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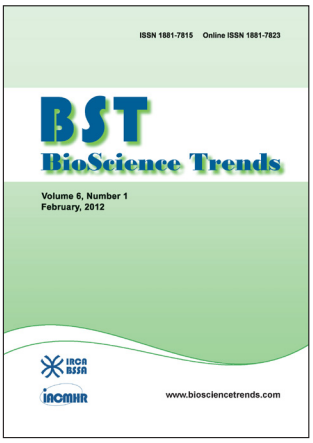

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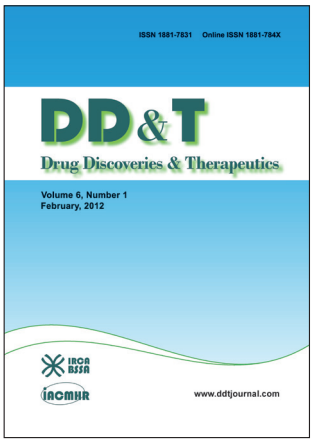

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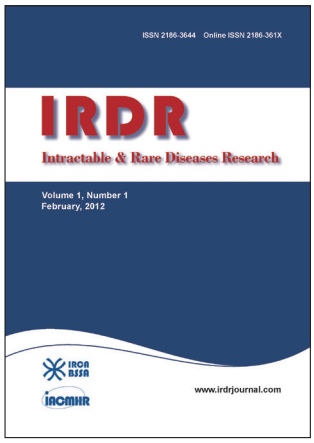

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Editorial and Head Office

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

E-mail: office@irdrjournal.com
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Recent deep learning models for dementia as point-of-care testing: Potential for early detection

Kenji Karako[1], Peipei Song[2,3,*], Yu Chen[1,*]

[1] Department of Human and Engineered Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan;
[2] Center for Clinical Sciences, National Center for Global Health and Medicine, Tokyo, Japan;
[3] National College of Nursing, Japan.

SUMMARY Deep learning has been intensively researched over the last decade, yielding several new models for natural language processing, images, speech and time series processing that have dramatically improved performance. This wave of technological developments in deep learning is also spreading to medicine. The effective use of deep learning in medicine is concentrated in diagnostic imaging-related applications, but deep learning has the potential to lead to early detection and prevention of diseases. Physical aspects of disease that went unnoticed can now be used in diagnosis with deep learning. In particular, deep learning models for the early detection of dementia have been proposed to predict cognitive function based on various information such as blood test results, speech, and the appearance of the face, where the effects of dementia can be seen. Deep learning is a useful diagnostic tool, as it has the potential to detect diseases early based on trivial aspects before clear signs of disease appear. The ability to easily make a simple diagnosis based on information such as blood test results, voice, pictures of the body, and lifestyle is a method suited to point-of-cate testing, which requires immediate testing at the desired time and place. Over the past few years, the process of predicting disease can now be visualized using deep learning, providing insights into new methods of diagnosis.

Keywords deep learning, dementia, prediction, point-of-cate testing

Deep learning has continued to attract attention since its performance exceeded that of humans in image recognition tasks at the ImageNet Large Scale Visual Recognition Challenge (ILSVRC2012), an image processing contest held in 2012 (*1*). Deep learning has been intensively researched over the last decade, yielding several new models for natural language processing, images, speech and time series processing that have dramatically improved performance. In the field of image recognition, ResNets capable of recognizing objects in basic image recognition tasks have emerged (*2*). Yolo has been proposed for object detection tasks (*3,4*), and it is capable of detecting object regions in an image in real time. As well as recognizing objects in images, deep learning is also capable of generating new images. Models for image generation have been proposed, first called GANs, that produce images similar to the training data (*5*), and recently images that might be mistaken for the real thing can now be produced. Stable Diffusion produces images that represent input text (*6*). Natural language processing has also made great strides with the emergence of bert (*7*) and transformer (*8*), which are generic models for languages. Chat GPT (*9*) has emerged in the past few years, and it can generate responses to questions as if they were answers from a person with actual expertise.

Recent developments in deep learning for diagnostic imaging technology

This wave of technological developments in deep learning is also spreading to medicine. The impact of the dramatic development of image-oriented models in the early stages of the development of deep learning technology has led to widespread research on the use of deep learning for image-based medical examinations. In particular, models have been proposed to detect various diseases by combining basic imaging diagnostics such as X-rays (*10*), CT scans (*11*), and MRI scans (*12,13*). Diseases are diagnosed with deep learning using images. As well as researching models using still images, detection models have also been studied for endoscopy (*14*) and ultrasonography (*15*), where video

is taken. These imaging models help to prevent doctors from missing anything and reduce the time to diagnosis.

The potential for deep learning in early disease detection

Although the effective use of deep learning in medicine is concentrated in diagnostic imaging-related applications, deep learning has the potential to lead to early detection and prevention of diseases. The detection of diseases requires an examination to analyze and detect physical aspects of disease, and methods of testing and diagnosis have been studied. Physical aspects of disease that went unnoticed can now be used in diagnosis with deep learning. Deep learning analyzes large amounts of data and it learns and identifies factors that are important to diagnosis so that subtle information and aspects of disease that went unnoticed can be identified. Deep learning therefore has the potential to detect aspects of disease in its early stage.

The current work looks at the potential for new methods of detecting dementia early using deep learning and possible applications of those methods.

Early models for detection of dementia using deep learning

One area where deep learning is being used effectively is in the early detection of dementia. There is no cure for dementia, and the condition needs to be addressing in its early stages to mitigate symptoms and delay progression. Deep learning identifies and analyzes the effects of dementia on physical and cognitive functions, facilitating its diagnosis. As shown in Figure 1, early detection is possible based on blood test results that reflects lifestyle factors that contribute to dementia and speech reflecting cognitive function. In addition, deep learning can also detect the effects of dementia in the face and voice, which are difficult to analyze.

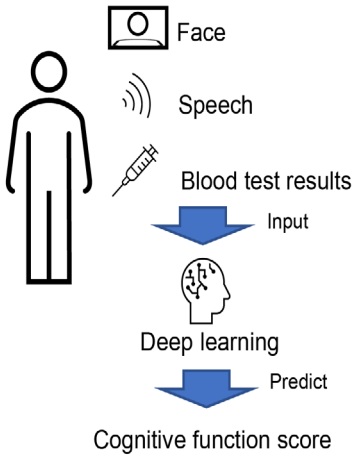

Figure 1. Deep learning predicts the cognitive status of patients based on the various effects of dementia on the body. A variety of information is used as input data, such as blood test results, voice signals, spoken text, and facial photographs.

The first approach to predicting cognitive function was predicting the Mini-Mental State Examination (MMSE) Score, which is used to assess cognitive function, based on blood test results reflecting lifestyle in order to detect dementia of lifestyle-related origin in its early stage (*16*). The proposed model uses a three-layer neural network model, which is a basic deep learning technique, to predict the MMSE Score using 23 items on a blood test performed on 202 elderly patients (73.48 ± 13.1) with various underlying diseases. Common items on the test such as the total blood cell count, red blood cell count, and the hemoglobin level are used. Although the training data were limited, the correlation coefficient between the predicted MMSE Score and the actual MMSE Score was 0.66, indicating a close correlation.

A model for predicting cognitive function based on speech has been proposed as a second approach. The risk of dementia is predicted based on speech when answering a few fixed questions or answering for about five minutes. This approach includes models that predict cognitive function based on the information contained in sound and models that convert speech into textual information and that predict the risk of dementia based on aspects of cognitive function evident in the text. In a model that predicts using speech information as it is (*17*), speech is treated as signals and deep learning extracts the phonetic features that emerge due to dementia. In a model that predicts dementia by converting speech into textual information (*18*), deep learning analyzes sentences that reflect the decline in comprehension and judgement occurring with dementia, and it evaluates cognitive functions. Although the information used in both methods of prediction is the same speech, the predictions focus on different aspects of dementia.

Once sound is converted to textual information, signals that should have been present are missing as a result of dementia. There are models that use both speech signals and textual information to predict the risk of dementia (*19*).

A final approach is to predict the risk of dementia based on images of the face. A model (*20*) has been proposed to predict whether there is cognitive decline or not based on photographs of the face taken without facial expressions. A deep learning model was created using data from 121 patients with cognitive decline and 117 normal individuals, and it performed well at discrimination with a sensitivity of 87.31%, a specificity of 94.57%, and an accuracy of 92.56%.

Effective use of deep learning as point-of-cate testing

Models have been studied to indirectly predict the risk

of dementia by examining the facial appearance, voice, or blood test results that exhibit aspects of dementia. Photographs of the face cost almost nothing, voice recordings take 10 minutes at most, and blood tests are performed during physical examinations. Therefore, the risk of dementia can easily be determined with little effort. In addition to the benefit of being able to determine the risk of dementia, models can also ascertain there is a risk of dementia before symptoms become apparent. Thus, deep learning can serve as point-of-cate testing (PoCT) that encourages an individual to undergo a thorough examination, although there are concerns about prediction performance (*21*).

Originally, deep learning had a black box problem, in which the prediction process was not evident from the outside, and what caused the accurate or inaccurate prediction of cognitive function was not known. Over the past few years, however, a method of revealing the prediction process called SHAP (*22*) has been proposed. It can also reveal which blood test results are responsible for the poor prediction of cognitive function. Such information would not only increase the transparency of the deep learning diagnostic process but could also be an important source of information for determining the appropriate course of action for each individual's condition. When predicting cognitive function based on blood test results, for example, knowing which blood test results had the greatest impact when poor cognitive function was predicted would allow the suggestion of a coping strategy tailored to that individual. In addition, revealing the prediction process could provide new insights into the early detection of dementia.

When deep learning models predict dementia based on photographs of the face and speech, analyzing regions of the face, facial expressions, and phonetic features that are particularly affected could help to detect changes due to dementia that were previously overlooked. This could help lead to new diagnostic methods.

Deep learning, which has developed rapidly in recent years, is being used not only as a means to help doctors diagnose diseases, but also as a completely new means with which to detect diseases early. Physical aspects of disease that went unnoticed can now be used in diagnosis with deep learning. Research on the early detection of dementia in particular is being conducted to predict aspects of dementia from multiple perspectives using information such as blood test results, voice, and photographs of the face. The available data are currently limited, but if data are collected in the future, then this could be an effective PoCT tool. Combining all of these predictive models based on blood test results, photographs of the face, and voice should lead to further improvements in performance. Deep learning has the potential to allow early detection of dementia and other diseases based on their physical aspects.

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Address correspondence to:
Peipei Song, Center for Clinical Sciences, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan.
E-mail: psong@it.ncgm.go.jp

Yu Chen, Department of Human and Engineered Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 227-8568, Japan.
E-mail: chen@edu.k.u-tokyo.ac.jp

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Mini-Review

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Hyperphagia in Prader-Willi syndrome with obesity: From development to pharmacological treatment

Qaddra Fahada Ab. Rahman, Nurul Farhana Jufri*, Asmah Hamid

Biomedical Science Program, Center for Toxicology and Health Risk Studies, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

SUMMARY Prader-Willi syndrome (PWS) is a rare genetic disorder due to lack of genes expression inherited from the paternal chromosome 15q11-q13 region usually from paternal deletions, maternal uniparental disomy 15 or imprinting defect. There are two different nutritional stages reported in an individual with PWS; first stage during infancy marked by feeding and growth difficulties and second stage where hyperphagia starts and leads to development of obesity. However, the exact mechanism of hyperphagia development, from having difficulties in feeding during early years to insatiable appetite after they grow is still unknown and is the focused in this review. The keywords used for literature search such as "Prader-Willi syndrome", "hyperphagia", "obesity", and "treatment" were used to create the search strings by using synonyms in order to retrieve the relevant records from PubMed, Scopus and Science Direct. The possible mechanism of hyperphagia can be classed into hormonal abnormalities such as increase in ghrelin and leptin from infancy to adulthood. Low level of hormones was observed in the thyroid, insulin and peptide YY at certain ages. Neuronal abnormalities contributed by Orexin A and brain structure alteration was documented at 4-30 years old. Treatment in the form of drugs such as livoletide, topiramate, and diazoxide could potentially alleviate these abnormalities and make hyperphagia less prominent in PWS. The approaches are important to regulate the hormonal changes and neuronal involvement as potentially controlling hyperphagia and obesity.

Keywords appetite, genetic disorders, hormones, neurodevelopment, overeating

1. Introduction

Prader-Willi syndrome (PWS) is a genetic neurodevelopmental disorder (*1*). The prevalence of this syndrome is 1/10,000-1/30,000 with approximately 350,000-400,000 individuals worldwide reported with this condition that occurs equally regardless of gender and race (*2*). PWS is due to loss of gene function in the region of chromosome 15 located at 15q11.2-q13 that is expressed in the paternal gene while the genes on the maternal copy are turned off or inactive in a condition known as genomic imprinting (*3,4*). Three different causes can lead to PWS. In most cases of PWS, about 70 percent of the cases occur when a paternal chromosome 15 segment is deleted. In another 25 percent of cases, individuals with PWS have two copies of chromosome 15 inherited from the maternal side instead of one copy from each parent, known as maternal uniparental disomy. Another 5 per cent is caused by a chromosome rearrangement called translocation or by a mutation or other defect that abnormally deactivates a gene on paternal chromosome 15. Thus, individuals with these chromosomal changes lose specific critical genes in this segment that leads to PWS.

PWS is characterized by severe hypotonia with difficulty in sucking and breastfeeding during infancy before progressing to overeating and the gradual development of morbid obesity from childhood to adulthood. They also have poor motor and language development as well as cognitive disabilities. PWS individuals have been reported with behavioural problems such as skin picking, difficulty with a change in routine, temper tantrums, obsessive, compulsive behaviours and mood fluctuations (*5*). In addition, they also suffer from endocrine abnormalities that are present in the form of genital hypoplasia, incomplete pubertal development and infertility in addition to a short physique related to growth hormone deficiency (*2*).

There are two different nutritional stages reported

in an individual with PWS; the first stage is marked by feeding and growth difficulties during infancy and the second stage is when the hyperphagia starts and leads to the development of obesity (*6*). Hyperphagia is the constant pathologic urge to consume food and constant hunger that can advance into dangerous food-seeking behaviour (*7*). This leads to the central feature of PWS individuals in which obesity is a primary phenotypic component (*8*).

PWS is the most commonly known cause of morbid obesity in humans. The annual mortality rate is estimated at 1-4% primarily due to complications of hyperphagia and obesity-related causes (*7*). The contribution of hyperphagia and obesity as a cause of death in PWS is often discussed as a consequence of cardiorespiratory failure. As this clinical manifestation is mainly presented in PWS, this review sought to summarize the different types of mechanisms in the manifestation of hyperphagia from infancy to adulthood and possible pharmacological treatment approaches for PWS individuals with hyperphagia.

2. Literature search

The keywords and synonyms were used to generate the search strings to retrieve relevant records from PubMed, Scopus and Science Direct (Supplemental Table S1). The search strings were combined using Boolean operators (AND, OR, NOT) by using the advanced search in the database to screen the relevant articles from 2016 to 2022. To limit the records retrieved to research papers, titles and abstracts were screened to eliminate books, reviews, conference papers, and other non-research publications. The titles and abstracts of the chosen original research in English were selected in this review. Then, relevant primary research articles were screened for relevant content to hyperphagia and obesity in PWS individuals in English. The references are classified based on the mechanism and treatment.

Based on the three chosen databases in this scoping review including Pubmed, Science Direct and Scopus, there were a total of 778 records retrieved which were Pubmed (n = 54), Science Direct (n = 424) and Scopus (n = 300). Then, 430 duplicates were removed. After the initial screening, there were 348 articles, 9 records were removed due to the articles detected were without titles along with authors and abstract in the EndNote system. Then, the total of 339 records, complete with title, names and abstract in the EndNote system were identified, 49 records were removed because it included books and unrelated articles. Finally, a total of 290 records were selected as research articles that had undergone secondary selection. A total of 258 articles were excluded due to hyperphagia or obesity that are not related to PWS and 11 articles were published in a language other than English. Finally, 21 studies including 10 mechanism studies and 11 treatment studies were included into the literature synthesis. The screening process was done by more than one person to avoid bias and contradiction in the selection of studies.

3. Potential mechanisms causing hyperphagia

PWS is a genetic neurological disorder due to loss of function on the long arm (q11–q13) of the paternal chromosome 15. The paternally expressed PWS region located on chromosome 15 contains genes encoding polypeptides such as *MKRN3*, *MAGEL2*, *NECDIN* and snoRNA (*2*). The critical gene for most PWS phenotypes involves the snoRNA gene, SNORD116 are affected (*9*). PWS baby could survive until adulthood, however they tend to develop into obesity resulted from hyperphagia. The prevalence of overweight and obesity in PWS is around 40% in children and adolescents (*10*), while this percentage tends to increase between 80% and 90% in adulthood (*11,12*). Although PWS individuals have poor nutrition and appetite during infancy, they experience uncontrolled appetite leading to weight gain after the age of 4 (*6*). While the exact mechanism has not yet been fully explained, the development of obesity is mainly related to dysfunction in the feeding centre of the hypothalamus and its hormones that lead to uncontrol food intake and energy expenditure (*13*). Disruption in the hypothalamic pathway of satiety control results in persistent and unsatiated appetite, hyperphagia and hunger-related eating behaviours. It is closely associated with hormones and neuronal abnormalities that can cause body composition to change and stimulate hyperphagia.

Hypothalamic neurons sense both neural and physiological signals and respond by releasing neurotransmitters and peptide neuromodulators into the brain (*14*). Hypothalamic neurons also regulate puberty, reproduction, stress, circadian rhythms, immune function, and more complex behaviours such as social behaviour. Furthermore, hormones are released from the endocrine glands to regulate physiology and behaviour. The dysfunction of human hypothalamic neurons and hormones have been linked to obesity, hypertension, mood disorders and sleep disorders (*15*). For instance, it helps to regulate body composition, that is composed of fat, bone and muscle in the body. However, an alteration in a high percentage of fat with less muscle mass is present in PWS instead of a normal body composition that consists of less fat and more muscle mass (*16*).

A deficiency of thyroid hormone levels in PWS was observed as early as infancy (*17*). About 20-30% of PWS patients have thyroid hormone deficiency (*2,19*). Low T4, T3 and thyroid stimulating hormone (TSH) levels are among the cause of the floppy baby syndrome and hypotonia. Apart from that, the reduction of thyroid hormone leads to a change in metabolic rate and the reduction of energy consumption (*17*). This makes PWS individuals more prone to develop obesity later as they age.

Next, an increase in ghrelin has been seen in PWS individuals since they were 5 weeks old (*18*). Ghrelin is a powerful orexigenic hormone, an appetite stimulant hormone that increases appetite and food intake. The stomach secretes ghrelin during fasting or when hungry. The individual will feel hungrier and the increment of hormone level will gradually decrease after eating. However, the ghrelin level will remain high even after food consumption in PWS. Hyperghrelinemia was also experienced as early as one year old, who was still in nutritional phase 1a, characterized by poor appetite and feeding (*19*). Ghrelin also is associated with playing an essential role in the adaptation of the fetus to intrauterine malnutrition as well as the growth of infants who are smaller than the average fetus, known as small for gestational age (SGA) (*20*). High levels of ghrelin have been observed in SGA and premature infants leading to the hypothesis that intrauterine growth restriction and low birth weight are the possible physiological effects of excessive ghrelin secretion (*20,21*). Therefore, it is possible that the high ghrelin levels observed in PWS infants are a physiological response to their birth weight being on average about 15% less than expected (*22*). Increased ghrelin leads to two different results according to age which is an adaptation of the fetus to malnutrition as well as the growth in small-sized babies, but the hormone acts as an appetite stimulant later in life (*21*).

The following mechanism is the increase of leptin which can be seen in two different studies; in seven-month-old subjects (before hyperphagia starts) and in adulthood (after the hyperphagia phase starts) (*1,23*). The function of leptin is to control the long-term balance between food intake and energy use (*18*). The small infant size at birth was associated with higher leptin levels in umbilical cord blood and in turn, was associated with higher body mass index weight gain at 4 years of age (*24*). Besides, the effect of high leptin in adults is associated with low Brain-Derived Neurotrophic Factor (BDNF). BDNF acts as a satiety signal guided by leptin-melanocortin signalling. In PWS, the neural circuits in this area become dysfunctional and cause a decrease in local BDNF levels. Low BDNF level causes less peripheral density and this in turn causes adiposity to secrete more leptin. A prolonged increase in leptin causes leptin resistance and subsequently lack of satiety, overeating and increased weight.

Low peptide YY (PYY) and insulin levels are also involved in hyperphagia and obesity of PWS. The function of PYY and insulin are to stimulate pro-opiomelanocortin (POMC) neurons and inhibit neuropeptide Y (NPY) follows by activation of melanocortin receptor 4 (MC4R) to induce satiety (*13*). As PWS individuals have low levels of PYY and insulin, NPY is released and prevents MC4R activation that leads to increased food intake (*26,27*). This causes failure of satiety control as α and β-MSH or MC4R fail to be activated (*28*).

In addition to hormones, other mechanisms from the study's findings involved neuronal abnormality *via* changes in brain structure. Changes in several brain areas (hypothalamus, amygdala, hippocampus, orbitofrontal and medial prefrontal cortex) play an important role in regulating abnormal food intake in PWS. Functional magnetic resonance imaging showed higher activity in reward/limbic regions (nucleus accumbens, amygdala) in subjects with PWS (*29*). Mainly, subjects with PWS exhibited greater food activation in limbic and paralimbic regions (hypothalamus, amygdala, hippocampus) and lower activation in cortical inhibitory regions (orbitofrontal cortex, medial prefrontal cortex) (*29,30*). In addition, reduced functional connectivity between the ventral striatum and limbic structures (hypothalamus and amygdala) was reported in subjects with PWS and it was associated with obsessive eating behaviour (*30*). These brain function studies show that hypothalamic control disorders, dysfunction in food reward-related circuit areas and impairments in inhibitory control areas contribute to hyperphagia and extreme obesity in PWS.

Low levels of Orexin A could be seen in subjects aged 5 to 11 years, a phase where hyperphagia has already started. The function of orexin is to stimulate appetite and increase food intake (*31*). These findings are consistent with the association of orexin with serious neurological dysfunction involving food-addictive behaviour (*32,33*). The dopamine-rich ventral tegmental area (VTA) and the nucleus accumbens (NA) function as behavioural regulators, behaviour driven by food reward and addiction. Both structures are heavily innervated by orexin neurons and express high levels of orexin receptors (*34*). Excessive orexin stimulation in the hypothalamus, as well as the VTA and NA contribute to hyperphagia by increasing the reward value of food in patients with PWS. Thus, the insatiable appetite and unusual eating-related problems exhibited by PWS patients indicate abnormalities in the orexin system. The findings are summarized in Table 1 and tabulated in Figure 1 according to nutritional phase and age in PWS.

4. Potential treatment to reduce hyperphagia

Several pharmaceutical companies are developing drugs to target the mechanisms (Table 2). Among the potential treatments to correct ghrelin abnormalities are AZP-531 and RM-853. Two primary forms of ghrelin are found in circulation, acylated ghrelin (AG) and unacylated ghrelin (UAG). Studies show that the ratio of AG to UAG is relevant to hyperphagia in PWS (*35*). A hypothesis has been made that when UAG levels are too low, it will cause a higher AG/UAG ratio, which then results and leads to the development of hyperphagia and obesity (*36*). Therefore, an approach to treat hyperphagia and obesity in PWS is *via* pharmacological alteration of the AG/UAG ratio, through an increase in UAG concentration. AZP-531, an amino acid peptide, is a stable UAG

Table 1. Mapping of evidence regarding the potential mechanisms of hyperphagia

Abnormality	Subject's age	Level of abnormality	Details	*Ref.*
Thyroid hormone	1 week–3 years	Decreased	—Function: Regulate whole body metabolism. —In PWS: ↓ in PWS resulting in altered metabolic rate and energy expenditure.	(*17*)
Ghrelin	5 weeks–36 years old	Elevated	—Function: Regulates short-term food intake, ↑ in hunger, ↓ after food intake. —In PWS: Persistently ↑ ghrelin even after food intake leads to weight gain. ↑ body fat.	(*18*)
Leptin	7 months–5 years old	Elevated	—Function: Help regulate the long-term balance between the body's food intake and energy use.	(*1*)
Insulin	Children (median age 11.35 years old)	Decreased	—Function: Stimulate POMC and inhibit NPY neurons leading to stimulation of MC4R to induce satiety —In PWS: ↓ PWS leads to MC4R not being stimulated.	(*26*)
Peptide YY	19–42 years old	Decreased	—Function: Induce satiety by stimulating POMC and inhibiting NPY resulting in activation of α and β-MSH and reducing gastric emptying. —In PWS: ↓ PYY in PWS causes loss of stimulating signal to POMC, fails to stimulate α and β-MSH.	(*27*)
Brain-Derived Neurotrophic Factor (BDNF) and leptin	30 adults	BDNF: Decreased Leptin: Elevated	—Function BDNF: act as a satiety signal. —Function Leptin: helps regulate the long-term balance between the body's food intake and energy use. —In PWS: BDNF signalling is compromised, ↓ and local BDNF levels, ↑ leptin, resulting in leptin resistance, leading to hyperphagia and obesity.	(*24*)
Ghrelin and Glucagon-like peptide-1	Adults, median age 27.5 years old	Ghrelin: Elevated GLP-1: Decreased	—Function ghrelin: Regulates short-term food intake, ↑ in hunger, ↓ after food intake. —Function GLP-1: is an appetite suppressor hormone. Elevated satiety signal concentrations could be a compensatory response to higher ghrelin levels. —In PWS: Persistently ↑ ghrelin even after food intake leads to weight gain. ↓ in GLP-1 secretion, resulting increase in gastric emptying rates.	(*23*)
Altered brain structure	Children, median age 7.2 years old	Cortical volume: Decrease White matter integrity: Reduced fractional anisotropy (FA) Grey matter volume: Decrease	—Cortisol volume = decreased cortical volume in the bilateral frontal, medial prefrontal cortex and anterior cingulate lead to dysfunctions in regulations of appetite, increased self-reported hunger and increased risk of overeating through an imbalance between cognitive and emotional processing. —White matter integrity and Gray matter volume: ↓ FA indicates ↓ white matter health. Grey and white matter damage were present in the brain regions associated with food intake in PWS.	(*30*)
Orexin A	5–11 years old	Elevated	—Function Orexin A: stimulates appetite and increases food consumption. —In PWS: Overstimulation of orexin signalling in the hypothalamus contribute to hyperphagia by increasing the food addiction.	(*31*)

analogue (*36*). AZP-531 (livoletide) treatment reduced waist circumference and fat mass, but no significant changes were detected in weight. Besides, no serious side effects were observed during the study, indicating that AZP-531 is well tolerated in PWS individuals (*37*). In addition, RM-853 [Ghrelin O-acyltransferase (GOAT)] is an enzyme that catalyzes the octanoylation of ghrelin (*38*). Inhibition of GOAT will inhibit the production of AG and block orexigenic and adipogenic effects. This restriction will also increase UAG levels. Ghrelin signalling through GOAT inhibition provides a potential therapeutic opportunity to treat hyperphagia and obesity (*39*).

