering, 2013	$100 million upfront + $220 million in milestone payments.	ALS, SMA
Biogen	AGTC	partnering, 2015	$124 million upfront + up to $1.1 billion in milestone payments	XLRS and XLRP

ALS, amyotrophic lateral sclerosis; DMD, Duchenne's muscular dystrophy; ERT, enzyme replacement therapies; HAE, hereditary angioedema; LAL, lysosomal acid lipase; MPS, Mucopolysaccharidoses; SMA, spinal muscular atrophy; XLRP, X-linked retinitis pigmentosa; XLRS, X-linked retinoschisis.

Table 2. Comparison of orphan drug policies in several major countries/regions

Country/region	USA	EU	Japan	Canada	Singapore	Australia	China	India	Taiwan
Orphan drug legislation	yes	yes	yes	no	yes	yes	no	no	yes
Orphan drug designation	yes	yes	yes	no	yes	yes	no	no	yes
Market exclusivity	7 years	10 years	10 years	no	10 years	no	no	no	10 years + 2 years
Financial incentives	yes	not at EU level	yes	yes	no	yes	no	no	yes
Reimbursement	yes	yes	yes	yes	yes	yes	limited, regional differences	no	yes

EU, European Union; USA, United States of America.

drugs, low reimbursement rates, and low incomes; this translates into less affordability for the majority of the Chinese rare disease patients (Table 2) (*5*). In addition, only a few drug companies in China are specializing in rare diseases mainly due to the lack of legislative incentives that have been deemed essential for the success of the orphan drug industry in the USA.

Since the Chinese Government launched its first pilot project in 2013 to improve healthcare for rare disease patients (*6*), significant progress has been made at every front in the fight against rare diseases in China (Supplemental Table S1) (*6-10*). The recent announcement of the first National Committee of Experts on Rare Disease Treatment and Patient Protection (*7*) is another encouraging sign that policy-makers and legislators have begun to recognize the impact of rare diseases and are starting to consider those diseases as a national healthcare priority.

There are currently only a handful of drug companies in China with in-house R&D programs devoted to rare diseases, although this number is expected to grow tremendously thanks partly to increased public awareness. Chipscreen is a successful example, since the China Food and Drug Administration (CFDA) recently approved Chipscreen's innovative cancer drug Chidamide for the treatment of peripheral T-cell lymphoma (PTCL)

Table 3. Orphan drug programs run by Chinese drug companies

Company	Drug name	Indication	Stage of development
Chipscreen	Chidamide	PTCL	launched in China
Shanghai Genomics	Aisi Rui, Etuary	IPF	launched in China
Hua Medicine	HME01	PD-LID, FXS	preclinical
Prosit Sole Biotechnology	multiple products	chronic norovirus infection, articular cartilage injury, refractory gout, lupus renal failure & uremia	preclinical

FXS, fragile X syndrome; IPF, idiopathic pulmonary fibrosis; PD-LID, Parkinson's disease – L-dopa-induced dyskinesia; PTCL, peripheral T-cell lymphoma.

(*11*). Several newly established innovative biotech companies have been actively working in the area of rare diseases (Table 3), *e.g*., Hua Medicine has been working on several rare diseases such as fragile X syndrome (FXS) and Parkinson's disease – L-dopa-induced dyskinesia (PD-LID) (*12*). Another new biotech company, Beijing Prosit Sole Biotechnology, has also devoted considerable resources to rare diseases including several rare immunological disorders (*13*). In addition, Shanghai Genomics has recently launched a drug for the treatment of idiopathic pulmonary fibrosis (IPF) (*14*).