Potential treatments to correct PYY and insulin abnormalities are topiramate, diazoxide and setmelanotide. Topiramate reduced the mRNA for NPY. As NPY stimulates food intake, increases motivation to eat and delays satiety, reducing its level could reduce food intake and increase metabolic rate (*40*). The next approach is *via* Diazoxide, a K^+-ATP channel agonist approved by the FDA for treating hypoglycemia, hyperinsulinemia and acute hypertension. Diazoxide has a therapeutic effect on PWS through insulin secretion from pancreatic β-cells, modulation of hypothalamic NPY, and activation of KATP channels in adipocytes (*41*). This study showed that oral diazoxide administration for 12 weeks reduced fat mass, lowered blood glucose, and increased endurance capacity. Although the effects of diazoxide on PWS-related hyperphagia are not yet well understood, current evidence suggests that diazoxide

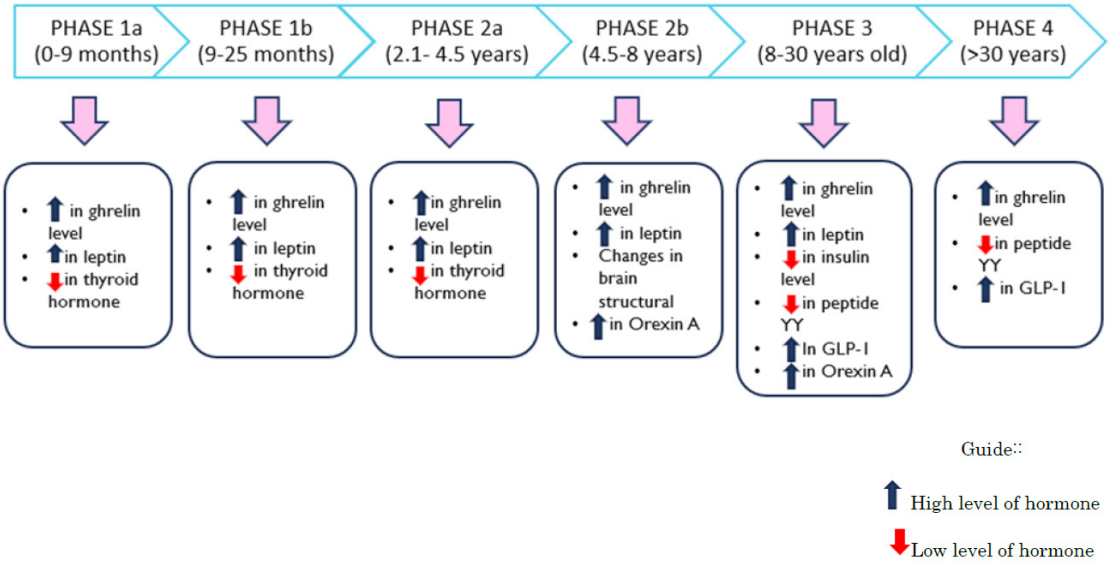

Figure 1. Summary of the hormonal and physiological changes involved in the development of hyperphagia from infants to adults in Prader-Willi syndrome. The changes are tabulated according to nutritional phase and age in Prader Willi syndrome.

Table 2. Mapping of evidence on potential treatments to reduce hyperphagia

Treatment	Mechanism of action	Advantages	*Ref.*
AZP-531 (Livoletide)	Decreases the appetite-stimulating effects of ghrelin.	Potential to address PWS-specific increase in ghrelin.	(*37*)
RM-853 (ghrelin o-acyltransferase (GOAT) inhibitor)	Inhibitor of an enzyme that catalyzes ghrelin octanolycation, thus resulting in reduced production of ghrelin.	Possible to modify food intake and prevent weight gain.	(*39*)
Topiramate	In the hypothalamus, topiramate increased mRNA for neuropeptide Y.	Possible reduced food intake acutely and increased metabolic rate. There were also significant reduction in leptin.	(*40*)
Diazoxide	K^+ ATP channel agonist that may exert therapeutic effects through the down-regulation of insulin secretion, modulation of hypothalamic neuropeptide Y concentrations, increased GABAnergic neuronal excitability, and activation of KATP channels in adipocytes.	FDA-approved drug for the treatment of hyperinsulinemia and hypoglycemia. Potential to treat hyperphagia.	(*41*)
Carbetocin	Carbetocin is an oxytocin analogue that has the exact mechanism and outcomes as oxytocin.	Have a longer half-life than oxytocin.	(*44*)
Beloranib	inhibits an enzyme methionine aminopeptidase 2 (MetAP2) that reduces hunger while stimulating the use of stored fat as an energy source	FDA-approved drug for the treatment of hyperphagia and weight loss.	(*45*)
Setmelanotide	Activates MC4R and this causes the inhibition of food intake.	Possibility to address an underlying defect in hunger circuits.	(*46*)
Oxytocin	Binds to G protein-coupled receptor and this leads to activation of several systems that regulate appetite.	Potential to replace the insufficiency of oxytocin in patients with PWS and may have positive effects on hyperphagia.	(*47*)
Tesofensine	serotonin-noradrenaline-dopamine reuptake inhibitor acts primarily as an appetite suppressant with related effects on fat oxidation and resting energy expenditure.	Potential treatment to reduce appetite, decrease food craving.	(*48*)
JD5037 (antiobesity drug candidate)	Restricted cannabinoid-1 receptor (CB1R) an antagonist that targets the overstimulated endocannabinoid system in PWS to reduce appetite.	Potential to treat obesity-related metabolic disorders without producing adverse nervous system effects.	(*49*)
CBDA-O-methyl ester (EPM301)	Enhance serotonergic 5-HT1A receptor activation following agonist binding.	Weight loss, increased ambulation, and improved glycemic and lipid profiles.	(*50*)

deserves further research attention. PWS individuals may also be responsive to the therapeutic activation of MC4R, which provides a rationale for treating obesity with setmelanotide. Setmelanotide is a potent and selective MC4R agonist for treating genetic disorders of obesity. It binds with high affinity to human MC4R, resulting in efficient MC4R activation that may potentially reduce hyperphagia associated with PWS (*42*).

Another study finds that targeting oxytocin, a hormone produced in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus, could better regulate food intake and satiety (*43*). Thus, the oxytocin analogue, carbetocin also has been introduced intranasally to PWS and shows positive effects because it could improve hyperphagia (*44*). The following approach is *via* appetite suppressant. Tesofensine, a serotonin-noradrenaline-dopamine monoamine reuptake inhibitor was co-administered with metoprolol (to reduce blood pressure) and showed that it could inhibit hunger feeling and food cravings by boosting the three neurotransmitters' activity. Meanwhile, Beloranib inhibits an enzyme methionine aminopeptidase 2 (MetAP2) that reduces hunger and induces weight loss (*45*). While JD5037, a cannabinoid-1 receptor blocker reacts on CB receptors function in inhibiting food intake and increasing satiety.

5. Conclusion

Based on this review, the mechanism of development of hyperphagia and obesity could be seen from hormonal changes (ghrelin, leptin, insulin, thyroid, PYY), Orexin A alteration and changes in brain structure. Several drugs have been shown to potentially ameliorate abnormalities of hormones, body composition and eating behaviour. However, these treatments need to be studied further because most still have no record of long-term safety and effectiveness in PWS individuals.

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*Address correspondence to:
Nurul Farhana Jufri, Biomedical Science Program, Center for Toxicology and Health Risk Studies, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, Kuala Lumpur 50300, Malaysia.
E-mail: nurulfarhana@ukm.edu.my

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Supplemental Data

Supplemental Table S1. List of search strings

No	Search String
Search string 1	Prader-Willi Syndrome OR Prader Willi Syndrome OR "Prader Willi Syndrome" OR Willi-Prader Syndrome OR PWS
Search string 2	Hyperphagia OR Binge-eating disorder OR "Binge eating" OR "Excessive eating" OR Polyphagia
Search string 3	Obesity OR Obese OR Overweight OR "Weight gain"
Search string 4	Treatment OR Management OR Intervention

Mini-Review

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Uncommon, overlooked and underreported causes of upper gastrointestinal bleeding

Sachin Mahendrakumar Chaudhary[1], Ajay Singh[2,*], Manisha Chavan[3], Arghadip Das[4], Pinank Kiritkumar Bathvar[1]

[1] Department of General Surgery, SMT. NHL Municipal Medical College, Ahmedabad, Gujarat, India;
[2] Department of General Surgery, Sri Ram MurtiSmarak Institute of Medical Sciences, Bareilly, Uttar Pradesh, India;
[3] Department of General Surgery, Kakatiya Medical College, RangamPeta, Warangal, Telangana, India;
[4] Department of General Surgery, Nilratan Sircar Medical College and Hospital, Kolkata, West Bengal, India.

SUMMARY Upper gastrointestinal bleeding (UGB) is a potentially fatal consequence of digestive disorders. There is a wide range of rare causes for UGB that can lead to misdiagnosis and occasionally catastrophic outcomes. The lifestyles of those who are afflicted are mostly responsible for the underlying conditions that result in the hemorrhagic cases. The development of a novel approach targeted at raising public awareness of the issue and educating the public about it could significantly contribute to the elimination of gastrointestinal bleeding with no associated risks and to a nearly zero mortality rate. There are reports of UGB related to Sarcina ventriculi, gastric amyloidosis, jejunal lipoma, gastric schwannoma, hemobilia, esophageal varices, esophageal necrosis, aortoenteric fistula, homosuccus pancreaticus, and gastric trichbezoar in the literature. The common feature of these rare causes of UGB is that the diagnosis is difficult to establish before surgery. Fortunately, UGB with a clear lesion in the stomach itself is a clear sign for surgical intervention, and the diagnosis can only be verified by pathological examination with the help of immunohistochemical detection of a particular antigen for a specific condition. The clinical traits, diagnostic techniques, and the therapeutic, or surgical options of unusual causes of UGB reported in the literature are compiled in this review.

Keywords upper gastrointestinal bleeding, uncommon cause, diagnosis, treatment

1. Introduction

Upper gastrointestinal bleeding (UGB) is a potentially fatal complication of gastrointestinal illnesses. Even though bleeding is a common symptom, the various reasons vary greatly. Bleeding is caused by several conditions and organ abnormalities in addition to the gastrointestinal tract itself. Therefore, for clinicians to properly diagnose and treat the disease, understanding the origin and characteristics is crucial (*1*). Peptic ulcers continue to be the most frequent cause of UGB, accounting for around half of all cases (*2-3*). UGB can also be brought on by esophageal varices, gastritis, gastric cancer, benign digestive tract tumors, use of blood thinners, or use of non-steroidal anti-inflammatory drugs (*2-10*) (Table 1). Despite advancements in medicine, endoscopy, intensive care units, and surgical management, the death rate of patients with UGB is 5-10% and has not improved much since 1945 (*11*).

Numerous case reports of uncommon causes of UGB have been published, which has expanded our knowledge of the etiology of the condition (*12*). However, UGB from rare causes can also contribute to misdiagnosis (*13*) and can have life threatening consequences. In this succinct review, we attempt to encapsulate uncommon causes of UGB (Table 2). This will increase our understanding of the causes of UGB, amplifying our ability to diagnose patients correctly and enhance the effectiveness of treatment through timely and more aggressive intervention.

2. Uncommon, overlooked, and underreported causes of UGB

2.1. Sarcina ventriculi

A rare gram-positive anaerobic bacteria, Sarcina ventriculi, does well in the stomach's acidic environment. Patients with delayed stomach emptying or gastric outlet obstruction frequently have the bacteria present

Table 1. Common causes of upper gastrointestinal bleeding

Cause / *Ref.*	Pathophysiology	History and clinical findings	Treatment
Peptic ulcer (*2-3*)	Commonly due to Helicobacter pylori infection or irritation from nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin or ibuprofen.	Many people with ulcers experience no symptoms. If symptoms do occur, they may include pain, often in the upper abdomen, nausea or vomiting, feeling of fullness or bloating.	Anti- H. pylori therapy and proton pump inhibitors (PPIs)
Mallory-Weiss tear (*5*)	Mucosal tears of the esophagus or fundus. The rip in the mucosa at the less flexible gastroesophageal junction is caused by a sudden rise in intra-gastric or intra-abdominal pressure.	Episode of hematemesis following a bout of retching or vomiting. Less common presenting symptoms include melena, hematochezia, syncope, and abdominal pain. The presence of hiatal hernia is a predisposing factor. Excessive alcohol use have been reported in 40-75% of patients.	5-30% patients require intervention, mostly endoscopic. Patients without risk factors for rebleeding or active bleeding at endoscopy can be managed conservatively.
Gastritis (*6*)	Inflammation of the stomach due to the use of NSAIDs, injury, inflammatory bowel disease (IBD). Overtime, gastritis can cause ulcers or damage parts of the stomach lining, leading to bleeding.	Majority of people with gastritis do not have any symptoms, but it can cause pain in the upper abdomen, feeling of fulness or bloating.	PPIs are administered to suppress stomach acid production.
Esophageal varices (*7*)	Portal hypertension from fibrotic liver parenchyma and dilation of collaterals.	Most common in people with liver disease, such as cirrhosis. Alcoholism and ascites may also be causes. Do not usually have symptoms unless the veins begin to bleed. The symptoms include stomach pain, vomiting blood, and bloody stools.	Beta blockers, nasogastric intubation, rubber band ligation, sclerotherapy, transjugular intrahepatic portosystemic shunt, balloon tamponade, and therapeutic endoscopy.
Arteriovenous malformations (*8*)	Congenital vascular malformations that are predisposed to rupture.	Painless bleeding in older patients (> 70 years), history of iron deficiency anemia.	Surgical resection of the involved bowel with complete resection of the nidus.
Dieulafoy's lesion (*9*)	Submucosal caliberpersistent artery anomaly mostly occurring in the upper part of the stomach, rarely in the antrum or the duodenum.	The bleeding stops temporarily with hypovolemic shock but recurs while resuscitation is achieved.	Endoscopic injection, cautery, ligation, embolization, and surgery.
Esophageal malignancy (*10*)	Bleeding from vasculature	Multiple previous episodes of bleeding, recent unintentional weight loss, history of alcohol or tobacco abuse.	Coagulation therapy with APC (argon-plasma coagulation) is used as a first choice. It enables the coagulation of superficially large areas of the bleeding tumor as well as the additional ablation of some of the intraluminal tumor mass.

(*14*). Gastric ulcers, emphysematous gastritis, gastric perforation, gastric adenocarcinoma, and pancreatic cancer have all been linked to the bacterium. It is quite uncommon for hematemesis to be the first sign of S. ventriculi infection in an otherwise asymptomatic patient. Epigastric stomach discomfort or spasms, abdominal distension, nausea, and later emesis are symptoms of S. ventriculi infection (*15*). Endoscopy demonstrates diffuse erythema, esophageal and stomach inflammation, a gastric ulcer, and food and bile retention. Endoscopic examination reveals a distinct, well defined border between normal and aberrant mucosae, a highly uncommon characteristic that suggests this illness. However, given the association with severe and life-threatening consequences, a multidrug treatment approach with metronidazole, ciprofloxacin, sucralfate, and pantoprazole has proven effective. Nonetheless, there currently needs to be a consensus regarding the best therapeutic agents or duration of treatments. Concomitant conditions and patient age are taken into account when adjusting the dose.

2.2. Splenic artery pseudoaneurysm

Though uncommon, UGB caused by a splenic artery pseudoaneurysm (SAPA) can potentially be fatal (*16*). Only a little more than 200 instances of SAPA have been reported so far (*2*). Pancreatitis, trauma, iatrogenic, post-operative reasons, and peptic ulcer are rare causes of SAPA. It is quite uncommon to have a SAPA eroded by a duodenal ulcer. The pancreatic duct is a typical location for bleeding, with some spilling into the peritoneal cavity, stomach, or even the colon (*17,18*). However, the rarity of SAPA fistulizing to the duodenal bulb makes precise diagnosis challenging. Massive hematemesis with an acute start and momentary loss of consciousness are the symptoms, but there is no acid reflux, stomach discomfort, or distension. Imaging methods, particularly computer tomography (CT) angiography and three-dimensional rendering, are helpful in this case for

Table 2. Uncommon, overlooked, and underreported causes of upper gastrointestinal bleeding (UGIB)

Cause / *Ref.*	Clinical Findings	Diagnostic Technique	Treatment	Caution
Sarcina ventriculae (gram positive anaerobic bacteria) (*14,15*)	Delayed stomach emptying or gastric outlet obstruction, gastric ulcers, emphysematous gastritis, gastric perforation, gastric adenocarcinoma, and pancreatic cancer are all possible causes. Hematemesis is a somewhat infrequent precursor to S. ventriculi infection in a patient who is otherwise asymptomatic. The symptoms include nausea, abdominal distension, epigastric pain or spasms, and eventually emesis.	Endoscopic examination reveals a clear, well-defined line between normal and aberrant mucosae, a fairly unusual feature that points to this condition.	Metronidazole, ciprofloxacin, sucralfate, and pantoprazole are part of a successful multidrug therapy regimen.	The dose is modified based on the patient's age and any coexisting conditions.
Splenic artery pseudoaneurysm (SAPA) (*17,18*)	Rare causes of SAPA include gastric ulcer, pancreatitis, trauma, iatrogenic, and post-operative reasons. The erosion of a SAPA by a duodenal ulcer is incredibly rare. The symptoms include severe hematemesis with an abrupt onset and brief loss of consciousness, although there is no acid reflux, stomach discomfort, or distension.	CT angiography to examine the bleeding veins. In the arterial phase of contrast-enhanced computed tomography (CT), the hematoma between the duodenal bulb and pancreas can be seen in both cross-sectional and coronal views.	Endovascular therapy or surgical intervention are recommended treatments for bleeding pseudoaneurysms.	Due to the atypical patient history and endoscopic results, both pharmacological and endoscopic techniques to diagnosis and hemostasis are disregarded.
Gastric amyloidosis (*20,21*)	Diarrhea, nausea, vomiting, weight loss, low hemoglobin, widespread abdominal pain, multiple episodes of melena, and GI bleeding.	In addition to bluish-black lesions and inflammatory lesions in the antrum with adherent blood clots, upper GI endoscopy also reveals blood clots in the fundus and body of the stomach.	Patients can be managed with the help of supportive care, such as intravenous proton pump inhibitors, intravenous steroids, fluid resuscitation, blood transfusions, *etc.*	The effectiveness of the treatment is dependent on the patient's age and comorbidities.
Jejunal lipoma (*22,23*)	The small intestine is where lipomas generally develop alone. When they are severe, UGIB, intussusception, and occlusion are rare. Persistent GI bleeding could be caused by a massive lipoma. The bleeding is caused by direct pressure from the lipoma or mucosal ulcers caused by mass expansion and regular peristalsis.	The video capsule endoscopy (VCE) can screen the whole small intestine and identify bleeding that has occurred in the proximal, middle, or distal third of the small intestine based on the length of the transit time of the capsule.	Surgical resection of the lipomas.	It can be difficult to identify the bleeding site by angiography or radionucleotide scan due to the intermittent or low rate of bleeding.
Gastric schwannoma (*24,25*)	Individuals between the ages of 30 and 50 are usually affected by gastric schwannomas, which are typically single lesions that arise from the lower curvature of the stomach. On histology, they are visible as prominent peripheral lymphocyte aggregates, either with or without a germinal core. Spindle cells constitute the majority of them, while epithelioid and unusual plexiform variations have also been reported.	Typically diagnosed through endosonography.	Surgical resection is the treatment of choice.	The muscularis propria of the stomach is where schwannomas most usually present as a nodular mass. As a result, they occasionally get misdiagnosed as neurofibromas and gastrointestinal stromal tumors.
Hemobilia due to hepatobiliary manipulation (*26,27*)	Although stomach ache, jaundice, and tarry stools are the usual symptoms, patients may also exhibit rectorrhagia and hypotension.	Computed tomography angiography.	Endovascular embolization is the primary treatment. Vascular or bile duct stent implantation is another option.	Always be on the lookout for patients who have UGIB, a history of biliary duct instrumentation or manipulation, or a previous diagnosis of biliopancreatic disease.

Table 2. Uncommon, overlooked, and underreported causes of upper gastrointestinal bleeding (UGIB) (continued)

Cause / *Ref.*	Clinical Findings	Diagnostic Technique	Treatment	Caution
Hemobilia due to choledocholiothiasis (*28,29*)	Among other things, iatrogenic, traumatic, vascular disease, neoplasms, inflammation, and gallstones can cause hemobilia. Usually, a large stone will erode the cystic artery or invade an adjacent artery or organ. There is a chance that it will cause severe hemobilia, which must be addressed severely.	The suggested initial diagnostic procedure is esophagogastroduodenoscopy (EGD), as it can find blood or clots at the ampulla of Vater in instances of hemobilia. The hepatic artery selective arteriography is the most conclusive test.	Endoscopic retrograde cholangiopancreatography (ERCP) or in some cases a laparoscopic cholecystectomy with bile duct exploration	During a preoperative radiological examination, large stones could be misconstrued for cancerous lesions. Therefore, surgeons should use conventional diagnostic algorithms and keep open surgery in mind early on rather than solely relying on endoscopic methods.
Inadvertant esophageal intubation (*30,31*)	Overdistension during endoscopy for the placement of a percutaneous endoscopic gastronomy tube has been documented to result in rips in the stomach mucosa in situations of malnutrition, old age, and gastric atrophy. When upper gastrointestinal bleeding occurs in severely ill patients who have unintentionally had esophageal intubation or cardiopulmonary resuscitation, this complication should be included in the differential diagnosis.	Capnography	Laparotomy	Abdominal imaging should be done to exclude stomach perforation in UGB subjects with a history of inadvertent esophageal intubation before upper endoscopy.
"Downhill" esophageal varices (*32,33*)	Blood from dilated veins brought on by superior vena cava blockage enters the azygous vein or inferior vena cava. They may be in the upper esophagus or the entire esophagus, depending on the severity of the obstruction above or below the azygous venous system, respectively.	Upper endoscopy provides for direct visualization of proximal varices and, if necessary, enables for intervention. Several imaging techniques, including a CT scan, can be used to determine the underlying reason of obstruction.	The main line of treatment concentrates on the underlying issue. The variceal band ligation procedure considerably reduces bleeding. Using a Sengstaken-Blakemore tube can potentially save lives in cases of uncontrolled bleeding	There needs to be a customised treatment plan. The underlying cause is the main focus of treatment.
Acute esophageal necrosis (*34,35*)	esophagus develops a distinct demarcation at the gastroesophageal junction and totally necrotizes along its circumference. The patients are typically elderly and critically ill, with common comorbidities like atherosclerotic cardiovascular disease, diabetes mellitus, hypertension, chronic renal insufficiency, and malnutrition. Patients often have hematemesis and melena in addition to upper stomach pain and systemic hypotension.	Endoscopy confirms distinct and conspicuous mucosal abnormalities.	Red blood cell transfusions, intensive fluid resuscitation, nothing by mouth (NPO orders), IV proton pump inhibitors, and other treatments are used.	Due to the serious underlying conditions that are present in most patients, acute esophageal necrosis can have a poor prognosis.
Aortoenteric fistula (AEFs) (*36*)	AEFs should be suspected in patients who have undergone aortic reconstruction surgery. Secondary AEFs often develop where a vascular graft's suture line meets the intestine, whereas primary AEFs are hypothesised to happen where the native aorta and GI tract interface. The graft's frequent pulsations against the duodenum wall could cause it to become ischemic, which would cause it to disintegrate and bleed. Sepsis that is concurrent is typically seen in secondary AEFs.	EGD and CT scans are the most useful tests to detect aortoenteric fistula.	Clot removal, graft revascularization, duodenorrhaphy, and omentoplasty should all be performed in conjunction with a laparotomy.	Based on the patient's medical history and a thorough physical examination, a proper diagnosis necessitates a high index of clinical suspicion.

Table 2. Uncommon, overlooked, and underreported causes of upper gastrointestinal bleeding (UGIB) (continued)

Cause / *Ref.*	Clinical Findings	Diagnostic Technique	Treatment	Caution
Homosuccus pancreaticus (HP) (*38,39*)	Clinical indicators include epigastric discomfort with sporadic, mild gastrointestinal bleeding. A blood clot develops as a result of pressure on the pancreatic duct, which causes pain. Following its expulsion into the gastrointestinal lumen, this blood clot causes sporadic episodes of melena and, less frequently, hematemesis.	Contrast-enhanced CT with angiography is a remarkable diagnostic technique for detecting structural pancreatic defects, and it typically yields an accurate diagnosis.	Interventional therapies, which have a high rate of success and a low mortality, are the first line of treatment for patients who are hemodynamically stable. Only patients with hemodynamic instability who have failed a prior, less invasive therapy undergo surgery.	HP may not always be found during EGD because to its anatomical position and sporadic bleeding.
Gastric Trichobezoar (*40,41*)	The clinical signs include asthenia, anaemia, melena, hematemesis with abdominal pain, and weight loss. Gastric bezoars can result in blockage, perforation, bleeding, and ulceration of the stomach.	Best diagnostic tool to detect trichobezoar is endoscopy.	The suggested course of treatment is minimally invasive procedures like laparoscopic extraction or endoscopic fragmentation and removal of trichomes. However, surgical extraction is the preferred treatment.	Endoscopic removal of all the debris necessitates multiple sessions and carries a risk of pressure sores and esophageal perforation.

surveying the bleeding veins. The hematoma between the duodenal bulb and pancreas is visible in the arterial phase of contrast-enhanced CT in both cross-sectional and coronal images. Both pharmacological and endoscopic approaches to diagnosis and hemostasis are excluded due to the atypical patient history and endoscopic findings. To treat the bleeding pseudoaneurysm, either endovascular therapy or surgical intervention are suggested.

2.3. Gastric amyloidosis

Another rare cause of UGB is gastric amyloidosis. The likelihood of gastrointestinal involvement in amyloidosis varies depending on the type, and it seems less common in AL amyloidosis (amyloid light chain or primary amyloidosis) (*19*). In a retrospective assessment of 769 patients with systemic amyloidosis, 1% of patients had clinically evident gastrointestinal (GI) amyloidosis (*20*). Presentations included diarrhea, nausea, vomiting, weight loss, and GI bleeding. It is uncommon for gastric amyloidosis to cause severe and deadly GI hemorrhage. The patient might also have low hemoglobin, widespread abdominal pain, and multiple episodes of melena. In upper GI endoscopy, blood clots are found in the fundus and body of the stomach, as well as bluish-black lesions and inflammatory lesions in the antrum with adhering blood clots. It is conceivable that multiple myeloma is the etiology of AL amyloidosis (amyloid light chain or primary amyloidosis) in these patients based on the number of biopsies they have undergone and their increased serum beta 2 microglobulin levels (*21*). Patients can be managed with supportive therapy such as intravenous proton pump inhibitors, intravenous steroids, fluid resuscitation, blood transfusions, *etc.* The patient's age and comorbidities determine the efficacy of the treatment.

2.4. Jejunal lipoma

The benign submucosal tumors known as jejunal lipomas are uncommon and frequently discovered by accident. The large intestine is the most common site, where they appear in 65-75% of cases, followed by the stomach. The majority of instances manifest in the sixth and seventh decades of life, with a slight female preponderance. Acute GI bleeding or intestinal obstruction may occasionally occur, however, their clinical behavior is typically quiet (*22*). In most cases, repeated gastroscopy and colonoscopy show no substantial abnormalities. Lipomas typically occur alone in the small intestine. UGB, intussusception, and occlusion are uncommon when they are significant. An enormous lipoma may result in persistent GI bleeding. The ulceration of the mucosa brought on by mass enlargement and regular peristalsis, or direct pressure from the lipoma, results in the bleeding. Jejunal lipomas have been documented in roughly 1% of cases (*23*). Due to the intermittent or

low rate of bleeding, identifying the bleeding source by angiography or radionucleotide scan can be challenging. Based on the capsule's transit duration, video capsule endoscopy (VCE) can screen the whole small intestine and pinpoint the bleeding caused to the proximal, middle, or distal third of the small intestines. When ambulatory investigation fails due to the issue of compliance, VCE might be used as an initial modality for evaluating cryptic GI bleeding in a hospital setting. Surgical resection of the lipomas remain the treatment of choice.

2.5. Gastric schwannoma

These uncommon gastrointestinal mesenchymal tumors, also known as schwannomas, neurinomas, and neurilemomas, most frequently manifest themselves in the stomach as a nodular mass in the muscularis propria (*24*). Because of this, they are occasionally mistakenly identified as neurofibromas and gastrointestinal stromal tumours. Gastric schwannomas are typically single lesions that develop from the stomach's lower curvature and frequently affect people between the ages of 30 and 50. They appear as conspicuous peripheral lymphocytic aggregates with or without a germinal center on histology. Although epithelioid and uncommon plexiform variants have also been reported, spindle cells make up the majority of them. They have positive immunohistochemical staining for vimentin and s-100 protein but negative staining for CD117, C-kit, and smooth muscle actin. Because they are benign tumors, gastrointestinal schwannomas can be surgically removed and have a decent prognosis (*25*). Gastric schwannomas are typically diagnosed with GI endoscopy, such as endosonography. Only a few examples of gastric malignant schwannomas have been documented in the literature due to their exceptional rarity. Further research is needed on the effectiveness of surgical resection and the postoperative prognosis.

2.6. Hemobilia due to hepatobiliary manipulation

Hemobilia accounts for less than 5% of instances of UGB (*26*). Any patient with GI bleeding who has recently undergone hepatobiliary surgery is at risk for hemobilia, which has an iatrogenic origin in the majority. Depending on the level of the bleeding, its clinical presentation varies. Patients may also present with rectorrhagia and hypotension, although the typical symptoms are stomach discomfort, jaundice, and tarry stools. In a majority of cases, these symptoms resolve on their own, without the need for further treatment. Patients with UGB, a history of biliary duct instrumentation or manipulation, or a previous diagnosis of biliopancreatic illness should always be suspects (*27*). The gold standard for diagnosing of hemophilia is an angiography; however, developments in computed tomography angiography have made this procedure less invasive and more accessible. Although vascular or bile duct stent implantation are other options, endovascular embolization is the primary treatment for these patients. The procedure is generally well tolerated and successful, with minimal rates of death and morbidity. Surgery is an infrequent choice for the treatment of hemobilia.

2.7. Hemobilia due to choledocholiothiasis

Hemobilia can be brought on by iatrogenic, traumatic, vascular illness, neoplasms, inflammation, and gallstones, among other things. In 25% of instances of cholelithiasis and 35% of cases of choledocholithiasis, microscopic bleeding occurs (*28*). Less than 1% of all cases of hemobilia that have been recorded involve macroscopic hemobilia. It typically occurs when a sizable stone enters an adjacent arterial or organ or erodes the cystic artery. It can occasionally result in profuse hemobilia, which needs to be taken seriously. Stones in common bile duct (CBD) can grow to substantial sizes without showing any other severe signs besides jaundice. Giant stones may be mistaken for malignant lesions during preoperative radiological examination. Therefore, instead of just depending on endoscopic procedures, surgeons should use traditional algorithms for diagnosis and keep open surgery in mind early on. Hepatolithiasis was frequently present in cases with sizeable common duct stones, which have been documented in multiple instances (*29*). Chronic obstruction of the pancreatobiliary tract can potentially cause hemobilia by causing inflammation, erosion, and fistulization with nearby vascular structures. For stones smaller than 2.5 cm, mechanical lithotripsy is used. Esophagogastroduodenoscopy (EGD) is the preferred initial diagnostic procedure for individuals with UGB because it can detect blood or clots at the ampulla of Vater in cases of hemobilia. The most confirming test is selective arteriography of the hepatic artery.

2.8. Inadvertant esophageal intubation

In cases of malnutrition, advanced age, and gastric atrophy, overdistension during endoscopy for the installation of a percutaneous endoscopic gastronomy tube has been reported to cause tears in the stomach mucosa (*30*). Patients receiving resuscitation and unintentional esophageal intubation have experienced stomach perforation due to an overdistension. Rapid air collection causes mucosal rips and, ultimately, iatrogenic gastric rupture in certain situations. The defect is often seen in the smaller curvature of the stomach (the area with the least elastance) during laparotomy or autopsy (*31*). Rapid gastric distension may change the angle of the antrum and result in the right hemidiaphragm compressing the cardia. These modifications enable air passage through the gastroesophageal junction at the pylorus. Before doing upper endoscopy in cases of UGB where a history of unintentional esophageal intubation is

documented, abdominal imaging should be used to rule out stomach perforation.

2.9. "Downhill" esophageal varices

The azygous vein or the inferior vena cava receives blood from dilated veins caused by superior vena cava blockage. Depending on the degree of obstruction above or below the azygous venous system, respectively, they are either in the upper esophagus or may involve the entire esophagus (*32*). Only 0.1% of esophageal variceal bleeding is caused by "downhill" varices (*33*). Lack of coagulopathy and submucosal and higher-located varices in the esophagus, away from erosive gastroesophageal reflux, may contribute to a lower risk of bleeding. There are no firm recommendations on how to identify and treat "Downhill" varices. An individualized treatment strategy is required. The primary course of treatment focuses on the underlying cause. Although the location of the banding is not well defined, variceal band ligation is efficient for reducing bleeding. The weakening of the proximal esophagus posterior wall and the general lack of serosa appear to increase the risk of bleeding or perforation. In the event of uncontrolled bleeding, using a Sengstaken-Blakemore tube may be lifesaving. Successful management can be achieved through awareness, quick diagnosis, and case-by-case care using available endoscopic, radiographic, and surgical techniques.

2.10. Acute esophageal necrosis

Acute esophageal necrosis, often known as "black esophagus", is a disorder that causes the esophagus to become completely necrotic across its circumference, with varied proximal extension and visible demarcation at the gastroesophageal junction (*34*). It is a rare cause of UGB that can be identified on endoscopy by distinct and conspicuous mucosal abnormalities. Less than 0.5% of occurrence is suggested, indicating an unusual clinical appearance. With common comorbidities such as atherosclerotic cardiovascular disease, diabetes mellitus, hypertension, chronic renal insufficiency, and malnutrition, the patients are typically elderly and severely ill. In addition to upper stomach pain and systemic hypotension, patients typically present with hematemesis and melena. Histopathologically, it shows severe mucosal necrosis along with ulceration and hemosiderin deposits. Necrosis may involve the muscularis propria in deeper layers, and vascular thrombosis may indicate ischemia damage. It helps to rule out iron pill damage and esophageal melanocytosis, respectively, when particular staining with Perl's Prussian blue and Fontana-Masson is negative (*35*). A biopsy is not typically required until clinically needed in atypical presentations like cytomegalovirus (CMV) or herpes, as the endoscopic findings support the diagnosis.

2.11. Aortoenteric fistula

Patients having a history of aortic reconstruction procedures should be on the lookout for aortoenteric fistulas (AEFs), a rare but sometimes fatal cause of UGB. Primary AEFs are thought to occur where the native aorta and GI tract communicate, whereas secondary AEFs typically develop where a vascular graft's suture line meets the intestine (*36*). The duodenal wall could become ischemic from the graft's repeated pulsations against it, which would then erode and bleed. AEFs seldom arise following aortic reconstruction; and incidence ranges from 1% to 4%. In secondary AEFs, concomitant sepsis is frequently observed. Any section of the gastrointestinal canal may be affected, but the duodenum's third portion accounts for 80% of cases, followed by its fourth portion, the jejunum and the ileum. Due to thrombus development, the first bleeding is frequently brief and self-limiting. Periods of bleeding can last for hours, days, or even weeks, building to a significant hemorrhage and hypovolemic shock in the end. Back discomfort or fever may be the primary symptoms in some persons. A correct diagnosis requires a high index of clinical suspicion based on the patient's medical history and a careful physical examination (*37*). To rule out alternative sources of upper GI bleeding in patients who have herald bleeding, EGD is typically performed initially. When a diagnosis is suspected, a laparotomy should be done along with clot removal, graft revascularization, duodenorrhaphy, and omentoplasty.

2.12. Homosuccus pancreaticus

The rare and potentially fatal condition known as Homosuccus pancreaticus (HP), also known as pseudohemobilia or wirsungorrhagia, causes UGI hemorrhage. It is primarily caused by bleeding from a pseudoaneurysm that enters the second part of the duodenum through the pancreatic duct. One case of HP is thought to occur out of every 1,500 (*38*). Less than 1.1% of instances of UGB are caused by rupture of pseudoaneurysm, a relatively uncommon but potentially fatal consequence of chronic pancreatitis that occurs in 6–8% of patients with pseudocysts (*39*). The average age of presentation is between 50 and 60 years old. Given its anatomical location and intermittent bleeding, HP may not always be detected during EGD. Clinical signs include epigastric pain linked to minor gastrointestinal bleeding that is progressive and intermittent. The pain is caused by pressure on the pancreatic duct, which leads to the secondary production of a blood clot. This blood clot is subsequently expelled into the gastrointestinal lumen, resulting in primarily intermittent episodes of melena, and less frequently, hematemesis. Laboratory tests reveal general findings such as iron deficiency anemia. Hyperbilirubinemia is also mentioned in relation to pancreaticobiliary reflux. The average number of days

between the onset of symptoms and HP diagnosis is more than 30. An excellent diagnostic method for identifying structural pancreatic anomalies is contrast-enhanced computed tomography with angiography, which in most instances, provides an accurate diagnosis. In patients who are hemodynamically stable, interventional therapies are the first line of treatment, with a high rate of success and decreased mortality. Surgery is only performed on hemodynamically unstable patients in whom a prior, less invasive therapy failed or was impractical, despite a death rate of 28 to 56% noted in these circumstances.

2.13. Gastric Trichobezoar

A Trichobezoar is a buildup of non-absorbable human hair in the stomach. It defines the Rapunzel syndrome when it spreads *via* the pylorus to the duodenum, jejunum, and colon (*1*). Less than 1% of the general population are affected by this unusual illness. 90% of Trichobezoar instances are seen in young women with long hair between the ages of 13 and 20, who also have Trichotillomania (the compulsive pulling out of hair) and Trichophagia (compulsion to swallow hair). The signs include asthenia, anemia, melena, hematemesis with abdominal pain, and weight loss. Gastric bezoars can result in blockage, perforation, bleeding, and ulceration of the stomach (*40*). Currently, minimally invasive treatments like laparoscopic extraction or endoscopic fragmentation and removal of trichomes are the recommended options for treatment. A risk of pressure ulcers and esophageal perforation comes with endoscopic removal of all the fragments, which calls for repeated sessions. So, the preferred treatment is still thought to be surgical extraction. In order to address comorbid illnesses that are frequently linked to the disease and prevent relapses, psychiatric counselling is also essential (*41*).

3. Conclusion

UGIB is a frequent emergency scenario that requires an immediate and precise diagnosis. Even while peptic ulcers and variceal bleeding are the most frequent causes of UGB and account for more than 80% of cases, there are still a wide range of uncommon, less frequently reported causes that also contribute to UGB with digestive tract hemorrhage. The mortality rate could be higher than 75% and some rare causes can be the reason for rapid and massive hemorrhage. That means that not all cases respond effectively to conservative treatment, and in some cases, immediate surgical intervention is the only effective treatment. It is also evident that not all bleeding origins can be found *via* endoscopy. Therefore, a more accurate diagnosis can be made by combining endoscopy with additional imaging detection methods including ultrasonography, CT scanning, and angiography.

For diagnosing rare situations, there are still some hints. The doctor's attention and emphasis should extend beyond just the digestive system. Since even rare diseases have distinctive traits of their own, a comprehensive physical examination and in-depth history inquiry help the doctor make the right diagnosis.

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*Address correspondence to:
Ajay Singh, Department of General Surgery, Sri Ram MurtiSmarak Institute of Medical Sciences, Bareilly, Uttar Pradesh, India.
E-mail: asinngh593510@gmail.com

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Review

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Treat to target and tight control: Could be a new approach in the treatment of sarcoidosis?

Senol Kobak*

Istinye University Faculty of Medicine, Liv Hospital, Department of Internal Medicine and Rheumatology, WASOG Sarcoidosis Clinic, Istanbul,Turkey.

SUMMARY Sarcoidosis is a chronic granulomatous disease with multisystemic involvement. Although it is accepted as a benign disease, it can sometimes cause life-threatening organ (heart, brain) involvement that determines the prognosis of the disease. There are conflicting opinions about the treatment of the disease. In the generally accepted treatment approach the "step-by-step" model has gained weight. According to this approach, corticosteroids (CS) drugs alone are preferred in the first step in patients who require treatment. In the second step, immunosuppressive drugs (IS) are used in patients who do not respond to CS and/or have contraindications to CS use, and biologics (TNF-alpha inhibitors) are used in the third step. This treatment approach may be valid in cases with mild sarcoidosis. However, although sarcoidosis is considered a benign and self-limiting disease in some major organ involvement, the "step-by-step" approach may be a treatment option that puts the patient's life in danger. In such selected patients, much more rigorous, early and combined treatment approaches that definitely include CS, IS or biologic drugs may be required. In selected sarcoidosis patients with high risk, early diagnosis, "treat-to-target" (T2T) and "tight control" follow-up of patients seems to be a rational approach. This article reviews the "step-down" treatment regimens in light of recent literature data and hypothesizes that the T2T model may be a probable new treatment approach in patients with sarcoidosis.

Keywords sarcoidosis, treat-to-target, tight control, remission, new approach

1. Introduction

Sarcoidosis is a chronic multisystem inflammatory disease characterized by granuloma formation. Although the pathogenesis of the disease is not clear, it is thought to develop as a result of immune system dysregulation triggered by an unknown antigen in people with a genetic background (*1*). Sarcoidosis is a heterogeneous disease that may be asymptomatic or may present with different clinical presentations (*2*). It can often cause lung, eye, skin and musculoskeletal system involvement. It can also present with different life-threatening organ involvement, which is rare but determines the prognosis of the disease (*3*).

The diagnosis is made in the presence of non-caseating granulomas in histopathology, exclusion of other possible causes, and existence of clinical and radiologic findings supporting sarcoidosis (*4,5*). The treatment of the disease is mainly based on case series and retrospective uncontrolled studies, rather than multicenter controlled prospective studies (*6*). This brings with it different and complex views in the literature. When the diagnosis of sarcoidosis is made, it must first be decided whether the patient needs any treatment or just follow-up. In cases with sarcoidosis requiring treatment all organs involved with sarcoidosis should be detected before starting treatment (*7*). Therefore, all system and organ scans may be performed and as an imaging method, positron emission computed tomography (PET-CT) may be preferred. This method shows the extent of the disease, the correct determination of the biopsy site to be taken, and disease activation (*8*). PET-CT imaging may help to determine asymptomatic bone involvement as well to change the stage of the disease and the associated treatment decision. In rheumatology practice, early diagnosis and treatment, and more frequent patient follow-up strategies have opened a new era in the treatment of these patients (*9*). This model provides better remission rates, less organ involvement, less radiological progression and destructive disease, and higher quality of life, especially in patients with rheumatoid arthritis (RA) (*10*). We hypothesize that a

similar approach in patients with severe sarcoidosis may affect disease control, organ damage, quality of life and mortality.

2. General treatment approaches in sarcoidosis

The treatment of sarcoidosis is as still complex and controversial as its diagnosis (*11*). The main reason for this is that treatment strategies are usually based on case series and expert opinion rather than randomized-controlled studies. While discussing the treatment of sarcoidosis, the involvement of organs affected by the disease and the presentation of the disease (acute/chronic), as well as gender, age, race, and presence of comorbid disease should be considered (*12*). Sarcoidosis is a disease that regresses spontaneously and mostly does not require treatment. While spontaneous remission is common in patients with stage 1 and 2 sarcoidosis (between 55–90%), spontaneous regression is rare (10–20%) in stage 3 (*13*). Acute sarcoidosis cases have a better prognosis compared to chronic patients, often do not require treatment and spontaneous recovery is usually seen. However, chronic disease and/or recurrent cases should be treated (*14*).

While the "wait and see" approach seems reasonable in asymptomatic and mild cases, different treatment approaches may be required in high-risk patients who must be treated (*15*) (Table 1). According to the European Respiratory Society (ERS) protocols, "step-by-step" strategies are recommended for the treatment of sarcoidosis (*16*). Corticosteroids (CS) drugs are the first step of treatment (*17*). In addition to their anti-inflammatory and immunomodulatory effects, they have been shown to correct impaired Th1/Th2 cytokine dysbalance in patients with sarcoidosis (*18*). It was observed that CS treatment in pulmonary sarcoidosis caused significant improvement in radiological improvement and respiratory functions (*19*). In another study, patients with spontaneous remission were compared with patients going into remission with CS use , and it was shown that relapses were more common in patients using CS (*20*). In the presence of eye involvement, neurosarcoidosis, cardiac and renal sarcoidosis, chronic locomotor system involvement (muscle, bone, joints) and hypercalcemia, CS indication is available (*21*).

CS drug indications arise in severe skin involvement and the presence of constitutional symptoms such as fever, fatigue, and weakness (*22*). While it has been accepted that CS are the first choice of treatment, the confusion of drug dosage and duration of treatment still continues. Immunosuppressive (IS) drugs are used in second-line treatment of sarcoidosis (*23*). Various IS drugs such as methotrexate (MTX), azathioprine (AZA), and hydroxychloroquine (HQ) are use especially in CS-resistant patients or in cases where steroids are not preferred due to side effects (*24-26*). In the third step of treatment, biological drugs such as TNF-alpha inhibitors (TNFi), rituximab (RTX), and tocilizumab (TCZ) are preferred (*27,28*). The most experience is available

Table 1. Low *vs.* high risk sarcoidosis patients according to demographic, radiologic, laboratory and clinical phenotype

Features	Low risk	High risk
Gender	male	female
Age	young	elderly
Ethnicity	Caucasian	Afro-American
Clinical phenotype	Löfgren's syndrome	chronic sarcoidosis
Pulmonary	acute alveolitis, normal PFT/DLCO	bronchial obstruction, pulmonary fibrosis, pulmonary hypertension, low PFT/DLCO
Neurosarcoidosis	facial nerve palsy, aseptic menengitis, isolated headache, vertigo	intracranial mass, spinal cord, optic neuritis, epilepsy
Eye	anterior uveitis, conjunctivitis, lacrimal gland swelling, pseudotumor	panuveitis, retinal vasculitis, orbital myozit, optic neuritis
Heart	pericarditis, supraventricular arrhythmia, intracardiac mass, valvulopathy	A-V blocks, ventricular tachicardia myocarditis, aneurisma, cardiomyopathy
Skin	erythema nodosum, skar sarcoidosis, makülopapüler lezyonlar hipo/hiperpigmentasyon	lupus pernio, alopesi
Musculoskeletal	arthralgia, acute arthritis, tendinitis	chronic arthritis, Jaccoud, dactilitys, sarcoid myopathy, bone lesions
Kidney	hypercalcemia, hypercalciuria	nephrocalcinosis, renal failure
Radiographic stage	stage 1/2	stage 3/4
Laboratory findings	low CRP, low TNF-A, HLA-DQB1*0201	high sIL-2R, hypercalcemia, hypercalciuria, high TNF-A, HLA-DQB1*1501

PFT: pulmonary function tests; A-V: atrio-ventricular; sIL-2R: soluble interleukine-2 receptor; TNF-A: tumor necrosis factor-alpha; DLCO: diffusion lung carbonmonoxide; CRP: C-reactive protein; HLA: human leukocyte antigen.

using TNFi (*29*). The studies have shown that TNFi is an effective option in different organ involvement (pulmonary, neurosarcoidosis, eye, skin, heart, muscle, joint, kidney, liver) (*30*). It should be noted that randomized controlled studies on the efficacy and safety of these drugs in patients with sarcoidosis are limited in the literature. The available data are mostly based on open studies, case reports and expert experience.

3. Principles of "treat to target and tight control" strategies: when and which patients

In RA patients, early diagnosis and treatment ensures early remission of the disease and prevents chronic complications (*31*). This treatment and follow-up model introduced about a decade ago has resulted in better remission rates, less organ involvement, less radiological progression and destructive disease, and a higher quality of life (*32*). The most important point of this model is early diagnosis, determining the treatment target and choosing the appropriate disease modifying anti-rheumatic drugs (DMARDs) to reach this target and initiating early treatment (*33*). The target in the treatment of RA was determined as complete remission and/or low disease activity, and this target was achieved with more frequent outpatient follow-up of the patient (*34*).

The diagnosis and treatment principles, disease activation scores, and remission criteria are clearly defined in RA, while these are not clear yet in sarcoidosis. The treatment in RA is determined in line with the results of randomized-controlled studies and meta-analysis. However these studies in sarcoidosis are insufficient, therefore conflict and debate continues in treatment principles. In order for this successful model in RA to be applied to patients with sarcoidosis, a new perspective on sarcoidosis is required. The paradigm that sarcoidosis is a "benign and mild" disease should be changed, and diagnostic criteria, disease activation scores, organ involvement, radiological imaging priority and remission criteria should be redefined. The concept of early diagnosis and early treatment in selected severe sarcoidosis should be supported by further studies to support the hypothesis that it can prevent complications of the disease and organ damage and improve quality of life. With the early diagnosis of sarcoidosis, organ involvement and complications may be prevented. As in rheumatic diseases lung involvement, if sarcoidosis lung involvement is detected and treated at an early stage, the development of fibrosis may be prevented. However, chest radiography recommended for diagnosis and still used in the Scadding classification is insufficient for the early diagnosis of pulmonary sarcoidosis (*35,36*). Contrast-enhanced thoracic CT seems to be essential for early diagnosis in pulmonary sarcoidosis (*37*). However the activation and extent of the disease is very important. Patients diagnosed as stage 1 pulmonary sarcoidosis or only skin sarcoidosis may also have other "hidden site" involvement such as heart and bone. Therefore, after the diagnosis of sarcoidosis is made, organ and system screening may be performed and "high risk" patients may be determined. Although it seems to be an expensive method, PET-CT may be an alternative method in terms of early diagnosis, disease extent, long-term disease follow-up, early initiation of TNFi and response to treatment (*38*).

After the early diagnosis and organ involvement are determined, the patients who should definitely receive treatment should be identified and treatment should be started as soon as possible (Table 2). Patients with sarcoidosis who have started treatment should be followed up more frequently ("tight control") , follow-up parameters (acute phase reactant, pulmonary function tests, *etc.*), drug efficacy and side-effect profile should be evaluated at each visit.

4. Rationale for early, aggressive and "step-down" treatment in sarcoidosis: some evidence

Sarcoidosis is a difficult to treat disease because "everybody has a different opinion" when treatment comes to the fore. The difficulty of treatment is not due

Table 2. Selected severe sarcoidosis patients which should be treated in accordance with "step-down" (combined drugs) strategy

Involvement	Diseases
Heart involvement	A-V blocks ventricular arrythmia myocarditis aneurisma cardiomyopathy
Neurosarcoidosis	intracranial mass spinal cord epilepsy parenchimal lessions
Lung involvement	bronchial obstruction acute active alveolitis ("ground-glass") pulmonary hypertension low PFT/DLCO progressive lung disease extended parenchimal infiltrations
Eye involvement	panuveitis retinal vasculitis orbital myozit optic neuritis unresponsiveness to local treatment
Kidney involvement	symptomatic hypercalcemia hypercalciuria acute renal failure
Musculoskeletal system involvement	chronic arthritis bone lessions dactylitis sarcoid myopathy
Chronic skin disease	lupus pernio
Progressive symptomatic extrapulmonary disease Sarcoidosis-associated fatique Small-fiber neuropathy	

PFT: pulmonary function tests; A-V: atrio-ventricular; DLCO: diffusion lung carbon monoxide.

to the difficulty of the disease, but to the fact that the treatment principles have not been clearly determined yet (*39*). The decision about "which patients" to treat depends on two main factors: the risk of death or organ failure, and the deterioration of quality of life (*40-42*). Compared with the general population, sarcoidosis causes an increase in mortality (*43*). The most common causes of death from sarcoidosis are lung and heart diseases (*44*). In addition to pulmonary and cardiac disease, neurological involvement and multiorgan sarcoidosis are most closely associated with poor outcomes (*45*). Features such as pulmonary hypertension, decreased lung function, and pulmonary fibrosis have been shown to increase the risk of death from pulmonary disease (*46*). Irreversible organ damage to the brain, eyes or kidneys due to sarcoidosis can also cause significant morbidity (*47*). Sarcoidosis-related fatigue and small fiber neuropathy are important findings that reduce the quality of life of patients (*48,49*). When evaluating all of these together, it is understood that severe multisystemic sarcoidosis is not a "benign" disease, but in some situation may be a fatal and damaging disease (*50*). It is a fact that has to develop new treatment approaches in order to prevent mortality and morbidity and improve the quality of life of patients (Table 3).