A point worth noting is that many academic institutions and major hospitals in China have been playing an important role in translational medicine for the treatment of rare diseases, and particularly in the area of cutting-edge technologies. As an example, the Shanghai Institute of Materia Medica (SIMM) of the Chinese Academy of Sciences recently announced that one of their orphan drug programs targeting pulmonary arterial hypertension (PAH) was approved by the CFDA to begin human clinical trials (*15*). With respect to cutting-edge technologies, Sichuan University's West China Hospital in Chengdu recently announced that they are preparing to conduct the world's first human trial using *CRISPR* gene editing technology (*16*). In addition, a team of scientists from Tongji Medical College have, in collaboration with FivePlus Molecular Medicine Institute in Beijing, successfully conducted a long-term trial of gene therapy in human patients with a rare genetic disorder known as Leber's hereditary optic neuropathy (LHON) (*17,18*), more than 10 years after the world's first gene therapy was approved in China (*19*).

Nonetheless, the orphan drug industry in China is still in its infancy with tremendous challenges to overcome. Compared to the USA and many Western countries with more mature industries as well as legislative and regulatory systems, currently, there are almost no large domestic pharmaceutical companies with significant resources allocated to rare diseases, although government funds allocated to rare disease research and DNA sequencing have increased significantly in China, along with funding from the private sector.

A major difference between the USA and China is that rare disease research and drug development are primarily driven by rare disease organizations and the private sector in the USA. Rare disease organizations played a critical role in the early stage of rare disease drug development and have been a nexus connecting patients, specialists, drug developers, and regulatory agencies. Rare disease organizations in China have only recently been established through grass-root efforts. One such organization, the Chinese Organization for Rare Disorders (CORD), has become highly influential as a patient advocacy group. Table 4 lists several major rare disease organizations in the USA, European Union (EU), and China.

Revolutionary technological advances in next-generation gene sequencing have enhanced our understanding of genetic risk factors and underlying genetic defects that are linked to rare diseases. Gene therapy has quickly emerged as an effective and powerful approach to treat or even potentially cure many rare diseases (Table 5).

In order to maximize and efficiently utilize available resources to successfully develop orphan drugs to treat rare diseases in China, a framework is presented here with several specific recommendations for drug companies interested in capitalizing on opportunities in the market:

1) Drug companies need to work closely with all stakeholders, including policy makers and regulatory agencies as well as rare disease communities, to create a healthy ecosystem as is essential for life science innovation.

2) Chinese drug companies interested in rare diseases should adopt a global outlook by eyeing the global market while operating in China and they should foster innovation through global collaboration by tapping into the intelligence and expertise of Western companies and joining forces with global partners.

3) Drug companies should decide areas to focus on by adopting a systematic approach with pre-defined criteria based on in-depth analyses prior to embarking on a program targeting a specific rare disease. The final decision should represent the best opportunities based on the information and resources available and focus on urgent, unmet needs that are medically addressable and commercially viable in China and the rest of the world. Several key criteria include:

i).Vast unmet needs with no or limited options available, and especially those with the greatest impact on China (*20*). Examples of major rare diseases in China include thalassemia, osteogenesis imperfecta, SMA, and Duchenne's muscular dystrophy (DMD) (*20*).

Table 4. Several of the major organizations dealing with rare diseases in the USA, EU, and China

Organization name	Type	Focus	Country /region	Link
National Organization for Rare Disorders (NORD)	umbrella	all rare diseases	USA	*http://rarediseases.org*
Global Genes	umbrella	all rare diseases	USA	*https://globalgenes.org*
EURORDIS	umbrella	all rare diseases	EU	*http://www.eurordis.org*
Chinese Organization for Rare Disorders (CORD)	umbrella	all rare diseases	China	*http://www.hanjianbing.org*
Rare Diseases International (RDI)	umbrella	all rare diseases	global	*http://www.rarediseasesinternational.org*
Cystic Fibrosis Foundation (CFF)	focused solely on CF	CF	USA	*https://www.cff.org*
Spinal Muscular Atrophy Foundation (SMAF)	focused solely on SMA	SMA	USA	*http://www.smafoundation.org*
Huntington Disease Society of America (HDSA)	focused solely on HD	HD	USA	*http://hdsa.org*
ALS Association (ALSA)	focused solely on ALS	ALS	USA	*http://www.alsa.org/*
SMA Europe	umbrella, with SMA patients and research organizations from countries across Europe	SMA	Europe	*http://www.sma-europe.eu*
European Huntington's Disease Network (EHDN)	umbrella, with HD patients and research organizations from countries across Europe	HD	Europe	*http://www.euro-hd.net*

ALS, amyotrophic lateral sclerosis; CF, cystic fibrosis; EU, European Union; HD, Huntington's disease; SMA, spinal muscular atrophy; USA, United States of America.