According to ERS recommendations, "step-by-step" treatment models are recommended in sarcoidosis (*16*). CS is the first step treatment option recommended to be started alone in all organ involvement. The second step is recommended to start IS drugs when unresponsive to CS treatment and/or CS-related side effects develop. It is known that the effects of DMARDs which are frequently used in rheumatology practice start late (average 1-3 months) (*51*). Considering the recurrent rates in sarcoidosis and the late onset of DMARDs, it would be more rational to start DMARDs and CS

Table 3. Summary of sarcoidosis treatment strategies

Sarcoidosis phenotypes	ERS/ATS/WASOG (Step-by-step strategy) 1. Fırst-line 2. Second line 3. Third line 4. Fourth line	Step-down strategy (combined drugs use) 1. Fırst-line 2. Second line 3. Third line 4. Fourth line
Pulmonary Sarcoidosis		
Low risk	1. CS	1. CS
Intermediate risk	1. CS 2. IS(MTX,AZA,LEF) 3. IFX, ADA 4. RTX, JAKi, RCI	1. CS 2. IS(MTX,AZA,LEF) 3. IFX, ADA 4. RTX, JAKi, RCI
High risk	1. CS 2. IS 3. IFX, ADA 4. RTX, JAKi, RCI	1. CS+IS(MTX,AZA,LEF) 2. IFX, ADA 3. RTX, JAKi, RCI - After sustained remission achieved step-down discontinue drugs
Skin	1. Topical/systemic CS 2. IS (MTX, AZA, HQ) 3. IFX,ADA 4. Apremilast, JAKi	1. Topical/systemic CS 2. IS (MTX, AZA, HQ) 3. IFX, ADA 4. Apremilast, JAKi - Combined drugs use in lupus pernio - After sustained remission achieved step-down discontinue drugs
Heart	1. CS+/-IS 2. IS(MTX, AZA,LEF) 3. IFX, ADA 4. CyA	1. CS+IS(MTX, CyA) +/-IFX 2. IFX, ADA - After sustained remission achieved step-down discontinue drugs
Neurosarcoidosis	1. CS 2. IS(MTX, AZA, MM,HQ 3. IFX, ADA	1. CS+IS (MTX, AZA, MM, HQ) 2. IFX, ADA - After sustained remission achieved step-down discontinue drugs
Sarcoidosis-associated fatigue	1. Exercize 2. Armodafinil/D-metylphenidate 3. Low-dose CS and/or MTX	1. Exercize + HQ(MTX)+low-dose CS 2. Sympthomatic drugs-Armodafinil/D-metylphenidate - After sustained remission achieved step-down discontinue drugs
Musculoskeletal system (chronic arthritis, myositis, bone)	1. CS 2. IS(MTX, AZA, MM,HQ 3. IFX, ADA	1. CS+IS(MTX, AZA, MM,HQ 2. IFX, ADA, JAKi - After sustained remission achieved step-down discontinue drugs
Eyes	1. CS eye drops 2. CS 3. IS(MTX, AZA, MM,HQ 4. IFX, ADA	1. CS eye drops 2. CS 3. IS(MTX, AZA, MM,HQ) 4. IFX, ADA - Combined drugs use(CS + IS +/- IFX) in severe form(retinal vasculitis/ panuveitis, optic neuritis, acute loss of vision) - After sustained remission achieved step-down discontinue drugs

ERS: European Respiratory Society; WASOG: World Association of Sarcoidosis and Other Granulomatous diseases; ATS: American Thoracic Society; MTX: methotrexate; CS: corticosteroid; AZA: azathyoprine; MM: mycophenolat mofetil; RTX: rituximab; HQ: hydroxychloroquine; IS: immunosuppresive drugs; IFX: infliximab; ADA: adalimumab; CyA: cyclophosphamide; JAKi: janus kinase inhibitor; LEF: leflunomide; RCI: repository corticotropine inhibitor.

drugs simultaneously, especially in cases with severe sarcoidosis.

CS, as with RA should be considered a "bridging therapy" (*52*). Early initiation of DMARDs may also prevent disease relapses. This approach may also protect against the side effects (osteoporosis, diabetes, hirsutism, cataract, hypertension, *etc*.) that develop due to chronic use of CS (*53*). There are few studies in the literature showing and supporting the early onset of DMARDs in sarcoidosis. Nagai *et al.* showed that MTX and CS combination therapy gave better results for left ventricular function than CS alone in patients with cardiac sarcoidosis (*54*). Arun *et al.* showed that early IS treatments with AZA, MTX and infliximab (IFX) could effectively improve clinical outcomes in patients with neurosarcoidosis (*55*). The treatment records of 3276 sarcoidosis patients registered in the RISE (Rheumatology Informatics System for Effectiveness) system were examined. Most patients (59.3%) received CS, while only 18.2% of patients received CS monotherapy. While MTX and HQ were the most commonly used coventional DMARDs, 12.1% of patients received one or more biological or targeted synthetic DMARDs, with the most common being TNFi (*56*). A randomized controlled trial comparing the efficacy and tolerability of prednisone and MTX as first-line therapy in pulmonary sarcoidosis (PREDMETH study) is ongoing (*clinicaltrials.gov*) (*57*). The study aims to show that MTX is as effective as prednisone as a first-line treatment for sarcoidosis, but with fewer side effects. Gavrysyuk *et al.* compared the efficacy, tolerability, and recurrent rates of MTX and methylprednisolone (MP) in 143 newly diagnosed patients with pulmonary sarcoidosis (*58*). They showed that MTX monotherapy was not significantly different from MP monotherapy in terms of efficacy and rate of serious adverse events. However, they reported a significant reduction in the incidence of treatment resistance and relapse rate in patients receiving MTX. Goljan-Geremek *et al.* showed that MTX as monotherapy in the treatment of chronic pulmonary sarcoidosis is as safe and effective as steroids and patients experience definite pulmonary function tests (PFT) improvement (*59*). Ballul *et al.* compare the efficacy of CS alone or associated with IS drugs for the prevention of relapse in cardiac sarcoidosis (*60*). The authors showed that the combination of CS with IS drugs might reduce the risk of cardiac relapse, as compared to CS alone. Cardiac sarcoidosis multi-center randomized controlled trial (CHASM CS-RCT) is an ongoing study evaluating the optimal initial treatment strategy for patients with active cardiac sarcoidosis (*61*). The authors hypothesize that a low dose prednisone/MTX combination will have non-inferior efficacy compared to standard dose prednisone and that these combinations may result in significantly better quality of life and few side effects compared to standard dose prednisone. The same approach applies to biologic drugs. Biological drugs such as TNFi are often reserved for severe cases of refractory sarcoidosis, as outlined in the ERS guidelines and take months sometimes years to get started (*62*). These drugs are generally used in treatment-resistant severe sarcoidosis cases, and there is not enough data in the literature on its use in early disease treatment. In one study, it was shown that there is less chance of restoration of intrinsic conduction and cardiac function if delays in effective treatment for cardiac sarcoidosis (*63*). Baughman *et al.* compared the treatment outcomes of IFX and MTX in pulmonary sarcoidosis and found that IFX was more likely to improve clinical status than MTX (*64*). Sohn *et al.* showed that the use of TNFi in patients with neurosarcoidosis resulted in earlier disease control and better prognosis (*65*). In one study, the high disease activity detected with 18-FDG-PET-CT was associated with the effectiveness of TNFi (*66*). In other words, in the presence of active disease detected by PET-CT, especially in cardiac involvement, early biological initiation may increase the treatment success. Simonini *et al.* showed superior efficacy of adalimumab (ADA) in the treatment of childhood resistant chronic uveitis (including sarcoid uveitis) when used as a first line drug (*67*). According to a Delphi consensus study on sarcoidosis treatment it revealed large variations in treatment strategies but recommended the use of IS drugs in disease and required prolonged treatment or as a steroid-sparing agent in patients with high risk of steroid toxicity (*68*). However, the Delphi study does not provide firm guidance on exactly when to initiate DMARDs (first-line as monotherapy, combination with CS, or after failed CS treatment). As the above-mentioned studies increase, there will undoubtedly be more researchers supporting early and targeted therapy models for sarcoidosis.

5. Conclusions

The treatment of sarcoidosis is still controversial, and the debate about this treatment is as old as the disease itself. In most patients with a mild course, the "step-by-step" approach recommended by the ERS guidelines should be preferred. In selected patients with severe organ involvement (heart, lung, neurosarcoidosis), it is obvious that early, aggressive and combined treatment will reduce the risk of morbidity and mortality. It may be possible to achieve early disease remission, decrease organ damage and relapses and improve quality of life in sarcoidosis with treat-to-target and tight control strategy. Although ERS recommendations are open to discussion and criticism, their strength is based on existing studies. Multicenter randomized controlled studies are needed to change these treatment recommendations and to identify new treatment strategies.

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**Address correspondence to:*
Senol Kobak, Istinye University Faculty of Medicine, Liv Hospital, Department of Internal Medicine and Rheumatology, WASOG Sarcoidosis Clinic, Canan sokak No:5 Istanbul, Turkey.
E-mail: senolkobak@yahoo.com

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Original Article

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A regionally adapted HRM-based technique to screen MMACHC carriers for methylmalonic acidemia with homocystinuria in Shandong Province, China

Haining Yang[1,2,3], Mian Li[1,2,3], Liang Zou[4], Hui Zou[5], Yan Zhao[1,2,3,*], Yazhou Cui[1,2,3,*], Jinxiang Han[1,2,3,*]

[1] Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;
[2] NHC Key Laboratory of Biotechnology Drugs (Shandong Academy of Medical Sciences), Ji'nan, Shandong, China;
[3] Key Lab for Rare & Uncommon Diseases of Shandong Province, Ji'nan, Shandong, China;
[4] Bone Biomechanics Engineering Laboratory of Shandong Province, Neck-Shoulder and Lumbocrural Pain Hospital of Shandong First Medical University, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan, China;
[5] Ji'nan Maternity and Child Care Hospital, Ji'nan 250000, Shandong, China.

SUMMARY Methylmalonic acidemia with homocystinuria (MMA-cblC) is an autosomal recessive genetic disorder of organic acid metabolism. Shandong, a northern province of China, has a significantly high incidence of about 1/4,000, suggesting a high carrying rate among the local population. The current study established a PCR technique involving high-resolution melting (HRM) to screen for carriers based on hotspot mutation analysis to further develop a preventive strategy to reduce the local incidence of this rare disease. Whole-exome sequencing of 22 families with MMA-cblC and a comprehensive literature review were used to identify MMACHC hotspot mutations in Shandong Province. Subsequently, a PCR-HRM assay based on the selected mutations was established and optimized for large-scale hotspot mutation screening. The accuracy and efficiency of the screening technique was validated using samples from 69 individuals with MMA-cblC and 1,000 healthy volunteers. Six hotspot mutations in the MMACHC gene (c.609G>A, c.658_660delAAG, c.80A>G, c.217C>T, c.567dupT and c.482G>A), which account for 74% of the alleles associated with MMA-cblC, were used to establish a screening technique. The established PCR-HRM assay detected 88 MMACHC mutation alleles in a validation study with 100% accuracy. In the general population in Shandong, the carrying rate of 6 MMACHC hotspot mutations was 3.4%. In conclusion, the 6 hotspots identified cover the majority of the MMACHC mutation spectrum, and the Shandong population has a particularly high carrying rate of MMACHC mutations. The PCR-HRM assay is highly accurate, cost-effective, and easy to use, making it an ideal choice for mass carrier screening.

Keywords carrier screening, methylmalonic aciduria, MMACHC, PCR-HRM, mutation

1. Introduction

Methylmalonic acidemia (MMA) is a severe autosomal recessive inborn error of organic acid metabolism. The estimated incidence of MMA ranges from 1/48,000 to 1/250,000 in different countries worldwide (*1*). Its incidence is particularly high, 1/4,000, in the population of Shandong, a northern province of China (*2*). MMA-cblC is MMA combined with homocystinuria caused by defects in cobalamin biosynthesis, and it is the most common form of MMA in China, accounting for approximately 70% of all MMA cases (*3-4*). Age of onset ranges from shortly after birth to late adulthood, with clinically heterogeneous manifestations that include vital organ damage, recurrent vomiting, mental retardation, and progressive developmental delay; some severe cases even lead to death (*5*).

However, two factors make MMA-cblC one of the few rare diseases with a better prognosis for most patients. First, due to the increasing prevalence of newborn screening based on tandem mass spectrometry (MS/MS), more infants are being diagnosed and treated early. Second, early supplementation with vitamin B12 and L-carnitine can have a significant therapeutic benefit, reversing some clinical phenotypes (*6*). However, a screening strategy to identify mutation carriers to

reduce prenatal incidence has not yet been explored. The apparent prevalence of MMA-cblC in Shandong Province suggests a significantly high carrying rate of MMA gene mutations in the general population. Therefore, the current study investigated hotspot mutations of the MMACHC gene and their prevalence in MMA-cblC, and it evaluated whether carrier screening is suitable for primary prevention of that condition in Shandong. This study also developed a cost-effective strategy that could be used in a large population.

2. Materials and Methods

2.1. Clinical samples

MMA-cblC was diagnosed according to previously reported criteria (*7-8*). Twenty-two patients diagnosed with MMA-cblC from January 2017 to January 2019 at the Jinan Maternity and Child Care Hospital (Shandong, China) and their parents were included in the spot mutation analysis using peripheral blood samples. Dry blood spots were obtained from 69 infants with MMA-cblC and one of the six hotspot mutations from January 2019 to January 2022 in Shandong, and those blood spots were used to develop and validate a PCR-HRM assay. Peripheral blood samples from 1,000 healthy volunteers in Shandong province were obtained between January 2019 to December 2021 as part of a study to screen for MMACHC mutation carriers. All the samples were collected with informed consent of the individual or his/her parent/guardian. This study was approved by the Institutional Ethics Committee of the Shandong Medicinal Biotechnology Centre

2.2. Whole exome sequencing and analysis

Genomic DNA was extracted from peripheral blood from 22 MMA-cblC pedigrees using the Vazyme Blood DNA Kit (Vazyme, China). A sequencing library was then constructed using the AmpliSeq™exome kit (Thermo Fisher Scientific, USA). Ion torrent S5 (Thermo Fisher Scientific, USA) was used to perform sequencing according to the manufacturer's instructions. FASTQ files were automatically generated from the sequenced original image data by base calling. Read pairs were concatenated and filtered to remove low-quality bases. Read alignments were performed against the hg19 reference genome. Variant calling and annotation was performed using the CLC Genomics Workbench (Qiagen, Germany) (*9*).

2.3. Establishment and optimization of the PCR-HRM assay

PCR-HRM primers were designed using the software Primer Express v3.0 in accordance with the reference sequence of the MMACHC gene (NG_013378.2) in NCBI. PCR primers specific for each hotspot mutation were designed to yield 100-150-bp products with a Tm between 58-60℃.

PCR-HRM was performed on a LightCycler 480 real-time PCR system (Roche Diagnostics, Germany). The total volume of the PCR reaction was 20 μL, including 10 μL of Roche Master Dye premix (2×), 1 μL of forward and 1 μL of reverse primers (4 μM) and 25 ng of the genomic DNA template. The reaction conditions were as follows: initial denaturation at 95℃ for 5 min, 45 cycles of denaturation at 95℃ for 10 s, annealing at 55℃ for 15 s, and extension at 72℃ for 10 s with fluorescence reading and single point acquisition mode. After amplification, melting curve analysis was performed in three steps: denaturation at 95℃ for 1 min, renaturation at 40℃ for 1 min, followed by continuous fluorescence reading mode at 65-95℃ with a rise rate of 0.02℃/s and data acquisition of 25 times/℃. Raw data were analyzed and normalized using the software supplied with the PCR machine. Negative controls were used as a reference curve to generate difference plots. The normalized melting curve and difference plots were then analyzed for mutation genotype.

2.4. Sanger sequencing

Sanger sequencing was used to validate the accuracy of HRM on an ABI-3730XL Genetic Analyser (Thermo Fisher Scientific, USA). The results were analyzed using the software Chromas.

3. Results

The flowchart for this study is shown in Figure 1. Hotspot mutations were first evaluated in 22 patients with MMA-cblC and their parents using whole exome sequencing. MMACHC mutations were found in both alleles in all patients (Table 1). Of them, 16 children were carriers of compound heterozygous variants and the remaining 6 children carried compound homozygous variants. In total, 9 known pathogenic variants in the MMACHC gene were identified. The most frequent mutation was c.609 G>A, which was detected in half of the patients (50.0%, 11/22). The second most common mutation, c.658_660delAAG, was detected in 10 (45.45%, 10/22). More than half of the pathogenic mutations were classified as nonsense and frameshift mutations.

The hotspot mutation list was then compared to two previous studies of the MMACHC mutation spectrum in Shandong Province (*1,10*). The top hotspot mutations in all three studies were c.609G>A, c.658_660delAAG, c.80A>G, c.217C>T, c.567dupT, and c.482G>A, representing 75.94% of MMACHC mutation sites. Therefore, these top hotspot mutations could be used as an ideal choice for massive carrier screening.

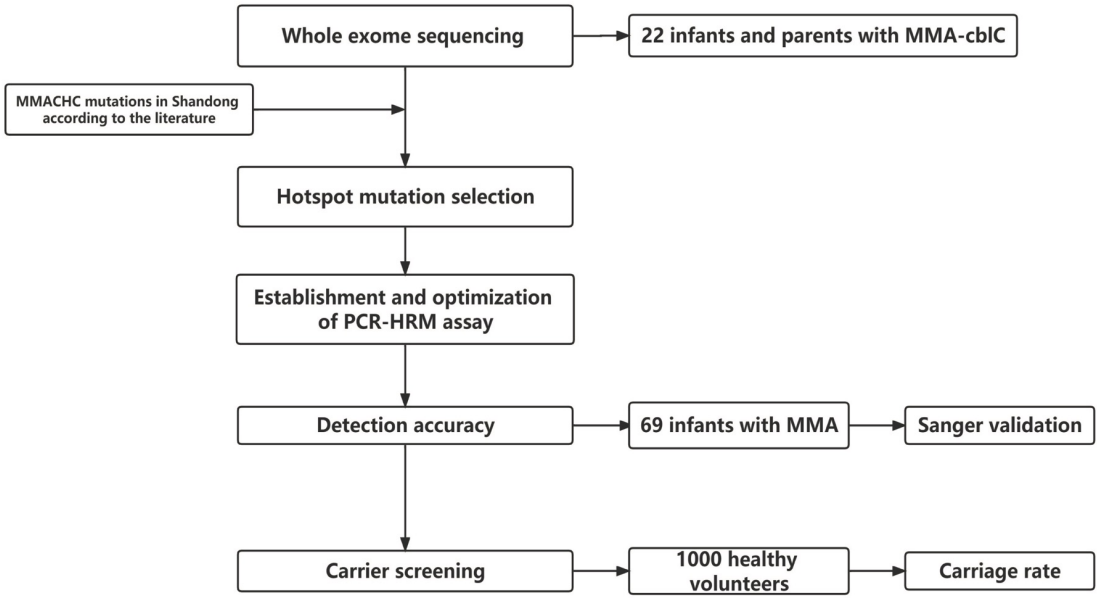

Figure 1. Flowchart for this study.

Table 1. Detailed information on the 22 MMA pedigrees for exome sequencing in this study

No.	Gene	Nucleotide changes	Amino acid changes	Mutation type	Source of variation	Homo./Het.
1	MMACHC	c.80A>G	p.Gln27Arg	Missense	Father	Het.
		c.609G>A	p.Trp203Ter	Nonsense	Mother	
2	MMACHC	c.658_660delAAG	p.Lys220del	Deletion	Parents	Homo.
3	MMACHC	c.217C>T	p.Arg73Ter	Nonsense	Father	Het.
		c.658-660delAAG	p.Lys220del	Deletion	Mother	
4	MMACHC	c.658-660delAAG	p.Lys220del	Deletion	Parents	Homo.
5	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Father	Het.
		c.622_627delTG	p.Val209fs	Frame shift	Mother	
6	MMACHC	c.445_446delTG	p.Cys149fs	Frame shift	Father	Het.
		c.615C>A	p.Tyr205Ter	Nonsense	Mother	
7	MMACHC	c.482G>A	p.Arg161Gln	Missense	Father	Het.
		c.567dupT	p.Ile190fs	Frame shift	Mother	
8	MMACHC	c.482G>A	p.Arg161Gln	Missense	Parents	Homo.
9	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Parents	Homo.
10	MMACHC	c.658_660delAAG	p.Lys220del	Deletion	Parents	Homo.
11	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Father	Homo.
12	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	-	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Mother	
13	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	-	Het.
		c.658_660delAAG	p.Lys220del	Deletion	-	
14	MMACHC	c.445_446delTG	p.Cys149fs	Frame shift	Father	Het.
		c.609G>A	p.Trp203Ter	Nonsense	Mother	
15	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Father	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Mother	
16	MMACHC	c.80A>G	p.Gln27Arg	Missense	Father	Het.
		c.609G>A	p.Trp203Ter	Nonsense	Mother	
17	MMACHC	c.217C>T	p.Arg73Ter	Nonsense	Mother	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Father	
18	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Mother	Het.
		c.626_627delTG	p.Val209fs	Frame shift	-	
19	MMACHC	c.445_446delTG	p.Cys149fs	Frame shift	Mother	Het.
		c.615C > A	p.Tyr205Ter	Nonsense	Father	
20	MMACHC	c.482G>A	p.Arg161Gln	Missense	Mother	Het.
		c.567dupT	p.Ile190fs	Frame shift	Father	
21	MMACHC	c.609G>A	p.Trp203Ter	Nonsense	Mother	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Father	
22	MMACHC	c.481C>T	p.Arg161Ter	Nonsense	Mother	Het.
		c.658_660delAAG	p.Lys220del	Deletion	Father	

Specific primers for the six hotspot mutations were then designed (Table 2), and a rapid PCR-HRM technique was established to detect mutations in 6 cases of MMA-cblC (Table 3). As shown in Figure 2, the melting peak and melting curve of each hotspot mutation site differed significantly different from the others, and those of the mutant and wild genotypes of each site were clearly distinct. The established PCR-HRM assay is fast, completed in half an hour, and data are easily analyzed using the software provided with the real-time PCR system.

To evaluate the accuracy of PCR-HRM techniques, PCR-HRM assays were performed on samples from 69 infants confirmed to have MMA-cblC and at least one of the six hotspot mutations. A total of 88 alleles of six selected MMACHC hotspot mutations were found. The PCR-HRM results were in 100% agreement with the Sanger sequencing of the complete exons (Table 4).

Finally, pilot screening for carriers of MMACHC hotspot mutations was performed on 1,000 healthy volunteers, and 34 MMACHC hotspot mutations were identified (Table 5). Therefore, results revealed a carrying rate of 3.4% for MMACHC in the Shandong population.

Table 3. PCR-HRM primers for *MMACHC* hotspot mutation sites

Mutation site	Name	Sequence (5′-3′)
c.80A>G	MMACHC-1F	CAGAGCTGAAGCAGAAGATCGA
	MMACHC-1R	CCCTAGAACAGCAGGAGGGATA
c.217C>T	MMACHC-2F	CACGCCTGCCATGTTTGAC
	MMACHC-2R	CCAGATGGTAGGCCACACACT
c.567dupT	MMACHC-3F	CCAGGGATAGAGGTGCCAGAT
	MMACHC-3R	TCACGCCAGTGGAAATTGAAG
c.482G>A	MMACHC-4F	CCAGCGCATATCAGGTGTGT
	MMACHC-4R	ATCCCTGGCAGCAGCACTAC
c.658_660	MMACHC-5F	CCTACTCGAAGGCTTCAATTTCC
delAAG	MMACHC-5R	CCGGGGAGGGAGAACTAGG
c.609G>A	MMACHC-6F	AGCTGACCGTATCGCCCTACT
	MMACHC-6R	AGTAGGCCTTCTGCTCTTCTGAGT

Table 2. Statistics on pathogenic *MMACHC* mutation sites according to the results of exosome sequencing and a literature review

Mutation site	This study ($N = 44$)		Li Y [10] ($N = 128$)		Wang F [1] ($N = 94$)	
	Allele number	Allele frequency	Allele number	Allele frequency	Allele number	Allele frequency
c.609G>A	13	29.55%	43	33.59%	52	55.43%
c.658_660delAAG	13	29.55%	13	10.16%	12	13.04%
c.567dupT	2	4.55%	6	4.69%	2	2.17%
c.80A>G	2	4.55%	15	11.72%	1	1.09%
c.217C>T	2	4.55%	1	0.78%	1	1.09%
c.482G>A	4	9.10%	19	14.84%	1	1.09%
Total	36	81.85%	97	75.99%	69	73.91%

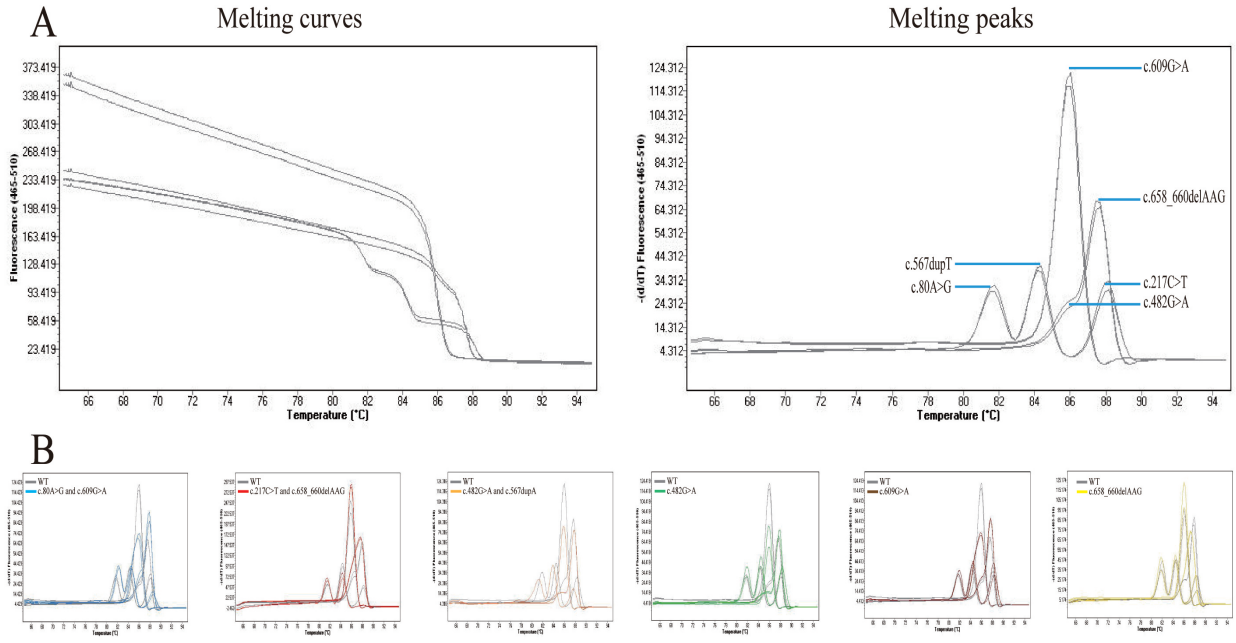

Figure 2. Performance of the established PCR-HRM technique in detecting mutations of the *MMACHC* gene. (A) The melting curves and peaks of hotspot mutation sites. **(B)** The melting peaks of six hotspot mutations in patients with MMA-cblC.