Table 5. Companies developing gene therapies and the diseases they treat

Company	Platform	Diseases treated
Spark Therapeutics	AAV-based gene therapy	rare forms of blindness, IRDs, such as RPE65-mediated IRDs (positive Phase III results), and choroideremia (Phase I/II on-going)
AveXis	AAV-based gene therapy	SMA (positive Phase I/II results)
AGTC	AAV-based gene therapy	rare ophthalmological disorders such as XLRS and XLRP (early clinical stages or IND-ready programs)
uniQure	AAV-based gene therapy	familial LPLD (Glybera® approved), hemophilia B (Phase I/II), Sanfilippo B (Phase I), and PD (Phase I) and other rare genetic diseases of the liver/metabolism, CNS, and cardiovascular system
Bluebird Bio	Lentivirus-based gene therapy	severe genetic disorders such as CALD (Phase II/II), transfusion-dependent β-thalassemia (also known as β-thalassemia major) (Phase II/II), and severe sickle cell disease (Phase I/II)
Regenxbio	AAV-based gene therapy	HoFH (Phase I/II trial); MPS type I & wet AMD (IND-ready)
Bamboo therapeutics	AAV-based gene therapy	rare genetic disorders such as GAN (Phase I/II on-going), DMD, and FA
Voyager Therapeutics	AAV-based gene therapy	rare CNS diseases, such as PD (Phase I/II on-going), ALS, and HD
Dimension Therapeutics	AAV-based gene therapy	rare genetic liver disorders, including hemophilia B (Phase I/II)
Ionis Pharmaceuticals	antisense -based therapy	HoFH (KYNAMRO® approved) & pouchitis (Alicaforsen approved) and a wide range of rare genetic diseases, including SMA (positive Phase III results) and HD (Phase II on-going)
Alnylam Pharmaceuticals	RNAi-based therapy	a wide range of rare genetic diseases, including hereditary amyloidosis ATTR (Phase III), hemophilia, and rare bleeding disorders (Phase II)

AAV, adeno-associated virus; ALS, amyotrophic lateral sclerosis; AMD, age-related macular degeneration; ATTR, TTR-related amyloidosis; CALD, cerebral adrenoleukodystrophy; DMD, Duchenne's muscular dystrophy; FA, Friedreich's ataxia; GAN, giant axonal neuropathy; HD, Huntington's disease; HoFH, homozygous familial hypercholesterolemia; IRDs, inherited retinal dystrophies; LPLD, lipoprotein lipase deficiency; MPS, mucopolysaccharidosis; PD, Parkinson disease; SMA, spinal muscular atrophy; XLRP, x-linked retinitis pigmentosa; XLRS, x-linked retinoschisis.

ii).Diseases with a more clearly defined history and progressive symptoms that respond to interventions with a clinically meaningful impact within a reasonable period. Additional features include biomarkers that can be used to predict disease progression, stratification of otherwise heterogeneous patient populations, and prediction of the patient response to treatment. Also critical are companion diagnostic kits based on reliable biomarkers that are available or easily developed.

iii). Easy access to local key opinion leaders (KOLs)/ hospitals in order to quickly identify and recruit patients for intervention trials and KOLs/hospitals who are willing to fiercely advocate on behalf of rare disease patients and drug companies.

iv). A rare indication with the same therapeutic target as a common disease is ideal, since it offers the potential for expanded indications in the future. This allows a company to build a portfolio based on multiple shots on goal with reduced risk and operational synergy.

4) Adopting a patient-centric approach, companies should create a corporate culture and business model by incorporating patients' perspective into program planning and execution. In fact, many rare disease patients and patient organizations have shown a strong desire and willingness to play a larger role in orphan drug development.

5) Drug companies should take advantage of favorable policies while establishing orphan drug R&D capacities in China. Historically, China has placed a high priority on biomedical research with strong governmental support and a favorable regulatory environment for cutting-edge technologies, such as gene and cell therapies (*16,19,21-23,24*). These technologies hold great promise for treating and even curing genetic diseases. The precision medicine initiative recently undertaken by the Chinese Government will no doubt further accelerate rare disease research in China.

6) Orphan drug development represents one of the best opportunities to create differentiated products to meeting vast unmet medical needs. Drug companies should embrace the recent CFDA reform favoring innovative development of drugs for a variety of medical needs, including rare diseases.

In conclusion, patients' needs should be the focus of coordinated national task forces and investigational networks on rare diseases, and these organizations need to be supported by a long-term strategy and sustained commitment from the Chinese Government. The recently released 13th National Five-Year Plan puts greater emphasis on healthcare and the pharmaceutical industry. The Plan specifically cites genetic research and precision medicine which will support and promote research and drug development for rare diseases. A systematic approach backed by national initiatives will pave the way for robust growth of the healthcare industry, including orphan drug development. With increasing government funding and support for innovative drug development, coupled with on-going regulatory reform, 'Made in China' orphan drugs may soon become a reality.

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(Received April 17, 2016; Revised September 1, 2016; Accepted September 2, 2016)

Supplemental Table

Supplemental Table S1: Highlights of major initiatives by Chinese government agencies in relation to rare diseases in China

- In 2009, Fast Approval by the CFDA – a fast approval process for drugs to treat several rare diseases – was implemented. A separate regulation specifies that drugs to treat rare diseases can fulfil fewer clinical trial requirements (*8*).
- Shanghai model: The Shanghai Rare Disease Society, founded in early 2011, also works to promote legislation, research, and insurance coverage for rare diseases. Over the past several years, the City of Shanghai has covered medical costs for treatment of 12 specified rare diseases (*8*).
- Qingdao model: In 2012, Qingdao, a coastal city in Shandong Province, approved a proposal to cover a capped amount of the treatment fees for all diseases, including rare diseases (*8*).
- In 2013, the China Rare Diseases Prevention and Treatment Alliance was established. The Alliance launched China's first pilot project at the national level to promote better healthcare for rare diseases. The Alliance established a national collaborative network involving more than 100 provincial and municipal medical facilities to implement this project. This network covers 13 provinces, which have a population of 0.7 billion (*6,8*)
- In January 2016, a National Committee of Experts on Rare Disease Treatment and Prevention was established under the leadership of the National Health and Family Planning Commission of the People's Republic of China in order to improve the management of rare diseases, to promote the standardization of diagnosis and treatment of rare diseases, and to ensure the basic medical needs of patients with a rare disease are met and their right to health is upheld (*7*).
- At the end of 2015, the CFDA announced that it would prioritize the review of new technologies and novel therapies for AIDS, tuberculosis, viral hepatitis, rare diseases, and cancer, and particularly for medicines developed for pediatric or elderly patients (*9*).
- In March 2016, the Ministry of Science and Technology of the People's Republic of China issued Guidelines for 2016 National R&D Programs Focused on Precision Medicine and other Key Projects (*10*).

CFDA, China Food and Drug Administration.

Letter

DOI: 10.5582/irdr.2016.01065

Frontal fibrosing alopecia treatment options

Raymond Fertig*, Antonella Tosti

University of Miami, Miller School of Medicine, Department of Dermatology and Cutaneous Surgery, Miami, FL, USA.