Table 4. Analysis of *MMACHC* mutation sites in 69 infants with MMA

Mutation site	Number (%)	Allele frequency (%)
c.80A>G	12	13.64
c.217C>T	1	1.14
c.567dupT	10	11.36
c.482G>A	11	12.50
c.658_660delAAG	19	21.59
c.609G>A	35	39.77
Total alleles	88	100

Table 5. Analysis of *MMACHC* mutation sites in 1000 healthy volunteers

Mutation site	Number	Carriage rate (%)	Allele frequency (%)
c.80A>G	3	0.3	8.82
c.217C>T	3	0.3	8.82
c.567dupT	1	0.1	2.94
c.482G>A	5	0.5	14.7
c.658_660delAAG	10	1	29.41
c.609G>A	12	1.2	35.29
Total alleles	34	3.4	100

4. Discussion

In autosomal recessive or X-linked recessive inheritance, all carriers have compound heterozygous pathogenic variants but do not exhibit any phenotypic characteristics. The aim of carrier screening is to identify asymptomatic individuals carrying the causative variant at an early stage so that appropriate fertility counselling can be provided. Tay-Sachs disease was the first genetic disease for which population-based carrier screening was implemented, and research has provided clear evidence that such efforts can dramatically reduce its incidence (*11-14*).

The causative gene for MMA-cblC was first identified as MMACHC by Lerner-Ellis *et al*. in 2006. According to the Human Gene Mutation Database (HGMD), 127 MMACHC mutation sites have been reported (*15*). MMACHC mutations vary by country and region. For example, c271dupA accounts for about 50% of MMACHC mutations in European populations, while c.331C>T is common in French-Canadian mixed-race populations and c.394C>T in Middle Eastern populations (*16,17*). In 2010, Liu *et al*. analyzed mutations of the MMACHC gene in children with MMA-cblC in China (*18*). The high incidence of the c.80A>G, C.609G>A and 658_660delAAG mutations, which account for more than 50% of the MMA alleles, may be related to the early founder effect of the Chinese population. The c.217C>T and c.482G>A mutations are also common in groups of Chinese patients with MMA-cblC, which may be related to the CpG island in the human genome (*19*). Although Shandong has a significantly higher incidence of MMA compared to other regions, the current analysis revealed that the spectrum of MMACHC mutations in Shandong is consistent with previous findings in the Chinese population. In light of the assay's substantial coverage of hot spot mutations, using a PCR-based screening technique with low throughput and a low cost is feasible.

This study found an MMACHC carriage rate of 34 per 1,000 in the Shandong population, similar to the finding in an earlier study (210/6800) (*10*). Compared to existing carrier screening techniques, the PCR-HRM technique established here is suitable for large-scale screening of pathogenic MMACHC gene carriers to reduce the incidence of MMA-cblC in Shandong Province because of its high efficiency, accuracy, low cost, short time, and limited workload in analyzing results. However, widespread promotion of this strategy for carrier screening still faces many challenges, including public awareness and education, informed consent, interpretation of results, and genetic counselling.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Jinxiang Han, Yazhou Cui, and Yan Zhao, Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences; NHC Key Laboratory of Biotechnology Drugs (Shandong Academy of Medical Sciences); Key Lab for Rare & Uncommon Diseases of Shandong Province, 6699 Qingdao Road, Ji'nan 250117, Shandong, China.
E-mail: jxhan9888@aliyun.com (JH); yzcui@sdfmu.edu.cn (YC); zhaoyan@sdfmu.edu.cn (YZ)

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Original Article

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Burden of illness seen in hereditary angioedema in Japanese patients: Results from a patient reported outcome survey

Beverley Yamamoto[1,2,3], Daisuke Honda[2,4,*], Isao Ohsawa[2,5], Kazumasa Iwamoto[2,6], Takahiko Horiuchi[7], Atsushi Fukunaga[8], Junichi Maehara[9], Kouhei Yamashita[10], Michihiro Hide[2,11,12]

[1] Graduate School of Human Sciences, Osaka University, Osaka, Japan;
[2] HAEJ registered NPO, Japan;
[3] HAEi Registered Charity, USA;
[4] Department of Nephrology, Chiba University Hospital, Chiba, Japan;
[5] Department of Nephrology, Internal Medicine, Saiyu Soka Hospital, Saitama, Japan;
[6] Iwamoto Dermatology & Allergology Clinic, Hiroshima, Japan;
[7] Department of Internal Medicine, Kyusyu University Beppu Hospital, Oita, Japan;
[8] Department of Dermatology, Osaka Medical and Pharmaceutical University Hospital, Osaka, Japan;
[9] Department of Acute Care & General Medicine, Saiseikai Kumamoto Hospital, Kumamoto, Japan;
[10] Department of Hematology, Kyoto University Hospital, Kyoto, Japan;
[11] Department of Dermatology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan;
[12] Department of Dermatology, Hiroshima Citizens Hospital, Hiroshima, Japan.

SUMMARY Hereditary angioedema (HAE) is a potentially life-threatening rare disease, which is mainly caused by the deficiency or dysfunction of C1-esterase inhibitor, and characterized by spontaneous, recurrent episodes of edema in various parts of the body including internal organs and the laryngeal area. Delayed diagnosis and treatment increase the burdens and risks of this condition. The current study aimed to understand the burden of illness for HAE patients in Japan before and after diagnosis through a patient reported outcome survey. A survey instrument was distributed to 121 adult patients with HAE by a patient organization via HAE treating physicians between July and November in 2016. Seventy patients (57.9%) returned the questionnaire. Patients reported high levels of medical resource utilization, including emergency procedures and services. Episodes of receiving laparotomy were somewhat less after diagnosis with HAE than before, but no apparent difference in episodes of tracheotomy between before and after the diagnosis. The economic burden, including direct and indirect medical costs, was highest before diagnosis, but still perceived as substantial after diagnosis. Patients reported disruption of work and school life, with 40% reporting that they miss 10 or more days from work or education per year. Sixty percent of patients reported that HAE affected their daily activities. We concluded that HAE is associated with considerable physical, social, economic and psycho-social burdens even after diagnosis, and that higher attack frequency is associated with a heavy disease burden for patients in Japan.

Keywords hereditary angioedema, burden of illness, Japan, patient reported outcomes, quality of life

1. Introduction

Hereditary angioedema (HAE) is a potentially life-threatening rare disease that is characterized by spontaneous and recurrent episodes of edema in various parts of the body, including internal organs and the laryngeal area (*1,2*). The frequency and severity of these episodes varies not only between patients, but also across a single patient's life course (*3*). Attacks are unpredictable, frequently painful and disfiguring, and may cause significant temporary disability (*4,5*). Symptoms are self-limiting, generally lasting between 1-4 days, but family history may include asphyxiation as a result of an untreated laryngeal attack (*3-6*).

HAE symptoms are the result of increased activity of plasma kallikrein which triggers a cascade leading to excessive bradykinin production. The background to this for the majority of patients is mainly the deficiency (Type I) or dysfunction (Type II) of C1-esterase inhibitor (C1-INH) (*1,2*). However, since the early 2000s a third type

of HAE has been described in the literature, HAE with normal C1-INH. Research into diagnostic biomarkers is ongoing, but this rare form of HAE is poorly understood. C1-INH function is normal but swelling is considered to be the result of excessive bradykinin production and/or function (*7-9*).

The prevalence of HAE has been estimated at 1 case per 50,000 people, with no reported bias among different ethnic groups (*3,4*). Based on population size, there would be an estimated 2,300-3,000 Japanese HAE patients, but unofficial data collated from case reports in Japan and from several pharmaceutical company databases suggests that the number of diagnosed HAE cases in Japan is between 500-550, meaning around 20% of the expected population with HAE.

In 2020, we reported on the results of the first patient reported outcome (PRO) questionnaire survey of Japanese patients covering a wide number of clinical topics, including the pathway to diagnosis, number of attacks and treatment, time from an attack to the treatment and then resolution, and satisfaction with treatment (*10*). This is the companion paper of reference 10, reporting the burden of illness for HAE patients in Japan both before and after diagnosis in the treatment environment at the time of the data collected, and patient perceptions of what they would like to see to increase their quality of life. Major categories of concern are medical resource utilization, physical, mental and socio-economic burdens on patients, as well as work, education and leisure impairments and restrictions to daily living. At the time that we conducted this PRO study, only one human plasma derived C1-INH concentrate was available as an acute attack treatment for HAE and this had to be administered in a hospital. Some patients were using tranexamic acid and attenuated androgens aiming for prophylactic treatment, but no medications for this designated by an international guideline for HAE had been approved in Japan (*10*). Our data-set offers an important baseline against which future studies can investigate the impact of a diversification of treatment options. At the same time, it offers a historic picture of the disease burden before self-administrated acute attack treatment and/or prophylactic treatment options were available.

2. Materials and Methods

In this study, we collected data between July and November 2016 from adult patients who had been diagnosed with HAE, as reported by Iwamoto, *et al*. in 2020 (*10*). Through discussion with physicians and patients involved in HAE practice, a questionnaire was developed with the goal of of understanding the burden and unmet needs of patients, and obtaining knowledge that would be useful in clinical practice. A total of 48 questions were generated through discussion to collect the necessary information while minimizing the burden on patients.

The survey was distributed to 121 patients between July and November in 2016 by a Japanese HAE patient organization (*https://haej.org*) via HAE treating physicians and analyzed in 2017. Patients without a confirmed diagnosis of HAE or patients under 20 years of age were excluded from the study. Patients completed the questionnaire and returned it to Anterio Co., Ltd., an organization specializing in pharmaceutical market research *(https://www.intage-healthcare.co.jp*). The data from the returned surveys were compiled by Anterio Co., Ltd.

After removing invalid responses from each questionnaire, the data were statistically analyzed using GraphPad Prism8 (GraphPad Software, San Diego, CA). Statistical significance between the period before and after diagnosis was calculated by the Wilcoxon matched-pairs signed rank test.

The protocol for this study was approved by the Ethics Committee of Hiroshima University (No. E_339) and was conducted on the basis of the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

3. Results

3.1. Demographics of respondents by gender

Of the 70 respondents, 78.6% of respondents were female (15 males, 55 females). The average age was 44.9 ± 18.8 years (mean ± SD, min-max, 8–84 years).

3.2. Segmentation by frequency of attacks

As attack frequency has been shown to be linked to the burden of disease and impact on health-related quality of life (HRQoL) (*11-14*), we divided patients into segmented subgroups based on reported attack frequency over the previous year. Patients reporting 20 or more attacks were categorized as a high frequency group (n = 19/70, 27.1%), those reporting 1-19 attacks were described as a low frequency group (n = 29/70, 41.4%), patients reporting a history of attacks, but none over the previous year were categorized as a no attack group (n = 7/70, 10.0%), and those reporting no attack onset were classified as an asymptomatic group (n = 7/70, 10.0%). An "unclassified" group was created to label those who did not indicate the number of attacks on average that they experienced in a year (n = 8/70, 11.4%).

While 78.6% of the sample were female, women made up 94.7% of those in the high attack group (n = 18/19). Male respondents were slightly over-represented in the low and asymptomatic groups, and more substantially over-represented in the no attack group (n = 7/29, 24.1%; n = 2/7, 28.6%; n = 3/7, 42.9%, respectively) (Figure 1).

3.3. Medical resource utilization

While the companion paper published elsewhere introduced a part of the data on medical resource utilization to compare the pre- and post-diagnosis periods, we expanded the analysis from the viewpoint of the burden of the disease for patients. In this report, we added attack frequency as an additional point of analysis, and we framed medical utilization as an indicator of the physical and psychological burden of HAE.

Patients were asked how often they visit the hospital or clinic where they receive regular care for HAE, whether for consultations or treatment. Answers to this question showed that there is a high utilization of medical resources for routine care of HAE. Eighteen out of 70 (25.7%) respondents reported visiting a medical facility every month, including 5 (7.1%) respondents who reported weekly visits. An additional 33 (47.1%) respondents reported that they went to their regular treating hospital or clinic once every 2–3 months. Patients in the high attack group reported higher usage of routine care with 8 out of 19 (42.1%) respondents reporting that they go weekly or twice monthly (Figure 2).

Utilization of emergency services was also commonly reported by patients, especially those in the high attack group. Patients were asked the approximate number of times they had used an ambulance for HAE symptoms, both before and after diagnosis (Figure 3A). Twenty-five out of 63 (39.7%) respondents reported having used an ambulance at least once in the pre-diagnosis period and 22 (34.9%) respondents in the post-diagnosis period. The mean number of uses by patients in the high attack group after diagnosis was 8.2 times compared to 2.2 times by patients in the low attack group. In the high and low attack groups, there were slightly higher utilization of emergency ambulance services after compared to prior to diagnosis (8.2 to 7.9, and 2.2 to 2.0, respectively). It is noteworthy that 2 out of 7 (28.6%) respondents in the "no attack" group reported having used an ambulance for HAE attacks both in the pre-diagnosed and post-diagnosed period.

Additional indicators of emergency medical resource utilization in this study were experience of tracheotomy or laparotomy. There were reports of both procedures in the pre-diagnosed and post-diagnosed periods. Two out of 63 (3.2%) respondents reported experience of a tracheotomy in the pre-diagnosed period, and this increased to 4 (6.3%) in the post-diagnosed period, with this concentrated in the low attack group (n = 3/29, 10.3%) (Figure 3B).

The opposite trend can be seen with reported incidence of laparotomy, with fewer incidences reported in the post-diagnosed period. Seven out of 63 (11.1%) respondents reported "one experience" of a laparotomy, one (1.6%) respondent reported "twice" and one (1.6%)

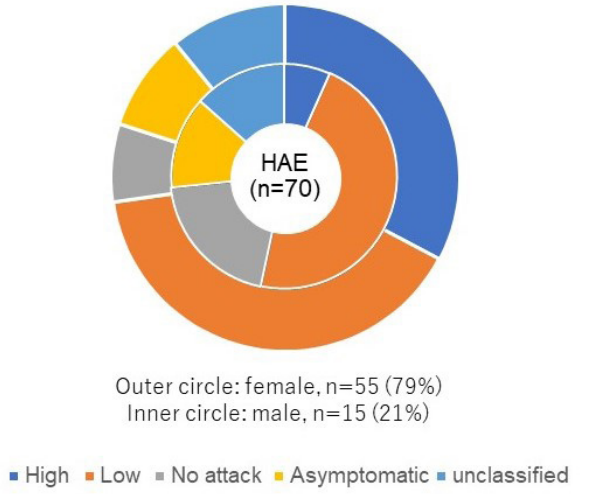

Figure 1. Demographics of respondents by gender and frequency of attacks. Of the 70 respondents, 55 (78.6%) respondents were female, and 15 (21.4%) were male. Those patients reporting on average 20 or more attacks were categorized as a high frequency group (n =19/70, 27.1%), those reporting 1–19 attacks were described as a low frequency group (n = 29/70, 41.4%), patients reporting a history of attacks, but none over the previous year were categorized as a no attack group (n = 7/70, 10.0%), and those reporting no attack onset were classified as an asymptomatic group (n = 7/70, 10.0%). An "unclassified" group was created to label those who did not indicate the number of attacks on average that they experience in a year (n = 8/70, 11.4%). Women made up 94.7% of those in the high attack group (n = 18/19). Male respondents were slightly over-represented in the low and asymptomatic groups, and more substantially over-represented in the no attack group (n = 7/29, 24.1%; n = 2/7, 28.6%; n = 3/7, 42.9%, respectively).

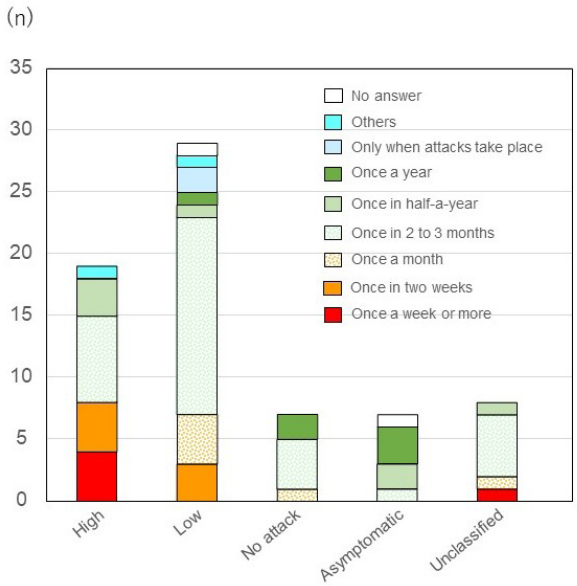

Figure 2. Frequency of visiting hospital or clinic where the patient receives routine care for HAE. Eighteen out of 70 (25.7%) respondents reported visiting a medical facility every month, including 5 (7.1%) respondents who reported weekly visits. An additional 33 (47.1%) respondents reported that they went to their regular treating hospital or clinic once every 2–3 months. Patients in the high attack group reported higher usage of routine care with 8 out of 19 (42.1%) respondents reporting that they go weekly or twice monthly. Abbreviation: HAE, Hereditary angioedema.

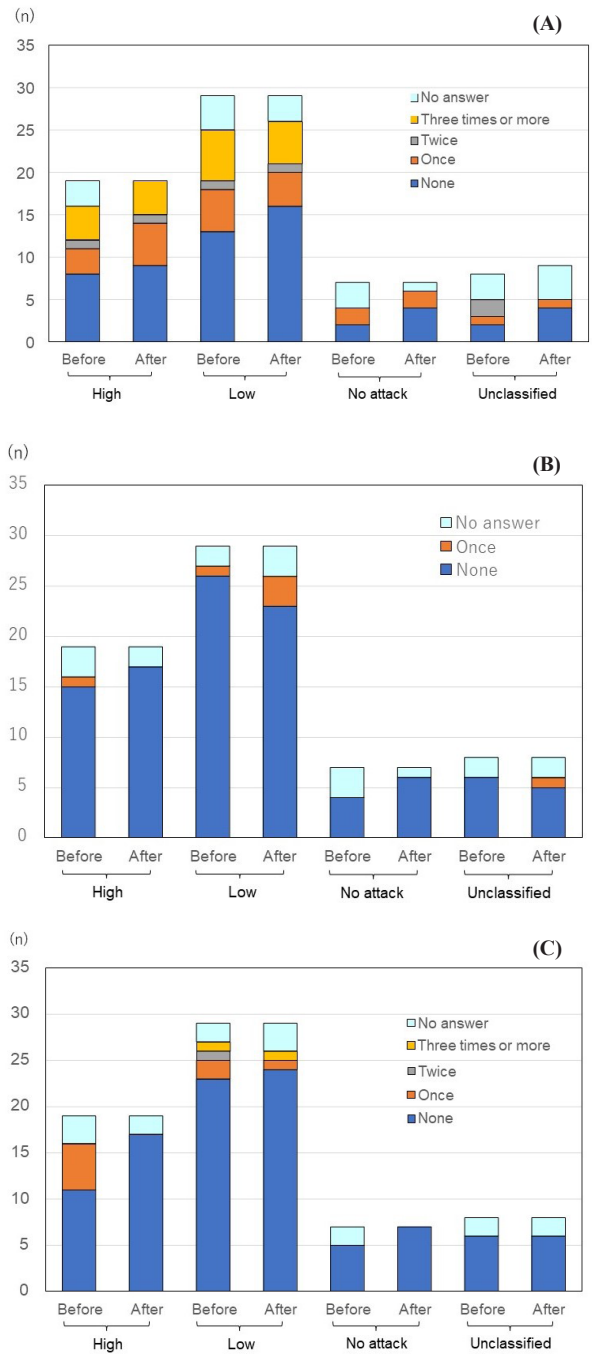

Figure 3. Utilization of emergency services and procedures. (A) Total episodes of being ambulanced to hospitals. Twenty-five out of 63 (39.7%) respondents reported having used an ambulance at least once in the pre-diagnosis period and 22 (34.9%) respondents in the post diagnosis period. It is noteworthy that 2 out of 7 (28.6%) respondents in the "no attack" group reported having used an ambulance for HAE attacks both in the pre-diagnosed and diagnosed period. **(B)** Total episodes of receiving a tracheotomy. Two out of 63 (3.2%) respondents reported experience of a tracheotomy in the pre-diagnosed period, and this increased to 4 (6.3%) in the post-diagnosed period, with this concentrated in the low attack group (n = 3/29, 10.3%). **(C)** Total frequency of ever receiving a laparotomy. Seven out of 63 (11.1%) respondents reported "one experience" of a laparotomy, one (1.6%) respondent reported "twice" and one (1.6%) respondent reported "3 times or more" in the pre-diagnosed period. The incidence of this surgery was higher in the high attack group, with 5 out of 19 (26.3%) respondents reporting at least once incidence. After diagnosis, only 2 out of 63 (3.2%) respondents report experience of a laparotomy. Abbreviation: HAE, Hereditary angioedema.

respondent reported "3 times or more" in the pre-diagnosed period. The incidence of this surgery was higher in the high attack group, with 5 out of 19 (26.3%) respondents reporting at least one incidence. After diagnosis, only 2 out of 63 (3.2%) respondents report experience of a laparotomy (Figure 3C).

3.4. Physical burden

Frequent hospital visits and the pattern of emergency medical resource utilization already point to the heavy physical burden of the disease. In our previous report by Iwamoto, *et al.*, we covered the time needed between onset of attack and treatment, but here we report on the time to get to hospital for regular check-ups for an outpatient appointment (*10*). For patients who regularly meet their doctor for a consultation or treatment, the commute time to the hospital creates both a physical and economic burden. The economic burden will be discussed below. Thirty-six out of 70 (51.4%) respondents are attending a medical institution regularly for HAE that takes more than 30 minutes to get to from where they live. Twelve (17.1%) respondents report that the journey time is more than one hour. Three out of 19 (15.8%) respondents in the high attack group reported time between 1 hour to 1.5 hours for them to reach their regular treating hospital. Some patients reported the physical burden not only of traveling to hospital, but also the long waits on arrival.

The coding of open-ended responses created two categories that related to the physical burden of the condition linked to frequent hospital visits. Six out of 70 (8.6%) respondents indicated in their response that they felt that the waiting time for hospital visits was too long. For the high attack group, 3 out of 19 (15.8%) respondents referred to the long waiting time as something that troubled them. Nine out of 70 (12.9%) respondents responded that their life would be improved if they could receive treatment at a local hospital. For the high attack group, 5 out of 19 (26.3%) respondents indicated that being able to get treatment at a local hospital would improve their lives.

As we reported in the previous analysis, approximately 55% of all attacks in the previous year had been treated (*10*). Here we further report that only 16 out of 47 (34.0%) respondents reported that they sought treatment every time they had an attack. Among the high attack group, one out of 19 (5.3%) respondents reported that they did not seek treatment for any attacks, and 3 (15.8%) respondents for less than 20% of attacks. The main reason given for not receiving treatment (multiple responses allowed), was that the symptoms were judged by the patient to be mild (n = 34/37, 91.9%). Other reasons given for not seeking treatment include the distance to the hospital (n = 7/37, 18.9%), the treating hospital not offering treatment at night (n = 4/37, 10.8%), going to the hospital to be treated would involve missing

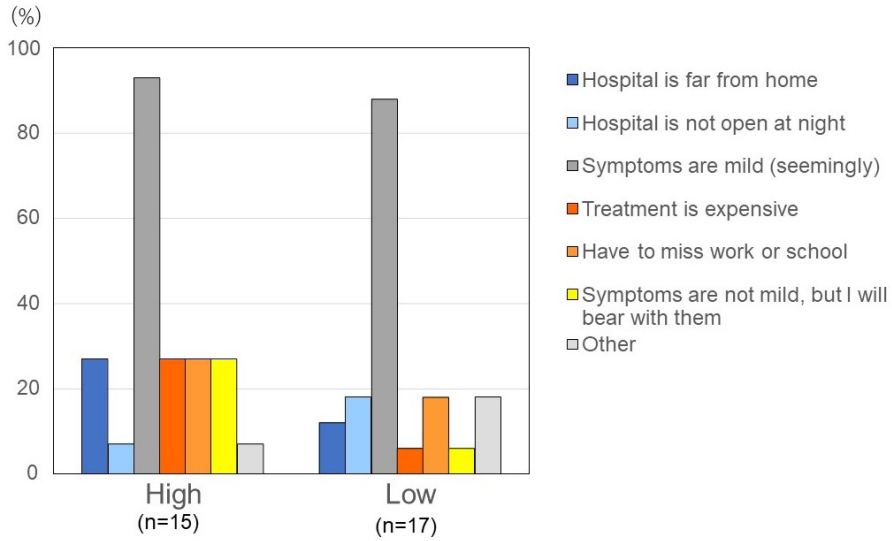

Figure 4. Reason for not seeking treatment for attacks. The main reason given for not receiving treatment (multiple responses allowed), was that the symptoms were judged by the patient to be mild (n = 34/37, 91.9%). Other reasons given for not seeking treatment include the distance to the hospital (n = 7/37, 18.9%), the treating hospital not offering treatment at night (n = 4/37, 10.8%), going to the hospital to be treated would involve missing work or school (n = 8/37, 21.6%) and the treatment was too expensive (n = 6/37, 16.2%).

work or school (n = 8/37, 21.6%) and the treatment was too expensive (n = 6/37, 16.2%) (Figure 4).

3.5. Economic burdens and associated impairments

Once a patient has been diagnosed with HAE, they can apply through their local health center for public remuneration under the Specified Disease Law. Patients were asked if they were currently receiving public reimbursement for out-of-pocket expenses. Fifty-five out of 70 (78.6%) respondents answered yes, but 14 (20.0%) respondents said that they were not receiving any public renumeration. All respondents of the high attack group (n = 19/19, 100%) and 22 out of 29 (75.9%) respondents in the low attack group were receiving public renumeration. Those in the no attack and asymptomatic groups were more likely to report that they do not receive public renumeration (n = 2/7, 28.6% and n = 4/7, 57.1%, respectively) (Figure 5). By age, those in their 50s and 30s were most likely to be receiving public renumeration (n = 12/13, 92.3% and n = 16/18, 88.9%, respectively), and those in their 60s the least likely (n = 5/8, 62.5%).

Patients who had experienced at least one attack (n = 62) were asked about HAE related out-of-pocket costs for treatment and tests per year, both in the pre-diagnosed and post-diagnosed periods. The mean out-of-pocket expenses per year were estimated to be 123,615 Japanese yen (JPY) (936.86 United States dollar – USD; conversion rate as of 6th February 2023, 1 JPY = 0.00758349 USD) prior to diagnosis, falling to 92,392 JPY (700.18 USD) after diagnosis in large part due to the public renumeration system. Those currently receiving financial renumeration report on average higher out-of-pocket expenses per year in the pre-diagnosed period (n = 52, 83.9%, a mean of 138,269 JPY; 1,048.22 USD)

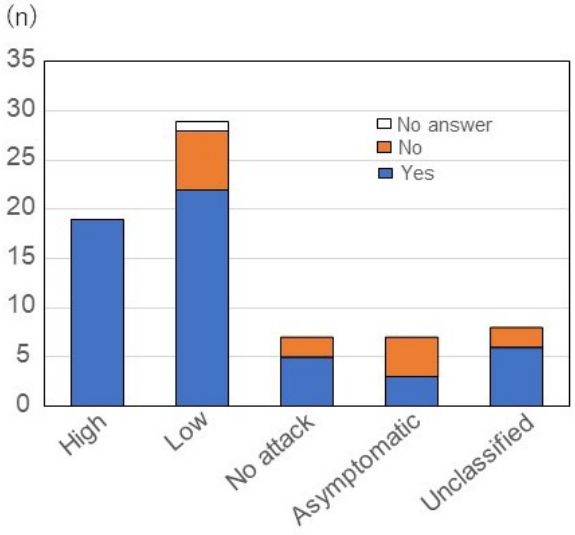

Figure 5. Patients receiving or qualified to receive public financial support for HAE treatment according to attack frequency. We found that all respondents of the high attack group (n = 19/19, 100%) and 22 out of 29 respondents (75.9%) in the low attack group were receiving public renumeration. Those in the no attack and asymptomatic groups were more likely to report that they do not receive public renumeration (n = 2/7, 28.6% and n = 4/7, 57.1%, respectively). Abbreviation: HAE, Hereditary angioedema.

compared to those not currently receiving financial support (n = 10, 16.1%, 64,050 JPY; 485.80 USD), but also in the post-diagnosed period (100,172 JPY; 759.78 USD compared to 59,070 JPY; 448.01 USD) (Figure 6).

Looking only at those who are currently receiving financial renumeration, the high attack group report higher out-of-pocket medical expenses on average than the low and no attack group both in the pre-diagnosed (mean of 209,444 JPY; 1,588.52 USD compared to

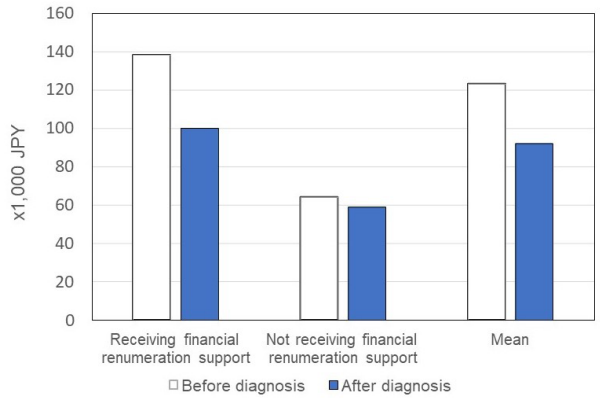

Figure 6. Out-of-pocket money for drug therapies/tests in a typical year. The mean out-of-pocket expenses per year was estimated to be 123, 615 JPY (938.08 USD) prior to diagnosis, falling to 92,392 JPY (701.14 USD) after diagnosis in large part due to the public renumeration system. Those currently receiving financial renumeration reported on average higher out-of-pocket expenses per year in the pre-diagnosed period (*n* = 52, 83.9%, a mean of 138,269 JPY; 1,049.16 USD) compared to those not currently receiving financial support (*n* = 10, 16.1%, 64,050 JPY; 485.98 USD), but also in the post-diagnosed period (100,172 JPY; 759.87 USD compared to 59,070 JPY; 448.19 USD). Abbreviation: JPY, Japanese yen; USD, United States dollar.

130,769 JPY; 991.58 USD and 5,000 JPY; 37.91 USD, respectively) and post-diagnosed periods (143,081 JPY; 1,084.91 USD compared to 92,328 JPY; 700.08 USD and 13,333 JPY; 101.09 USD, respectively).

Patients were also asked the estimated yearly out-of-pocket costs associated with receiving surgery or for ambulance use for HAE-like or HAE symptoms in the pre- and post-diagnosed period. This showed a dramatically higher average expenditure in the pre-diagnosed (*n* = 63, 102,543 JPY; 777.50 USD) compared to the post-diagnosed period (*n* = 63, 30,291 JPY; 229.68 USD).

Those currently receiving pubic renumeration (*n* = 52, 83.9%) reported a dramatic decrease in out-of-pocket expenses due to surgery and ambulance use in the post diagnosed period; before diagnosis an average of 111,034 JPY (841.92 USD) decreasing to 30,203 JPY (229.03 USD) after diagnosis. For those currently not receiving public renumeration (*n* = 10, 16.1%) the decrease was less dramatic with the average out-of-pocket costs in the pre-diagnosed period 50,800 JPY (385.20 USD) declining to 37,000 JPY (280.56 USD) in the post-diagnosed period.

3.6. Psycho-social burdens and areas of unmet need

Due to the lack of self-administered options for acute attack treatment at the time of the survey, seeking treatment resulted in work, education and leisure impairments due to the time involved in a hospital visit. As reported in the previous section, patients make frequent visits to their treating hospital and 27 out of 55 (49.1%) respondents reported that they had been hospitalized on average for "one or more days" per year. In addition, 18 out of 47 (38.3%) respondents reported missing "10 or more days" from work/school because of HAE attacks.

We were able to gain insights retrospectively on the longer-term impacts of HAE on work and school by asking about three periods: the time between onset of symptoms and first seeking medical advice; the period between first seeing a physician or other health care practitioner and being diagnosed; the period after diagnosis. This showed that the highest mean time for days missed from work or school per year was in the period after seeking medical advice and before diagnosis (*n* = 45, mean days lost 17.7 days). After diagnosis this was reduced to a mean of 11.9 days lost (*n* = 47) per year.

The coding of open-ended question data offered some insight into the mental health burden of HAE for Japanese patients at the time of the survey. Eleven out of 70 (15.7%) respondents reported issues that we coded as 'mental anxiety' due to HAE. This was highest in the high attack group (*n* = 6/19, 31.6%%), but was also notable in the low attack and non-attacks groups (*n* = 4/29, 13.8% and *n* = 1/7, 14.3%, respectively).

Another category retrieved from the data were statements that we coded "being troubled by attacks" (*n* = 11/70, 15.7%), which we also interpreted as a mental health burden. Statements suggesting patients were troubled by attacks were seen more frequently in the high attack group (*n* = 7/19, 36.8%), but were also present in the low attack (*n* = 3/29, 10.3%) and no attack (*n* = 1/7, 14.3%) groups.

Anxiety around HAE attacks impacts whether a patient feels able to work or not, and if they do seek employment it impacts the kind of work they feel they can do. One patient reported that she/he cannot take a night shift or any shift where there are few workers in case she/he gets sick. The responses also show that patients may alter their treatment-seeking behavior when an attack occurs, *e.g.* holding out until the evening to avoid losing pay or bothering co-workers. This behavior could result in delayed treatment, which is associated with a higher level of attack morbidity. While in school, an attack can involve missing school or spending time in the infirmary which leads to missed classes (Table 1).

Patients were asked whether HAE impacted their ability to make plans, or restricted travel and daily life activities. Roughly equal numbers of patients responded that 'they try not to travel' and 'daily life activities are restricted'. The percentage reporting that they were unable to make plans, restricted travel and daily life activities was considerably higher for the high attack group. Seventeen out of 19 (89.5%) respondents reported that they restricted their daily activities, compared to group (*n* = 8/29, 27.6%) for the low attack group (Figure 7).

Patients were asked what changes would improve

Table 1. Impacts of HAE on work and school, open-responses

About work and school:

- I want to work, but I can't when thinking about what I might do if an attack takes place. I would trouble others around me.
- HAE affects the type of work I can do, how often I can go out, the amount I can eat, and what I can do before needing to rest.
- I suddenly become unable to perform any work.
- I can't do a night shift or work where there are not many other workers [in case I am ill]
- I can't afford to keep missing days from work. So I go to the hospital at night more often, which burdens my children.
- When attacks occur, I have stomach pain and vomit and can't do any house work. I need help from family. I'm scared about attacks.
- I miss school, or sometimes have to rest at the school infirmary.
- I can get a swelling just from holding or running around with my child.
- I can't look after my children, I can't do the housework. [when I have an attack]

Abbreviation: HAE, Hereditary angioedema.

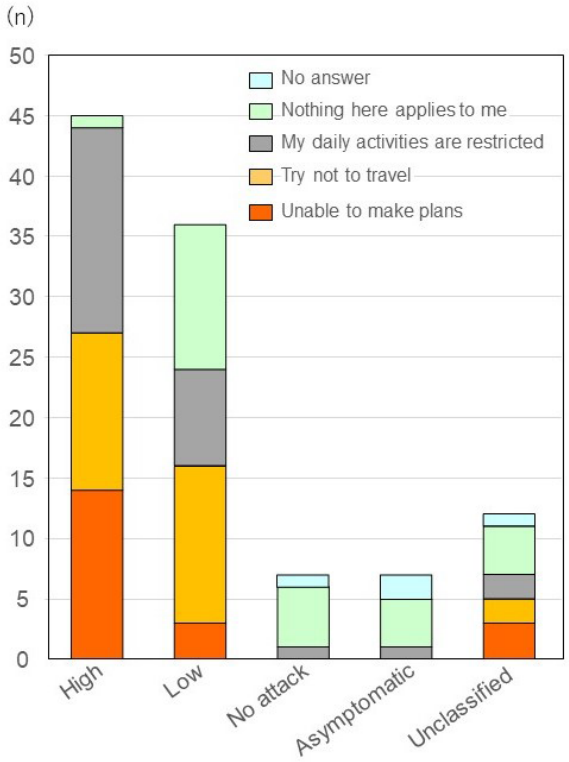

Figure 7. Daily activity impairments. Patients were asked whether HAE impacted their ability to make plans, or restricted travel and daily life activities. Roughly equal numbers of patients responded that "they try not to travel" and "daily life activities are restricted". The percentage reporting that they were unable to make plans, restricted travel and daily life activities was considerably higher for the high attack group. Seventeen out of 19 respondents (89.5%) reported that they restricted their daily activities, compared to group (n = 8/29, 27.6%) for the low attack group. Abbreviation: HAE, Hereditary angioedema.

their lives. Our coding of these open-ended responses reveals several areas where patients would like to see change. Ten out of 70 (14.3%) respondents reported that being able to self-administer medications for their acute attack would improve their lives. In the high attack group, 7 out of 19 (36.8%) respondents described that self-administration would improve their lives. Access to C1-INH concentrate for prophylactic treatment options was also indicated as something that would bring improvements to their lives by 7 out of 70 (10.0%) respondents, and 4 out of 19 (21.1%) respondents in the high attack group. Ten out of 70 (14.3%) respondents also wished for greater awareness of the condition among physicians, this figure rising to 4 out of 19 (21.1%) respondents in the high attack group (Table 2).

Patients were also asked hypothetically whether they would want to self-inject, if plasma derived C1-INH concentrate was authorized for home use for acute attack therapy and prophylactic use. Thirty-seven out of 51 (72.5%) respondents patients answered that they would want to self-inject for acute attack therapy, and 30 out of 51 (58.8%) respondents did so if it were available for prophylactic therapy. In the high attack group, this intension was even higher; 16 out of 18 (88.9%) respondents said that they would want to self-inject for acute attack treatment and 13 (72.2%) respondents for prophylactic therapy.

4. Discussion

Through a PRO survey instrument designed in consultation with patients, we were able to clarify the burden of HAE in key domains, including medical resource utilization, physical, psycho-social and economic burdens, and associated work and school impairments. We also elicited responses about what changes might reduce these burdens. Through this instrument we were able to clarify not only the current burden of illness for patients at the time of the survey, but also the impact in the pre-diagnosed period, which is something that most HAE burden of illness research has not attempted to do.

In keeping with other HAE burden of illness studies, we found that the heaviest burden in all domains was experienced by those with a higher attack frequency. This burden was evident both in the pre- and post-diagnosed periods. As comparable survey instruments have not been used in the various HAE burden of illness studies that have been carried out to-date, it is hard to precisely compare Japanese patients with their counterparts overseas (*11-20*). However, our study confirms that there is a very high level of work, school and activity impairment for HAE patients in Japan at the time of this survey, which is consistent with studies carried out in the

Table 2. Mental health burden of HAE

Items	Total (*n* = 70)	High (*n* = 19)	Low (*n* = 29)	No attack (*n* = 7)	Asymptomatic (*n* = 7)	Unclassified (*n* = 8)
Troubled with attacks	11	7	3	1	0	0
Mentally worried	11	6	4	1	0	0
Want access to self-injection, treatment at home	10	7	3	0	0	0
Raise awareness among doctors	10	4	4	1	0	1
Want access to a hospital that would offer treatment nearby	9	5	3	1	0	0
Want new products and formulations	9	5	3	0	0	1
Prophylactic use of Berinert or a drug that has preventive effect	7	4	1	0	1	1
Not enough information	4	0	4	0	0	0
Waiting time is too long	6	3	2	0	0	1
Supporting costs for genetic tests for family	4	2	2	0	0	0
Scared of going on trips	5	1	4	0	0	0
Concerned about the side effects of drugs	4	1	2	1	0	0
Wish to have no more attacks; Want a cure	3	1	0	0	1	1
Financial burden	4	2	1	1	0	0
Impact of treatment is unknown; Have unexplained symptoms	3	0	3	0	0	0
Cannot get my medication at a pharmacy	1	0	1	0	0	0
Burden on family	1	0	1	0	0	0
How to deal with throat swelling	1	0	0	0	0	1
A third-party counselor specializing in HAE	1	0	0	0	1	0
Nothing at this moment	4	0	2	1	1	0
No answer	18	1	9	2	3	3

Abbreviation: HAE, Hereditary angioedema.

United States and Europe (*11-20*).

We can also confirm that patients are frequent users of medical resources, both routine care and emergency care, which creates physical, mental and economic burdens for the patients and their caregivers. Our results show that for many patients, especially those with frequent attacks who make regular trips to the hospital, these visits often require a long travel time and then a long waiting time for treatment. It is notable that in overseas studies on the burden of illness of HAE patient, the issue of travel time to receive treatment is not raised, possibly due to the high prevalence of self-administration. Nevertheless, a systematic review of studies looking at the association between travel times to receive treatment for a variety of conditions, but not HAE, and health outcomes of countries in the global north found that 83 out of 108 (76.9%) studies revealed a negative relationship. The longer the travel time, the worse the health outcome and the higher the level of non-attendance for required treatments (*21*). While none of these studies were concerned with HAE, the study conclusions alert us to the possible HRQoL-related implications of longer travel times.

The survey instrument was able to clarify, to some extent, the psycho-social and economic burdens of HAE and the associated impairments on work, education and leisure activities, as well as the direct financial costs associated with treatment and the extent to which they were ameliorated by the public remuneration system.

The system of public renumeration for rare disease patients greatly reduces the direct costs associated with medical resource utilization. Yet, it is noteworthy that 14 out of 70 (20%) respondents of patients overall had not applied for public numeration. While 6 out of 29 (20.7%) respondents in the low attack group reported they were not receiving public renumeration, all patients in the high attack group were receiving public renumeration.

Patients in the high attack group were shown to have shouldered the heavier direct medical costs during the pre-diagnosed period. These results would suggest that those with heavier medical resource utilization history have a higher motivation to file for public renumeration to offset the economic burden. Nevertheless, we need greater insights into why some patients do not apply for public renumeration and the impact of this on how they access effective acute attack treatments.

While public renumeration reduces the financial burden of direct medical costs, we saw that some patients perceive the costs of receiving treatment for each attack as too high and this influences treatment seeking behavior. When we consider the economic burden of HAE, we also need to factor in lifetime costs, including those in the pre-diagnosed period, in which patients experience multiple misdiagnoses with high out-of-pocket expenses. This result confirms that an early diagnosis is not only important to reduce the risks of mortality and morbidity associated with undiagnosed HAE but also reduce the economic burden (*13*). It goes without saying that until a definitive diagnosis has been reached, an HAE patient cannot apply for public remuneration.

Due to the lack of self-administered options for disease specific treatments at the time of the survey, seeking treatment for HAE attacks resulted in work, education and leisure impairments due to the time involved in a hospital visit. Patients make frequent

visits to their treating hospital and this is not only time consuming, but results in time lost from work and educational activities.

Patients in the study pointed to three areas of possible improvement to reduce the burden of the disease. First, they called for self-administered HAE treatments, both for acute attack and to prevent attacks. Second, they called for the authorization of C1-INH concentrate, the only authorized disease-specific available treatment at the time of the survey, for prophylactic use. Finally, they called for greater awareness of the disease by physicians. The first two requests point to patients' desire to reduce the burden of being treated and, in the case of prophylactic care, reducing the frequency of attacks. Self-administration of acute attack and prophylactic medications has shown to reduce the burden of HAE in studies conducted outside of Japan (*16*).

The limitations of this study lie in the use of a non-validated survey instrument. Nevertheless, conversely, the strengths of this study are that the survey instrument was designed in consultation with patients and physicians, and that the content reflects key concerns that were generated in the consultation process. In addition, the survey was carried out in a treatment environment where only one disease-specific treatment was authorized, human plasma derived C1-INH concentrate, solely for an acute attack. Treatment required the patient to visit a designated hospital to receive intravenous administration from a physician or a nurse. Other studies on burden of illness have been carried out in environments where patients had access to a wider variety of treatments and to self-administration, largely in the United States and Europe (*11-20*). Second, patients were asked to recall attack history, medical resource utilization and direct economic costs in the pre-diagnosed period that varies by patient in length of time and distance from the present day. The reliance on retrospective reporting is another limitation. Nevertheless, it did allow us to clarify the cumulative impact of the disease in broad brush strokes. Even if in future, the burden is reduced by the introduction of new treatments and/or widening the access to the current treatment by the authorization of self-administration, the historical accumulation of impairments and costs will not be eliminated. A third limitation is that the patient population included in this study might be biased to those attending a specialist clinic on a regular basis or at least on an irregular basis.

It is important to note that HAE treatment environment in Japan has undergone rapid change over the last five years. Human plasma derived C1-INH concentrate has been available for acute attack treatment since 1990, but it became available for short-term prophylaxis as well in the first quarter of 2017, after our survey was administered. For both indications, treatment must be carried out in a hospital setting under the supervision of a physician. Additionally, subcutaneous administration of a bradykinin-B2-receptor antagonist (icatibant) and its self-administration was approved for acute attack in the final quarter of 2018. In the first quarter of 2021, an oral plasma kallikrein inhibitor (berotralstat) was approved for prophylactic indications, followed by the approval of monoclonal antibody against plasma kallikrein (lanadelumab) in Japan as treatment options for long-term prophylaxis to prevent attacks. In the context of this improving treatment environment, our data-set offers an important baseline against which future studies can investigate the impact of these improvements in HAE treatment options on the burden or illness experiences by patients. It also offers a historic picture of the disease burden before self-administrated acute attack treatment and/or prophylactic treatment options were available.

In conclusion, based on a PRO survey this paper revealed that HAE patients in Japan experience heavy burdens of illness in the physical, social, economic, and psycho-social domains, causing a decrease in HRQoL. As the burden of disease in these domains was shown to be even higher before than after diagnosis, we can conclude that early diagnosis is a necessary condition to reduce the lifetime burden of this disease. Patients felt that access to self-administered treatments would reduce the physical and social burdens associated with frequent hospital visits. We identify this as an area of unmet need at the time of the survey. Results of this study should be a basis for comparison in coming studies after the introduction of self-administered modalities for acute attack and prophylactic treatments.

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or participated in advisory boards for CSL Behring and Takeda/Shire. K.Y. has received speaker/advisor honoraria from CSL Behring and Takeda Pharmaceutical Company Ltd. M.H. has received honoraria from CSL Behring, Takeda and Torii Pharmaceutical Company Ltd, consulting fee from BioCryst, KalVista and Pharvaris.

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**Address correspondence to:*
Daisuke Honda, Department of Nephrology, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8677, Japan.
E-mail: dhonda@chiba-u.jp

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Original Article

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Telotristat Etiprate alleviates rheumatoid arthritis by targeting LGALS3 and affecting MAPK signaling

Ling Zhang[§], Yanwen Lin[§], Xinrui Xu, Huihui Liu, Xiangyu Wang, Jihong Pan[*]

Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences; Key Lab for Biotech-Drugs of National Health Commission, Ji'nan, Shandong, China.

SUMMARY Rheumatoid arthritis (RA) is one of the most widespread chronic immune-mediated inflammatory diseases characterized by continuous erosion of bone and cartilage by synovial hyperplasia. Telotristat Etiprate is an inhibitor of tryptophan hydroxylase, a rate-limiting enzyme in the biosynthesis of serotonin. Telotristat Etiprate can be used in the treatment of carcinoid syndrome. The purpose of this study was to explore the effect of Telotristat Etiprate on RA and its mechanism. We investigated Telotristat Etiprate in collagen-induced arthritis (CIA) model mice and in rheumatoid arthritis synovial fibroblasts (RASFs). Results showed that Telotristat Etiprate had anti-inflammatory effects both *in vitro* and *in vivo*, can inhibit the invasion and migration of cells, inhibit the formation of pannus, and induce cell apoptosis. Transcriptome sequencing (RNA-seq) and mass spectrometry analysis showed that Galectins-3 (LGALS3) could be a newly identified target of Telotristat Etiprate, affecting the phosphorylation of the MAPK signaling pathway through UBE2L6, thereby improving RA.

Keywords rheumatoid arthritis, Telotristat Etiprate, LGALS3, MAPK signaling pathway, UBE2L6

1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, destructive, systemic autoimmune disease characterized by synovial hyperplasia and leukocyte exudation leading to joint destruction (*1,2*). Rheumatoid arthritis synovial fibroblasts (RASFs) are major components of vascular membrane proliferation and actively participate in joint inflammation and cartilage and bone destruction through contact interactions, and production of inflammatory mediators and matrix degrading enzymes (*3*). RASFs resist apoptosis, exhibit more adhesion and invasion properties, evade contact inhibition, cells migrate and colonize elsewhere, infiltrate, and destroy joints in immunodeficient mice (*4-6*). Notably, this aggressive phenotype persisted after multiple cell passages *in vitro*, suggesting that synovial fibroblasts in RA were persistent, if not irreversible. The joints of RA patients produce excessive amounts of inflammatory molecules, such as cytokines and matrix metalloproteinases, which promote the destruction of cartilage and bone (*7,8*).

The goal of RA treatment is to minimize symptoms such as pain and swelling, prevent skeletal deformities, and maintain daily function. However, RA is currently mainly treated with anti-rheumatic drugs (DMARDs), which are painkillers used to help reduce pain (*9*). DMADRs have been found to improve symptoms, reduce joint damage, are usually started early in the disease and can help about half of people get remission, improving overall prognosis. However, due to the lack of long-term experience in the use of these drugs, which can only relieve but not treat the pain, the treatment process is often accompanied by a large number of toxic side effects (*10*). Therefore, we urgently need to find a small molecule inhibitor for targeted improvement of RA, from analgesia to pain treatment, and fundamentally relieve the pathological symptoms of RA.

Due to synovial hyperplasia, bone and cartilage are constantly eroded, leading to the increase of some pro-inflammatory cells (such as macrophages, plasma cells, B cells, T cells, *etc.*), which activate NF-KB, MAPK, JAK/STAT, PI3/Akt/mTOR and other inflammatory signaling pathways, and produce a large number of inflammatory cytokines (IL-6, IL-8, IL-1α, IL-1β, CCL2). Based on the above inflammatory signaling pathways, we purchased 1092 inhibitors from selleck's inhibitor library for screening. After layer upon layer screening, we finally selected Telotristat Etiprate, which had the greatest inhibitory effect, and further explored its mechanism of action to provide a theoretical basis

for RA treatment. Telotristat Etiprate is an inhibitor of tryptophan hydroxylase, a rate-limiting enzyme involved in 5-ht biosynthesis (*11,12*). The US Food and Drug Administration and the European Commission recently approved Telotristat Etiprate. This is a Category 2A recommendation in the National Comprehensive Cancer Network's Clinical Practice Guidelines for treating Carcinoid syndrome (CS) diarrhea that is inadequately controlled by Somatostatin Analogs (SSA) therapy (*13*).

In this study, first , collagens induced arthritis (CIA) model mice were used to explore whether Telotristat Etiprate has any effect on RA. Next, the effect of Telotristat Etiprate treatment on the biological function of RASFs was detected *in vitro*, and the mechanism of its action was explored by RNA-seq and mass spectrometry, thus providing a basis for the treatment of RA.

2. Materials and Methods

2.1. Collection of synovial tissues

Synovial tissues were collected during knee joint replacement surgery from patients with RA (n = 14; five males, nine females, aged 35 to 75 years, mean age of 55 years). Synovial tissues used in this study were provided by the Shandong Provincial Hospital. All patients fulfilled the 1987 American College of Rheumatology revised criteria for RA diagnosis. Written informed consent was obtained from each patient, and all samples were de-identified for research use. The Ethical Committee of the Shandong Academy of Medicinal Sciences approved this study (approval number 2019–02).

2.2. Collagen-induced arthritis (CIA) model

The CIA model is a well-established mouse model for human RA. The CIA model was induced by tail injection of 9-week-old male mice with 2 mg/mL bovine type II collagen (Chondrex, Washington, USA) and complete Fredrlich adjuvant (Sigma-Aldrich, Mannheim, Germany) 1:1 emulsifier (200 μL). After 21 days, the mouse tails were injected with bovine type II collagen and Sigma-Aldrich 1:1 emulsifier (200 μL). Mice were monitored for arthritis symptoms once a day. After swelling was identified in all four paws, mice were treated with Telotristat Etiprat for 1 month (intraperitoneal injection, 20 mg/mL), and paw inflammation relief was observed and recorded. Scores were based on the degree of swelling and erythema around the joint, ranging from 0 to 3, no signs of erythema or swelling; 1, erythema in only one joint; 2, erythema and swelling areas found in only one joint; 3, severe erythema and swelling extending from one joint to both joint areas. The total score for each mouse was added to the four paw scores, and the maximum possible score for each mouse was 12 points.

2.3. Cell acquisition and stimulation

Synovial tissue was soaked and digested with type II and III collagenase at 37°C for 6–8 h in 5% CO_2. Cells were cultured in DMEM (Gibco) complete medium containing 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin and were used in passages from 3 to 7 generations.

We screened a number of commonly used inflammatory factors for RASF stimulation, including lipopolysaccharide (LPS), IL-1β, IL-1α, IL-6, and TNF-α, but multiple trials found that IL-1β stimulation was most effective. RASFs were inoculated on cell culture plates and stimulated with IL-1β (10 ng/mL) for 24 h. Telotristat Etiprate was dissolved in 1 mM DMSO to reach a final concentration of 50 ng/mL in DMEM complete medium.

2.4. RNA extraction and qRT-PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) according to the reagent instructions, and RNA was reverse transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (Vazyme). qRT-PCR was performed using a LightCycler 480 (Roche, Basel, Switzerland) according to the following protocol: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min for 40 cycles. Upstream primers and downstream primers are shown in Table 1. All primers were synthesized by Beijing Genomics (Beijing, China). Each sample was analyzed in triplicate, and expression changes of target genes were calculated by the $2^{-\Delta\Delta Ct}$ relative quantitation method.