Summary **Frontal fibrosing alopecia (FFA) is a rare dermatologic disease that causes scarring and hair loss and is increasing in prevalence worldwide. FFA patients typically present with hair loss in the frontal scalp region and eyebrows which may be associated with sensations of itching or burning. FFA is a clinically distinct variant of lichen planopilaris (LPP) that affects predominantly postmenopausal women, although men and premenopausal women may also be affected. Early diagnosis and prompt treatment are necessary to prevent definitive scarring and permanent hair loss. Data from retrospective studies indicate that 5-alpha-reductase inhibitors (5aRIs) are effective in stabilizing the disease. In our clinical experience, we have seen optimal results treating FFA patients with oral finasteride in conjunction with hydroxychloroquine, topical calcineurin inhibitors (tacrolimus) and excimer laser in patients with signs of active inflammation.**

Keywords: Frontal fibrosing alopecia (FFA), treatment, finasteride

The prevalence of frontal fibrosing alopecia (FFA) is increasing worldwide and early diagnosis and prompt treatment are necessary to prevent definitive scarring and permanent hair loss. The main objective of treatment is to reduce inflammation and prevent disease progression. Disease activity is best evaluated with dermoscopy with peripilar casts being a good indicator of progression.

The lack of randomized clinical trials does not allow for definitive conclusions to be made regarding optimal treatment for FFA, but available evidence gives some guidance as to potential effective treatment approaches. Data from retrospective studies indicate that 5-alpha-reductase inhibitors (5aRIs) are effective in stabilizing the disease. A retrospective multicenter study in 355 FFA patients concluded that 5-alpha-reductase inhibitors were the most effective treatment modalities for FFA (*1*). In this study population, which represents the largest FFA study cohort to date, the 5aRIs finasteride and dutasteride were utilized in approximately one-third of patients, with improvement noted in 47% of patients and stabilization observed in 53% of patients (*1*). Furthermore, in a systemic review measuring treatment response of 114 FFA patients, 45% of patients treated with finasteride or dutasteride showed a favorable clinical response (*2*). We first reported the efficacy of finasteride in FFA patients 12 years ago (*3*) and still widely utilize this medication when treating patients. In fact, a recent case report indicates that 5aRIs may induce hair re-growth in some patients (*4*).

To date, FFA patients are mainly treated with combination medical therapy which has proven to be the favored course of action in our experience. We treat a large number of FFA patients in our academic clinical practice and have seen the best results using oral finasteride in conjunction with hydroxychloroquine, topical calcineurin inhibitors (tacrolimus) and excimer laser in patients with clinical or dermoscopic evidence of active inflammation. Four retrospective studies have evaluated the effectiveness of hydroxychloroquine in the treatment of FFA (*1,5-7*). In one such study, 15 FFA patients treated with hydroxychloroquine experienced a 73% reduction in signs and symptoms of FFA at 6-month follow up (*5*). Limited case reports support usage of tacrolimus in FFA patients (*8,9*). In our clinical experience, excimer laser is very effective in reducing inflammation and peripilar casts in patients with active disease. At least one study has confirmed the efficacy of excimer laser, showing success in treating 13 patients

Released online in J-STAGE as advance publication September 5, 2016.

**Address correspondence to:*
Dr. Raymond Fertig, University of Miami, Miller School of Medicine, Department of Dermatology and Cutaneous Surgery, Address: 1475 NW 12th Ave, 2nd Floor Miami Florida, 33136, USA.
E-mail: raymondfertig@gmail.com

with lichen planopilaris and achieving a significant reduction in clinical signs of inflammation (*10*). In this study, excimer laser treatments were performed twice per week with a cumulative mean dose of 4,300 mj/cm^2 (*10*).

Intralesional and topical steroids are commonly used by dermatologists to treat active disease, but they should be used with caution in FFA patients as they can worsen skin atrophy which is a hallmark of this disease. In addition, topical minoxidil is helpful and should be considered as female pattern hair loss is commonly associated with FFA. Positive results of combination therapy using minoxidil were seen in one small study of 8 FFA patients that showed halting of disease progression in 50% of FFA patients treated with minoxidil (2% concentration twice per day) and finasteride therapy (2.5 mg/day) following 12 to 18 months of treatment (*3*). Again, combination therapy seems to be the optimal choice for FFA patients, with finasteride serving as the core treatment to arrest disease inflammation, along with hydroxychloroquine, tacrolimus and excimer laser. Minoxidil should then be considered to increase hair volume.

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(Received August 17, 2016; Accepted August 25, 2016)

IRDR
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Pearl City Koishikawa 603
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