2.5. ELISA

Cells were treated with Telotristat Etiprate or DMSO. After 6 h, the proinflammatory factor IL-1β was added for stimulation. After 24 h, the supernatant was collected, and the secretion of IL-6, IL-8, and CCL2 was detected by ELISA (MultiSciences). After drug treatment in mice, serum was collected, debris was removed by centrifugation, and the secretion of inflammatory factors, such as IL-6, IL-8, IL-1α, and COMP, was detected by ELISA.

2.6. *In vitro* cell invasion and migration assay

After diluting matrix glue with serum-free and antibiotic-free DMEM at a ratio of 1:7, 100 μL were added to the upper chamber of the TransWell (each chamber) and incubated at 37°C with 5% CO_2 for 1 h. RASFs were treated as described above and inoculated with 10,000 cells in the TransWell upper chamber (DMEM complete medium with 2% FBS), and complete medium containing 15% FBS was added to the surface of the

Table 1. Primers used for qRT-PCR

Primer name†	Primer base sequence (5′to 3′)
GAPDH	Forward: TGATGACATCAAGAAGGTGG
	Reverse: TTACTCCTTGGAGGCCATGT
IL-6	Forward: CCACCGGGAACGAAAGAGAA
	Reverse: GAGAAGGCAACTGGACCGAA
IL-8	Forward:CAGTTTTGCCAAGGAGTGCTAA
	Reverse: AACTTCTCCACAACCCTCTGC
CCL2	Forward: AGAGGCTGAGACTAACCCAGA
	Reverse: TTTCATGCTGGAGGCGAGAG
IL-1α	Forward: AGATGCCTGAGATACCCAAAACC
	Reverse: CCAAGCACACCCAGTAGTCT
IL-1β	Forward: ATGATGGCTTATTACAGTGGCAA
	Reverse: GTCGGAGATTCGTAGCTGGA
PDGFRL	Forward: CAAGAACAAGCGTCCAAAAGAAC
	Reverse: AGCGACCTTTATCCAGCACTT
PLAT	Forward: AGCGAGCCAAGGTGTTTCAA
	Reverse: CTTCCCAGCAAATCCTTCGGG
LGALS3BP	Forward: AGGTACTTCTACTCCCGAAGGA
	Reverse: GGCCACTGCATAGGCATACA
HSPB3	Forward: ATAGAGATTCCAGTGCGTTACCA
	Reverse: CAGGCAGTGCATATAAAGCATGA
CD274	Forward: GGACAAGCAGTGACCATCAAG
	Reverse: CCCAGAATTACCAAGTGAGTCCT
UBE2L6	Forward: TGGACGAGAACGGACAGATTT
	Reverse: GGCTCCCTGATATTCGGTCTATT
OAS1	Forward: AGCTTCGTACTGAGTTCGCTC
	Reverse: CCAGTCAACTGACCCAGGG
NR4A1	Forward: ATGCCCTGTATCCAAGCCC
	Reverse: GTGTAGCCGTCCATGAAGGT
BATF2	Forward: CACCAGCAGCACGAGTCTC
	Reverse: TGTGCGAGGCAAACAGGAG
ANGPTL1	Forward: AGAAAGGAAAGCCGTAACATGAA
	Reverse: TCCCTGTATCTTGTTGCCATCT
HERC5	Forward: ATGGGCTGCTGTTTACTTTCG
	Reverse: TTCCCAGTTGTCCATCTTTTCC
OASL	Forward: CTGATGCAGGAACTGTATAGCAC
	Reverse: CACAGCGTCTAGCACCTCTT
RSAD2	Forward: CAGCGTCAACTATCACTTCACT
	Reverse: AACTCTACTTTGCAGAACCTCAC

†All primers are targeted against human genes.

lower chamber. After 18 h of incubation, cells invading the lower surface were stained and counted (three repeat views for each treatment condition) to calculate the average number of invaded cells.

RASFs were inoculated in six-well plates and treated in accordance with the above methods. After 24 h, RASF medium was replaced with serum-free and antibiotic-free medium and cell scratching was performed. RASF migration was recorded at 0, 3, 6, 12, and 24 h.

2.7. Apoptosis was detected by flow cytometry

In brief, RASFs were treated as described above, digested with trypsin, stained with Annexin V-fluorescein isothiocyanate (FITC) (10 μg/mL), and propidium iodide (10 μg/mL) (Sigma) according to manufacturer's protocol and analysed with a FACSCalibur machine (BD Biosciences). Annexin V+/+ cells were considered apoptotic cells.

2.8. Apoptosis was detected by TUNEL assay

The TUNEL method was used to detect RASF apoptosis. Cells in good condition were inoculated into 96-well plates for drug treatment, washed with PBS three times (3 min each), fixed with 4% paraformaldehyde, and permeabilised with 0.2-0.5% Triton X-100. Cells were then stained with TUNEL and DAPI and immediately observed under a confocal fluorescence microscope. Green fluorescence (apoptotic cells) was observed at 520 nm, and DAPI blue fluorescence was observed at 460 nm. Each group was set up with two repeating holes, and the experiment was repeated three times.

2.9. Histological analysis

A micro-CT system (Quantum GX, USA) was used to scan mouse paws, observe the joint microstructure, and accurately analyse the same area of mouse joints. After immobilisation in 4% paraformaldehyde for 24 h, claws were decalcified in 15% ethylenediamine tetraacetic acid (EDTA) for about a month. Claws were embedded in paraffin and cut into 5-μm sagittal sections. The sections were stained with H&E and Safranin O-Fast Green FCF.

2.10. Western blotting analysis

Cells were collected and lysed with RIPA lysate and protease inhibitors. The cleavage materials were separated by electrophoresis through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) and transferred to a PVDF membrane with a pore size of 0.45um. Then, western blotting was performed with a specific primary antibody and incubation was conducted overnight at 4°C. After washing with TBST, the membrane was incubated with each corresponding secondary antibody at 37°C for 1h and detected using ECL Plus detection system (Thermo Scientific, Pittsburgh, PA, USA). The following primary antibodies were used in this experiment: anti-P38 MAPK, anti-P44/42 MAPK, anti-SAPK/JNK MAPK, anti-P65 NF-NB, phospho- P38 MAPK, phospho-P44/42 MAPK, phospho-SAPK/JNK MAPK, phospho-P65 NF-KB, anti-PLD3, anti-LGALS3, anti-YKL140, anti-YWHAG, anti-CD11C, anti-CASP3, anti-PRDX3, anti-RAS2, anti-CASP3, anti-DNAJA1.

2.11. Immunofluorescence microscopy

After RASFs were treated in 48-well plates, the cells were slowly washed with PBS 3 times (3 min each), and fixed with 4% paraformaldehyde. After rinsing with PBS 3 times (3 min each), the RASF cells were sealed with 5% bovine al-bumin (BSA) for 1h at 37°C, and the cells were incubated with specific monoclonal antibodies at 4°C overnight. After rinsing with PBS 3 times, the fluorescence secondary antibody was incubated and the nuclei were stained with 4 ',6 '-Diamidino-2-phenylinndole (DAPI).

2.12. Small interfering RNA (siRNA) and adenovirus transfection and RNA sequencing

SiRNA and negative control were derived from RuiboBio (Guangzhou Development Zone, Pennant District, Guangzhou, China). SiRNA and negative controls were transfected into RASFs using the HiPerFect co-transfection reagent, following manufacturer's instructions. The sequence of siRNA was designed and synthesized by RuiboBio (Guangzhou, China), and the most effective sequence (as shown in Table 2) was used in the following experiment. RASFs (8×10^4 cells cultured in 6-well plates or 2×10^4 cells cultured in 24-well plates) were infected with UBE2L6 adenovirus or Empty adenovirus by ADV-HR (ViGeneBiosciences) as per manufacturer's instructions. For RNA sequencing, TNF-α and IL-1β stimulation after Telotristat Etiprate treatment for 24 h, total RNA was collected by Trizol method to construct a cDNA library for RNA transcriptomic sequencing by lC-Bio Technologies Co., Ltd (Hangzhou, Zhejiang, China). Gene Ontology (GO) enrichment analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to evaluate the bioactivity and signaling pathway distribution of differentially expressed Genes (DEGs).

2.13. Drug affinity responsive target stability (DARTS) assay and mass spectrometry (MS)

The tissues of RA patients (1.5 to 2.5g) were ground in liquid nitrogen and lysed on ice for 20 minutes with t-Per ™ histone extraction kit (Thermo Scientific, Waltham, MA, USA) containing protease and phosphatase inhibitors. 10 X TNC buffer (1M Tris-HCl, 5mL; 5M NaCl, 1mL; 1M CaCl2, 1mL; double distilled water, 3mL; pH7.4) was added to the cell lysate (total protein 5 mg/mL), to the desired concentration, and gently stirred. Telotristat Etiprate and protein lysates were incubated at room temperature for 1h to fully bind the target protein to the ligand, then treated with protease (dissolved in a 1 X TNC buffer) at a ratio of 1:100, 1:500, 1:1,000, 1:2,500, 1:5,000 (wt/wt), and digested at room temperature for 15 minutes. After the protein lysis products were incubated with drugs and digested by protease, 5 X protein loading buffer was added before boiling for 5-7 min. SDS-PAGE and Coomassie bright blue staining showed the bands. Finally, the gels with obvious changes were analyzed by MASS spectrometry (BGI, Beijing, China).

Table 2. siRNA sequences

siRNA name†	sequence (5′to 3′)
NR4A1	GGGACTGGATTGACAGTAT
LGALS3BP	GGACCTGTATGCCTATGCA
UBE2L6	GCTGGTGAATAGACCGAAT
HSPB3	GCACGGTTTTATCTCAAGA
PDGFRL	GCACCAAAGACGCAGTCTA

†All sequences are targeted against human genes.

2.14. Statistical analysis

Data analysis was performed by two-factor analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test performed using GraphPad Prism 7 software (La Jolla, CA, USA). Results were expressed as the mean ± SEM of five different experiments. The differences in central tendency were statistically significant. Results were considered statistically significant at a p value of < 0.05; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively.

3. Results

3.1. Improvement of inflammatory phenotype in collagen-induced model mice treated with Telotristat Etiprate

To establish an animal model of CIA, arthritis was induced in DBA/1J mice and treated by intraperitoneal injection of Telotristat Etiprate (20 mg/mL) once every day for a month. We included the following three groups: mice without CIA induction ($n = 6$), mice that did not receive drug treatment after CIA induction ($n = 6$), and mice that received drug treatment after CIA induction ($n = 6$). Two observers measured the degree of paw swelling daily and assigned a score (Figure 1, A and B). As shown in Figure 1C, the difference between front and rear paws was significant before and after treatment (swelling subsided and erythema decreased). Serum was obtained from fresh blood, and the expression of IL-6, IL-8, IL-1α, and COMP was detected by ELISA. We found that the expression of serum inflammatory factors after Telotristat Etiprate treatment was significantly reduced (Figure 1D). Micro-computed tomography (micro-CT) was used to image mouse paws, and it was evident that the joints of treated mice were significantly improved compared with those of the untreated mice (Figure 1F)This also, showed that trabecular separation (Tb.Sp, mm) was reduced, and trabecular thickness (Tb. Th, mm) and the bone volume to bone volume fraction (BV/TV, %) rise were exported for reconstruction using the manufacturer's software, Caliper Analyze (Figure 1E). Finally, aematoxylin and eosin (H&E) (Figure 1G) and Safranin O-Fast Green FCF (Figure 1H) staining and analysis demonstrated an improvement in synovial hyperplasia, monocyte infiltration, and bone destruction in mice treated with Telotristat Etiprate.

3.2. Telotristat Etiprate improves the arthritic phenotype of RASFs after treatment

To investigate the role of Telotristat Etiprate in RA, we first analysed the expression and secretion of inflammatory factors in RASFs, as RA is characterised by

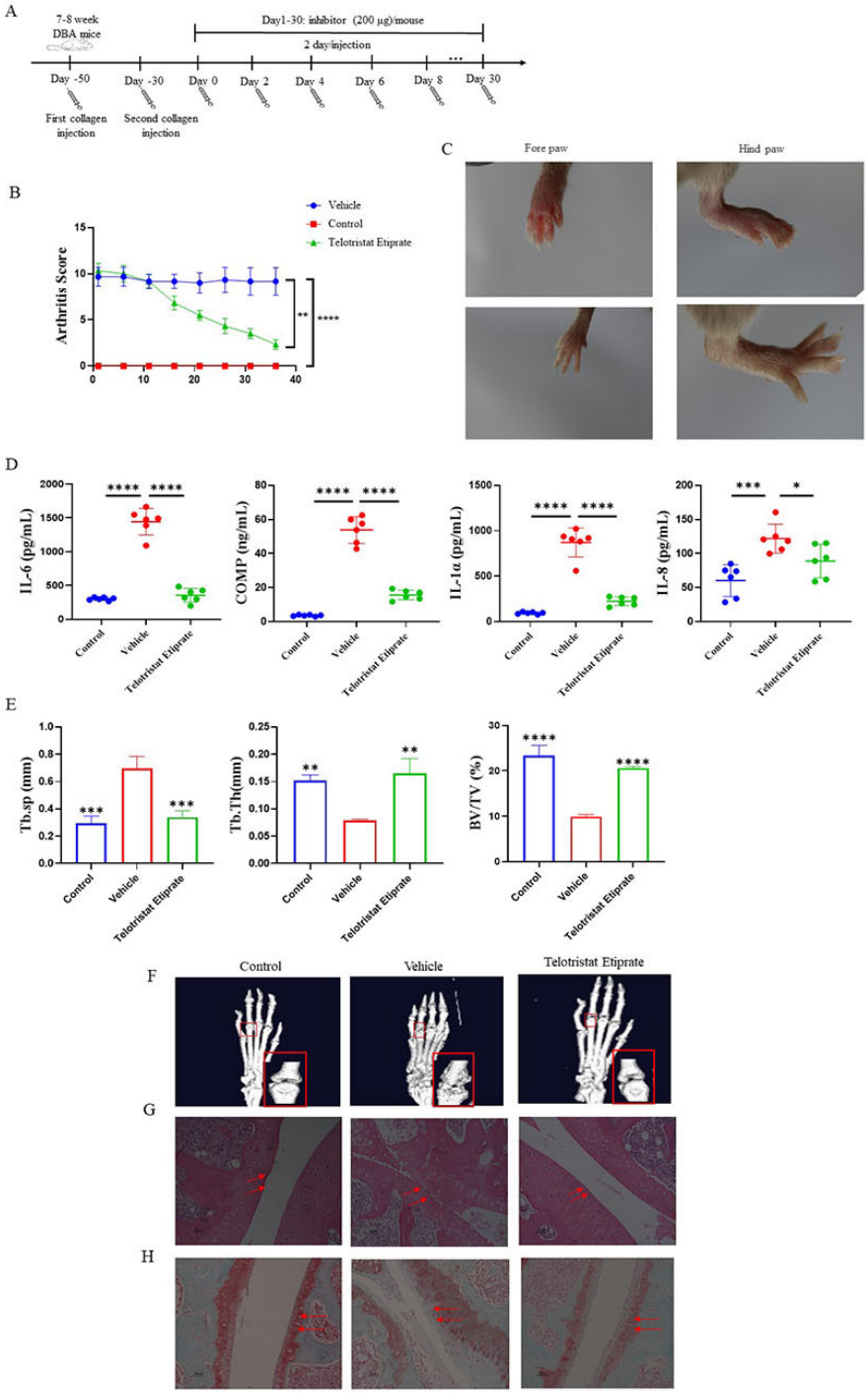

Figure 1. Improvement of inflammatory phenotype in collagen-induced model mice treated with Telotristat Etiprate. The following three groups were studied: 1. Contro group: mice without CIA induction (n = 6); 2, Vehicle group: mice injected with DMSO as control after CIA induction but not treated with Telotristat Etiprate (n = 6); 3, Telotristat Etiprate group, mice treated with Telotristat Etiprate after CIA induction (n = 6). **(A)** The overall scheme of animal experiments. **(B)** Two observers rated paw swelling (0-3 for each paw) on the first day of treatment after induction of arthritis. **(C)** Changes in swelling degree of paw (hind paw and fore paw) of mice before and after treatment. **(D)** Serum was extracted from fresh blood of mice, and the secretion of inflammatory cytokines in serum was detected by ELISA (IL-6, IL-8,IL-1α, COMP). **(E)** Analysis of mouse paws by micro-CT, trabecular separation (Tb.Sp, mm), trabecular thickness (Tb.Th, mm) and the bone volume to bone volume fraction (BV/TV, %). **(F)** Micro-CT imaging showed significant improvement in the degree of joint damage in the treated mice. **(G)** and **(H)** The histological changes of the joint were detected by HE staining and Muscovy solid green staining. All results are presented as the mean ± SEM of three independent experiments performed in triplicate; *p <0.05, **p < 0.01, ***p < 0.001.

joint inflammation. After 6 h of treatment with different concentrations of Telotristat Etiprate (1, 10, 50, and 100 ng/mL), the proinflammatory factor IL-1β (10 ng/mL) was added to stimulate the cells. After 24 h, the mRNA expression levels of *Il6*, *Il8*, and *Ccl2* were assessed (Figure 2, A and B). Cell survival rate was detected by

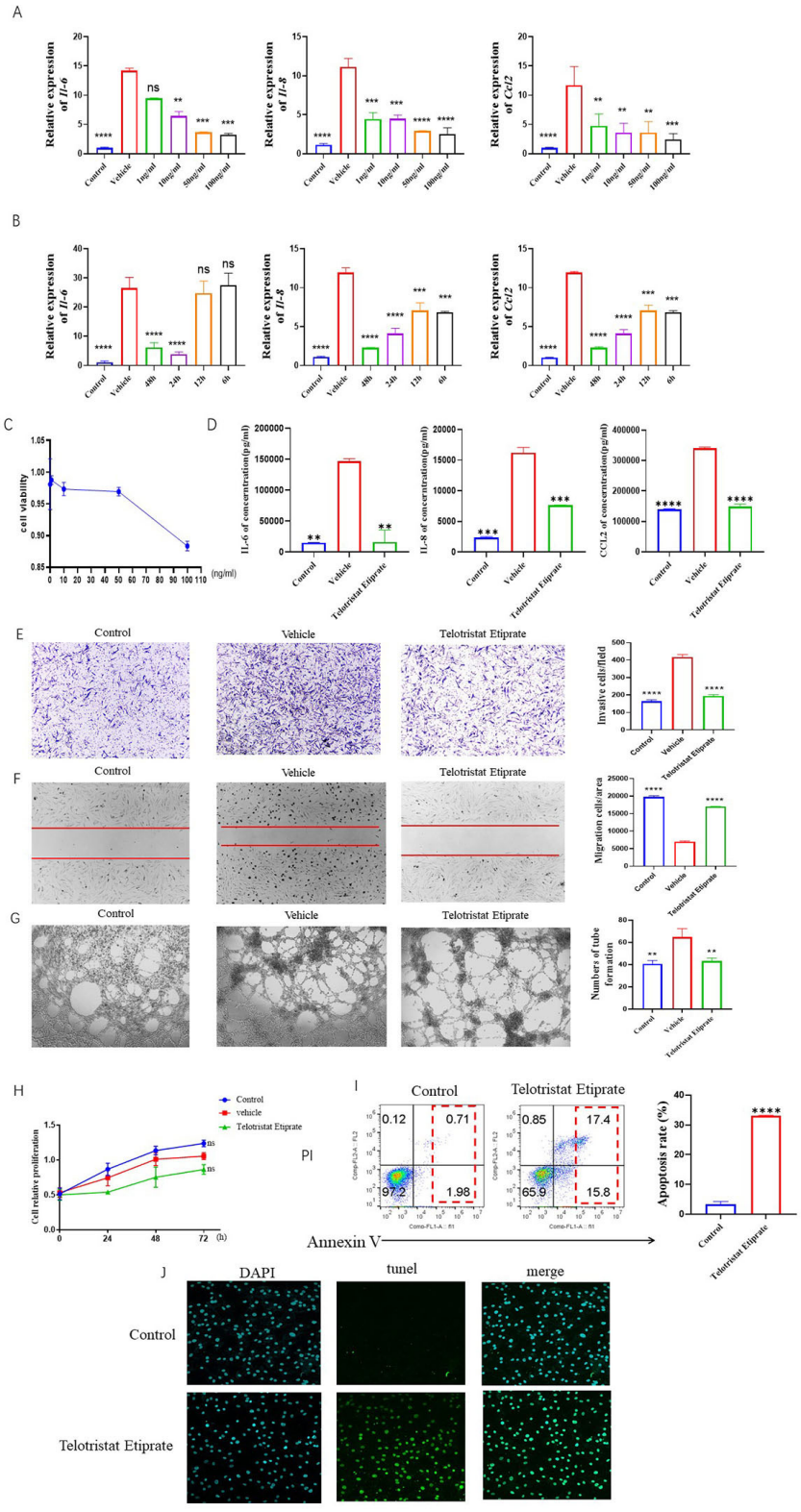

Figure 2. Telotristat Etiprate improves the arthritic phenotype of RASFs after treatment. (A) and **(B)** The optimal timing and concentration of drug action were investigated by qRT-PCR. **(C)** The survival rate of cells at different concentrations (0, 1, 10, 50, 100 ng/mL) was detected by CCK8 method. **(D)** After treatment with Telotristat Etiprate, cell supernatant was taken and the secretion of inflammatory cytokines (IL-6, IL-8, CCL2) was detected by ELISA. **(E)** Cell invasiveness was detected by the Transwell method. **(F)** Cell migration ability was detected by cell scratch method. **(G)** The effect of Telotristat Etiprate on RASF angiogenesis was detected by CRL-1730 cell assay. **(H)** The effect of Telotristat Etiprate on cell proliferation was detected by CCK8 assay. **(I)** Cell apoptosis was detected by flow cytometry. **(J)** TUNEL assay was used to further confirm Telotristat Etiprate induced apoptosis (green fluorescence). All results are presented as the mean ± SEM of three independent experiments performed in triplicate; *p <0.05, **p < 0.01, ***p < 0.001.

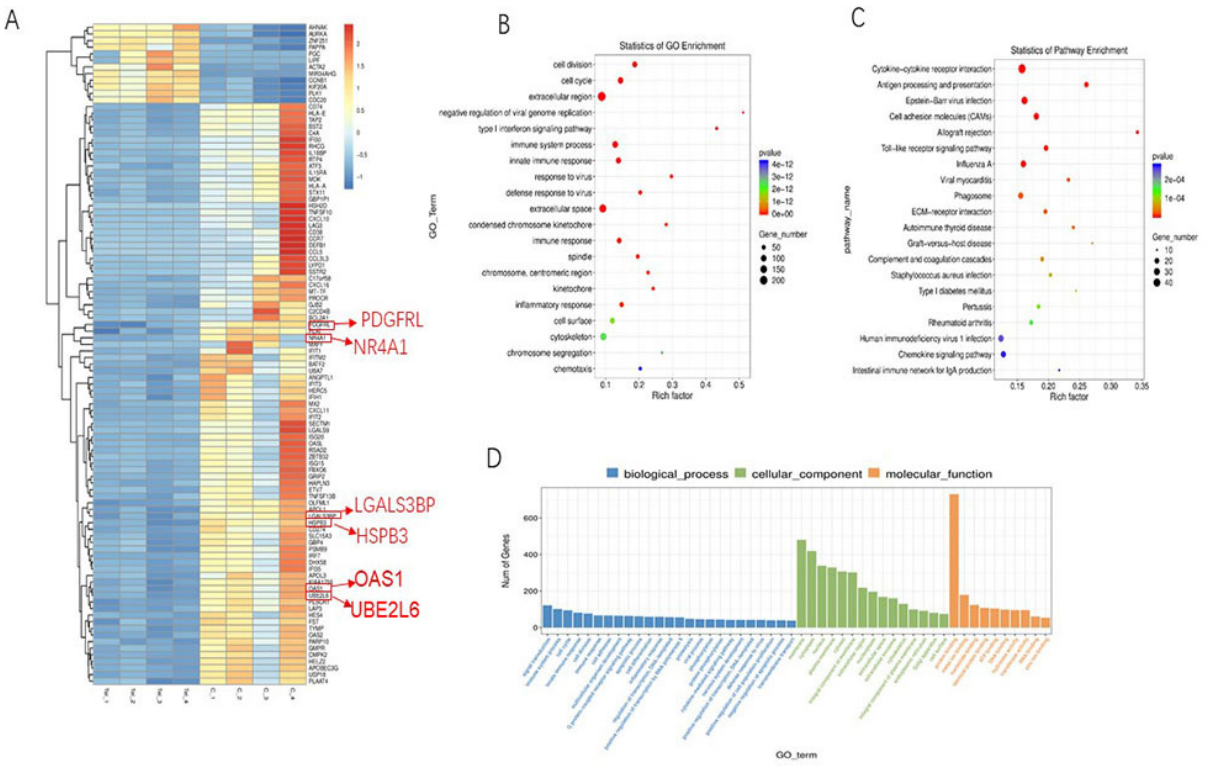

Figure 3. RNA sequencing. (A) Differentially expressed genes (DEGs) in Telotristat Etiprate treated RASFs activated by TNF-α and IL-1β were up-regulated in 562 genes and down-regulated in 597, of which the first 100 genes were significantly differentially expressed. **(B)** and **(C)** Enrichment analysis of GO classification and KEGG pathway was conducted. **(D)** In biological processes, 102 genes are involved in immune system processes, 82 genes are involved in endogenous immune responses, and 59 genes are involved in inflammatory responses.

the CCK8 method and was found to decrease when the drug concentration was 100 ng/ml (Figure 2C). However, the drug was most effective in inhibiting the expression of inflammatory cytokines and the cell survival rate was high when treated with 50 ng/mL for 24 h. The following experiments were carried out in accordance with this action time and concentration. In order to further explore the effect on the secretion of inflammatory factors in RASFs, supernatant from cells treated with the drug (50 ng/mL, 24 h)was tested for inflammatory cytokines (IL-6, IL-8, CCL2) by ELISA The results showed that it also had a certain inhibitory effect on the secretion of inflammatory factors (Figure 2D).

RASFs not only secrete a large number of inflammatory factors but also have oncology characteristics and stronger invasion and migration abilities than normal synovial cells. To investigate the invasion and migration ability of RASFs treated with Telotristat Etiprate, we used the TransWell assay and cell scratch method. The results showed that the addition of Telotristat Etiprate did inhibit the invasion and migration of cells in the experimental group compared with the control group (Figure 2, E and F). In addition, in the presence of Telotristat Etiprate, RASFs also significantly decreased their angiogenesis promoting ability (Figure 2G).

Cell proliferation was detected by the CCK8 method, and Telotristat Etiprate treatment had no significant effect on cell proliferation (Figure 2H). Flow cytometry and TUNEL method were used to detect the effect of Telotristat Etiprate on RASF apoptosis. Compared with the control group, the proportion of early apoptosis and late apoptosis in the experimental group after Telotristat Etiprate treatment was significantly increased (Figure 2I), which suggests that RASF apoptosis may be induced. In order to further verify, TUNEL method was also used to explore, as shown in Figure 2J. Compared with the control group, the apoptotic ability of Telotristat Etiprate was significantly enhanced (green fluorescence). It was further confirmed that Telotristat Etiprate may promote apoptosis of RASFs.

3.3. Telotristat Etiprate affects MAPK signaling pathway by inhibiting UBE2L6

Differentially expressed genes (DEGs), 562 up-regulated and 597 down-regulated, in Telotristat etiprate-treated RASFs activated by TNF-α and IL-1β were identified by RNA-seq analysis The first 100 genes were significantly differentially expressed (Figure 3A). Then, enrichment analysis was performed for GO classification (Figure 3B) and KEGG pathway (Figure 3C). In biological processes, 102 genes are involved in immune system processes, 82 genes are involved in endogenous immune responses, and 59 genes are involved in inflammatory responses (Figure 3D). qRT-PCR was used to verify the significantly differentially expressed genes on the

heat map, and the results showed that after Telotristat Etiprate treatment, the expression of genes related to inflammatory immune response was reduced (Figure 4A). Interestingly, it was found that these genes with reduced expression were related to MAPK and NF-KB signaling pathways. Therefore, western blot was used to detect changes in phosphorylation levels of MAPK and NF-KB signaling pathways in Telotristat Etiprate treated cells. The results showed that the key proteins involved in MAPK signaling pathway, P38, P44/42 and SAPK/JNK, were phosphorylated. However, P65, a key protein involved in the NF-KB signaling pathway was not phosphorylated (Figure 4B). Therefore, we preliminarily believe that Telotristat Etiprate treatment can affect the change in the MAPK signaling pathway.

We selected from the genes in the MAPK signaling pathways that showed the greatest decrease in expression after Telotristat Etiprate treatment (*Lgals3bp*, *Na4a1*, *Hspb3*, *Pdgfrl*, *Ube2l6*). The expression of inflammatory cytokines (*Il-6*, *Il-8*, *Il-1α*, *Il-1β*, *Ccl2*) at mRNA level was detected by qRT-PCR. The results showed that silencing these genes significantly reduced the expression of inflammatory cytokines compared with the control group. Among them, silencing UBE2L6 had the most significant inhibitory effect (Figure 4C). Next, ELISA (Figure 4D) and qRT-PCR (Figure 4E) were used to detect the effects of UBE2L6 overexpression or silencing (by adenovirus) on the expression and secretion of inflammatory cytokines (IL-6, IL-8, IL-1α, IL-1β, CCL2) in cells. The results showed that, overexpression of UBE2L6 by adenovirus increases the expression and secretion of inflammatory cytokines, while silencing UBE2L6 causes a decrease. Preliminarily, it is considereds that UBE2L6 can affect changes in the MAPK signaling pathway. To further explore, western blot was used to detect the phosphorylation of the MAPK signaling pathway after UBE2L6 was overexpressed or silenced by adenovirus. It was found that UBE2L6 could also cause phosphorylation of the MAPK signaling pathway after treatment. The effect was even more significant after IL-1β stimulation (Figure 4F). Therefore, we found that Telotristat Etiprate treatment in RASFs could affect the MAPK signaling pathway and inhibit the expression and secretion of inflammatory cytokines by inhibiting the expression of UBE2L6.

3.4. LGALS3 is a new target of Telotristat Etiprate

To elucidate the molecular basis of Telotristat Etiprate's effect on RASFs, we isolated the protein portion bound to Telotristat Etiprate. Through the implementation of Drug Affinity Responsive Target Stability (DARTS) experiments and mass spectrometry, PLD3, LGALS3, YKL140, CD11C, CASP3, RRAS2, DNAJA1, PRDX3 and YWHAG were identified as potential candidates (Figure 5A). Then, using a series of the proportion of protease and cell lysis (1:100, 1, 500, 1:1,000, 1:2,500, 1:5,000) for the DARTS samples to conduct western blot analysis, Telotristat Etiprat was found to protect PLD3, LGALS3, CD11C and YWHAG (Figure 5B). Interestingly, we found that LGALS3 is involved in the MAPK signaling pathway processes. Therefore, we preliminarily concluded that Telotristat Etiprate plays a biological role by binding to target protein LGALS3.

3.5. Telotristat Etiprate combine with LGALS3 to inhibit UBE2L6 and affect MAPK signaling pathway

In order to further explore the influence of LGALS3 on the MAPK signaling pathway, we used FHPI (0, 1, 10, 50, 100 ng/mL), an inhibitor of MAPK signaling pathway, and western blot method and found that the expression of LGALS3 decreased when the inhibitor FHPI was added. In addition, the inhibition effect was more significant when IL-1β was added (Figure 5C). Next, we further found through immunofluorescence that in the presence of MAPK inhibitor FHPI, the expression of LGALS3 decreased and also showed a concentration dependence (Figure 5D). Therefore, we believe that Telotristat Etiprate can affect the MAPK signaling pathway after binding to target protein LGALS3. Then, we further investigated whether LGALS3 could affect the expression and secretion of inflammatory cytokines in RASFs. qRT-PCR (Figure 5E) and ELISA (Figure 5F) experiments showed the expression and secretion of inflammatory cytokines in RASFs were decreased after silencing LGALS3, indicating that LGALS3 can indeed affect the expression and secretion of inflammatory cytokines in cells. In conclusion, Telotristat Etiprat can combine with target protein LGALS3 to inhibit UBE2L6 expression, thereby affecting the MAPK signaling pathway, inhibiting the expression of inflammatory cytokines in RASFs, and providing some relief of rheumatoid arthritis.

4. Discussion

Once a patient is diagnosed with rheumatoid arthritis, the overall treatment goal is to achieve a complete remission or at least a significant reduction in disease activity within approximately 6 months to prevent joint damage, disability, and systemic manifestations of rheumatoid arthritis (*14,15*). The importance of timely and targeted treatment of RA is highlighted by the fact that 80% of patients who are inadequately treated will develop a dislocated joint and 40% will be unable to work within 10 years of onset (*16*). The most common way to treat the disease is with anti-rheumatic drugs (DMARDs) (*17,18*). However, these drugs are not a complete cure and often cause serious side effects due to lack of long-term use experience. Fortunately, there is growing recognition that drugs can work by targeting multiple proteins. In addition, biological pathways and networks are abundant and powerful, so affecting only a single target can easily

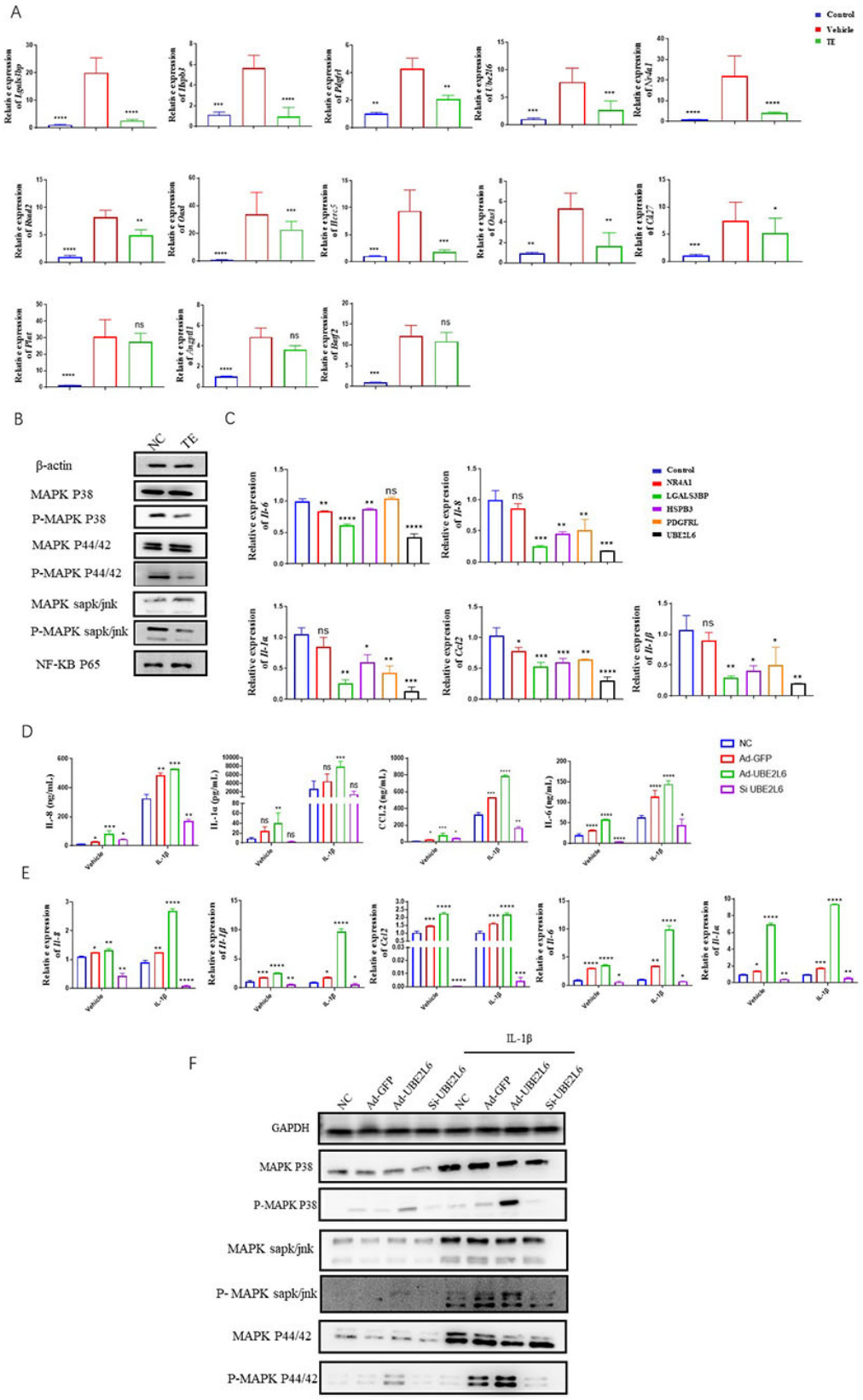

Figure 4. Telotristat Etiprate affects MAPK signaling by inhibiting UBE2L6. (A) qRT-PCR was used to verify the significant differentially expressed genes in RNA-SEQ sequencing results. **(B)** Western blot was used to investigate the effects of Telotristat Etiprate on phosphorylation of MAPK and NF-KB signaling pathways. **(C)** Silencing genes related to MAPK signaling pathway and detecting its effect on the expression of inflammatory cytokines in cells. **(D)** and **(E)** The effects of UBE2L6 silencing and adenovirus overexpression on the expression and secretion of inflammatory cytokines in cells were investigated by ELISA. **(F)** Western blot analysis of the phosphorylation of MAPK signaling pathway by UBE2L6 silencing and adenovirus overexpression. All results are presented as the mean ± SEM of three independent experiments performed in triplicate; *p <0.05, **p < 0.01, ***p < 0.001.

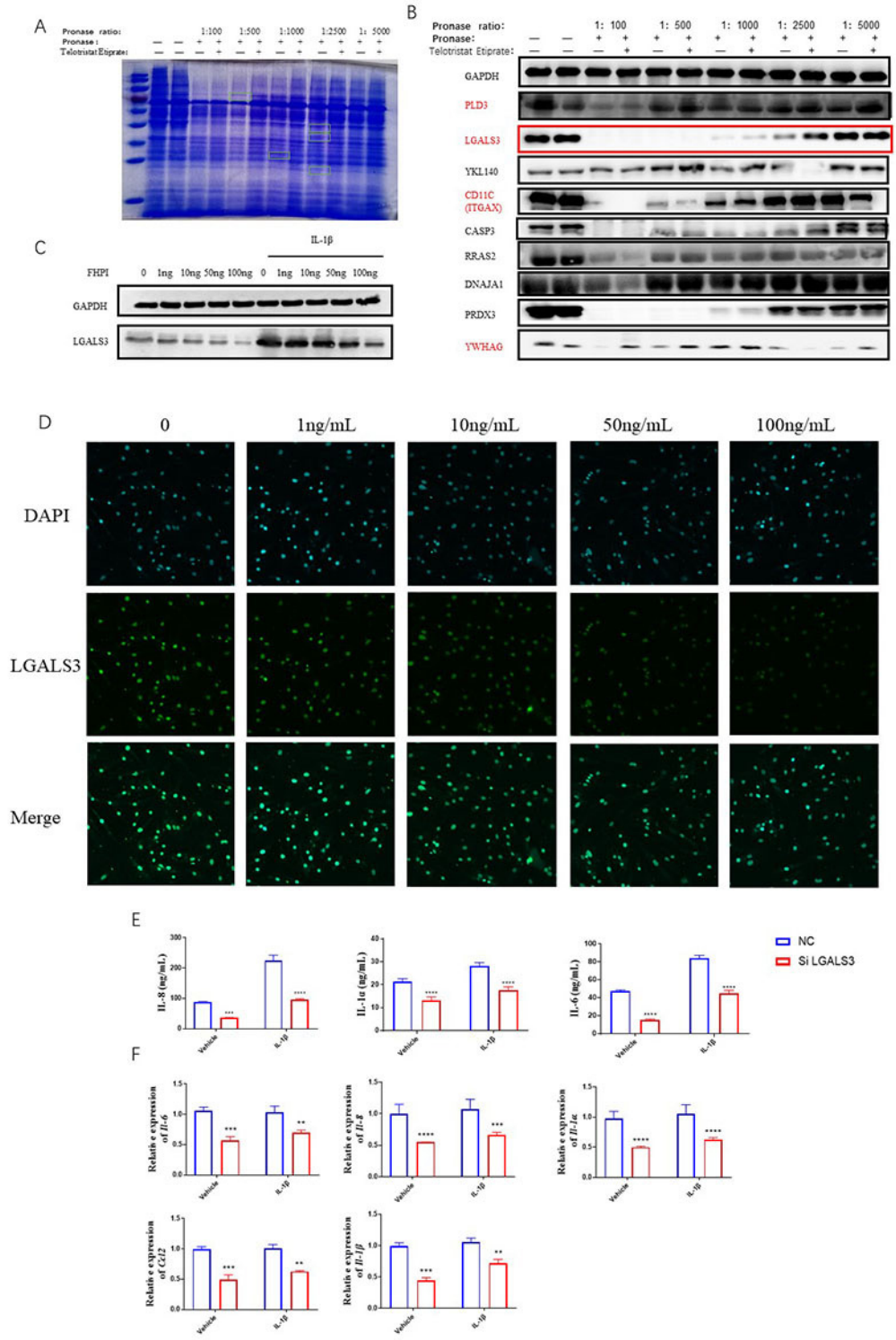

Figure 5. Binding of Telotristat Etiprate with LGALS3 inhibited UBE2L6 from affecting the MAPK signaling pathway. (A) Protein lysates were separated by electrophoresis after DARTS experiment and stained with Coomassie bright blue dye for mass spectrometry sequencing analysis. **(B)** Western blot was used to verify the results of mass spectrometry analysis. **(C)** Adding MAPK signaling pathway inhibitor FHPI (0, 1, 10, 50, 100 ng/mL) to explore its effect on LGALS3 expression. **(D)** Further study of the inhibition of the MAPK signaling pathway by immunofluorescence can reduce the expression of LGALS3 in a concentration dependent manner. **(E)** and **(F)** LGALS3 was silenced to detect its effects on the expression and secretion of inflammatory cytokines in cells. All results are presented as the mean ± SEM of three independent experiments performed in triplicate; *p <0.05, **p < 0.01, ***p < 0.001.

fail to produce the desired therapeutic effect (*19-21*). In short, we can develop drugs to treat RA using models that predict multiple drug-target interactions.

Telotristat Etiprat is a tryptophan hydroxylase (TPH) inhibitor used in the treatment of carcinoid syndrome. Many neuroendocrine tumors secrete

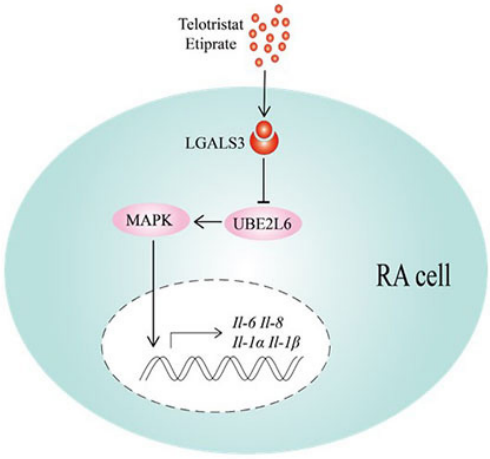

Figure 6. Telotristat Etiprate can improve RA by inhibiting UBE2L6 expression and affecting the MAPK signaling pathway after targeted binding with LGALS3.

5-hydroxytryptamine (5-HT) into the bloodstream, causing many symptoms, especially diarrhea. Telotristat Etiprat inhibits TPH, thus reducing the production of 5-HT (*22*). It has been reported that mice lacking 5-HT transporter (SERT; SERTKO mice) inactivated 5-HT and were oversensitive to both experimentally induced colitis and spontaneous colitis due to interleukin-10 (IL-10) deficiency (*23,24*). In contrast, mice deficient in tryptophan hydroxylase (TPH1), the rate-limiting enzyme for 5-HT biosynthesis by intestinal chromatin cells, were resistant to experimentally induced colitis. Thus, when these mechanisms, or the resulting inflammation, become overactive or dysfunctional, it may be advantageous for intestinal chromaffin cells depleted of serotonin to decouple the serotonin drive to inflammation. In addition, serotonin is thought to have a pro-inflammatory effect in animal models. When administered with a reversible inhibitor of tryptophan hydroxylase, a rate-limiting enzyme synthesized by serotonin, inflammation in arthritis-induced mice was significantly reduced by 30-40% (*25*). In this study, Telotristat Etiprat treatment reduced the invasion and migration ability of RASFs, induced apoptosis of cells, and also inhibited the expression of inflammatory cytokines. Telotristat Etiprat significantly improved joint swelling and erosion in mice in a collagen-induced arthritis (CIA) model. However, through RNA-SEQ and mass spectrometry sequencing results, we found that Telotristat Etiprat could target LGALS3 to inhibit UBE2L6 expression in RA, thereby affecting the MAPK signaling pathway and alleviating RA.

Galectins-3 (LGALS3) is controversial in terms of proinflammatory or anti-inflammatory activity, depending on a variety of factors, including its intracellular or extracellular localization and the target cells involved in these processes (*26*). Although it may help with inflammation by clearing apoptotic neutrophils, it exhibits pro-inflammatory effects primarily by enhancing the activation of macrophages, Dendritic cells, mast cells, natural killer cells, and T and B lymphocytes (*27*). LGALS3 is considered a pro-inflammatory mediator in both RA patients and animal models. LGALS3 mRNA and protein were detected in synovium, while LGALS3 binding protein was mainly expressed at the site of bone destruction (*28*). RASFs express high levels of CD51 and CD61 integrins, which bind to cartilage oligomic matrix proteins alone or by forming αVβ3 complex (Vitronectin receptor) to induce LGALS3 secretion (*29*). The externalization of this lectin affects the morphology and persistence of joint inflammation by inducing local fibroblasts to secrete pro-inflammatory cytokines (including IL-6, GM-CSF and MMP-3) and chemokines (such as CCL2, CXCL8, CCL3 and CCL5) (*30*). LGALS3 stimulates RASF's secretion of IL-6, mediated by Toll-like receptors -2, -3, and -4, amplifying the pro-inflammatory effect (*31*). In this study, Telotristat Etiprat was found to have a protective effect on LGALS3, and silencing LGALS3 reduced the expression and secretion of inflammatory cytokines in RASFs. These results suggest that LGALS3 can be used as a new target of Telotristat Etiprat to improve RA. In addition, LGALS3 is related to the MAPK signaling pathway (*32*), which corresponded with our RNA-seq sequencing analysis results, further indicating that Telotristat Etiprat could affect the MAPK signaling pathway by binding to LGALS3.

UBE2L6, an E2 ubiquitin/ISG15 binding enzyme, plays a decisive role in targeting c-MyC proteasome degradation by interacting with E3 ubiquitin ligase, thereby inhibiting cell proliferation and xenograft tumor growth (*33*). After Telotristat Etiprat treatment, transcriptome sequencing analysis showed that the expression of UBE2L6 was most significantly decreased in RASFs, and the MAPK signaling pathway was phosphorylated, suggesting that Telotristat Etiprat may affect the MAPK signaling pathway by inhibiting UBE2L6 expression. To confirm this idea, we found that the expression and secretion of inflammatory cytokines (IL-6, IL-8, IL-1α, IL-1β, CCL2) in cells can be inhibited/promoted by silencing and adenovirus overexpression of UBE2L6. In addition, UBE2L6 silencing or adenovirus overexpression can also lead to phosphorylation of the MAPK signaling pathway. Therefore, we believe that Telotristat Etiprat can inhibit the expression of UBE2L6 by combining with LGALS3, thus affecting MAPK signaling pathway and improving RA (Figure 6).

In conclusion, this study found that Telotristat Etiprat may play a certain role in improving RA (by inhibiting the expression and secretion of inflammatory cytokines in cells, inhibiting invasion and migration, inhibiting angiogenesis, and inducing apoptosis). In addition, through RNA-SEQ and mass spectrometry analysis, we found that Telotristat Etiprat could inhibit UBE2L6 expression and affect the MAPK signaling pathway

through targeted binding with LGALS3, thus improving RA.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Jihong Pan, Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, 6699 Qingdao Road, Ji'nan 250062, China.
E-mail: panjihong@sdfmu.edu.cn

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Correspondence

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Type XV osteogenesis imperfecta: A novel mutation in the *WNT1* gene, c.620G >A (p.R207H), is associated with an inner ear deformity

Jicai Zhu[1], Kai Liu[1], Shan He[1], Zihao Yang[2], Jiaying Song[3], Yan Ju[3], Caiyun Xiong[3], Guomei Zhang[1], Wen Yang[3], Chunhui Tang[1,*]

[1] Department of Pediatrics, The First People's Hospital of Yunnan Province, Medical School & Affiliated Hospital, Kunming University of Science and Technology, Kunming, Yunnan, China;
[2] Medical School & Affiliated Hospital, Kunming University of Science and Technology. Department of Radiology, The First People's Hospital of Yunnan Province, Kunming, Yunnan, China;
[3] Medical School & Affiliated Hospital, Kunming University of Science and Technology. Department of Pediatrics, The First People's Hospital of Yunnan Province, Kunming, Yunnan, China.

SUMMARY The Wnt signaling pathway is vital in encouraging bone growth. *WNT1* gene mutations have been identified as the major cause of type XV osteogenesis imperfecta (OI). Described here is a case of complex heterozygous *WNT1* c.620G>A (p.R207H) and c.677C >T (p.S226L) OI caused by a novel mutation at locus c.620G >A (p.R207H). The female patient had type XV OI, distinguished by poor bone density, frequent fractures, a small stature, skull softening, lack of dentine hypoplasia, a brain malformation, and obvious blue sclera. A CT scan of the temporal bone revealed abnormalities of the inner ear, necessitating a hearing aid 8 months after birth. There was no family history of such disorders in the proband's parents. The proband inherited complex heterozygous *WNT1* gene variants c.677C>T (p.S226L) and c.620G>A (p.R207H) from her father and mother, respectively. Presented here is a case of OI with inner ear deformation caused by c.620G>A (p.R207H), which is a novel *WNT1* site mutation. This case broadens the genetic spectrum of OI and it provides a rationale for genetic testing of mothers and a medical consultation to estimate the risk of fetal illness.

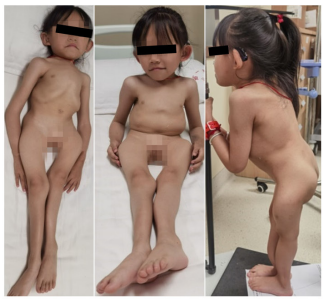
Keywords type XV osteogenesis imperfecta, heterozygous mutation, *WNT1*, inner ear dysplasia

Osteogenesis imperfecta (OI) is a condition with various morphologies caused by insufficient type I collagen production (*1*). It is distinguished by brittle bones and frequent fractures, which may result in bone abnormalities (*2*). Mutations in the *WNT1* gene generate type XV OI, which is responsible for severe (gradually deformed) recessive OI (*3*). Classic *WNT1* signaling is essential for optimal bone growth and maintenance of bone homeostasis. Patients with *WNT1* mutations have lower bone mineral density, more fractures, a shorter stature, and blue sclera (*4-9*), and some also had cognitive difficulties, developmental delays, and central nervous system problems. Over the past few years, congenital ptosis has been described in a few cases (*8,10,11*). No congenital hearing impairment (inner ear hypoplasia) or encephalomalacia has been reported in children heterozygous for the *WNT1* mutation. Described here is a female patient with type XV OI and impaired hearing, cranial softening, frequent fractures, and no family history. The female toddler had a complex heterozygous mutation of the *WNT1* gene. The location of c.677C >T (p.S226L) has been reported, but c.620G >A (p.R207H) is novel.

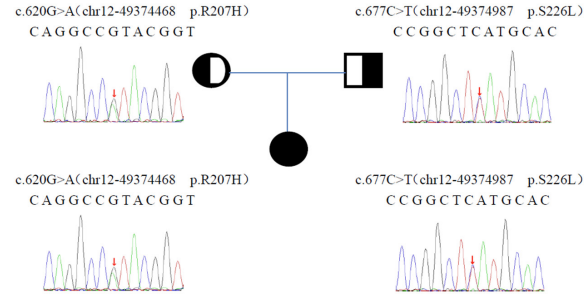
1. Clinical manifestations in a rare case

The proband (Figure 1A, B) was 8.5 years old and born to her mother, G1P1, after 39 weeks of pregnancy at the age of 22. She was born *via* cesarean section at Kunming Children's Hospital, weighed 2.6 kg, and measured 50 cm. There was no history of asphyxia or family history of asphyxia. A general medical examination of the proband revealed a height of 93 cm (-3SD), a weight of 14 kg (-3SD), a brain circumference of 50 cm, and physical asymmetry (left side length: 94 cm, right side length: 97.5 cm, left lower limb: 47.5 cm, right lower limb: 49 cm). Hearing loss was apparent, necessitating hearing aids, and both lower limbs have sustained several fractures. The proband was able to walk after one year of age, but she is still unable to walk alone.

(A)



(B)



(C)

The gene locus of c.677C >T (p.S226L) was analyzed by protein function prediction software

REVEL	D(0.8)	Polyphen2	Probably_damaging(0....	MutationTaster	Disease_causing(1)	MCAP	P(0.19950235)
SIFT	Damaging(0.001)	LRT	D(0)	GERP	Conserved(4.44)	dbscSNV	-
SPIDEX	-	SpliceAI	-	ClinPred	0.98719072		

The gene locus of c.620G >A (p.R207H) was analyzed by protein function prediction software

REVEL	D(0.931)	Polyphen2	Probably_damaging(0....	MutationTaster	Disease_causing(1)	MCAP	P(0.43134233)
SIFT	Damaging(0)	LRT	D(0)	GERP	Conserved(4.94)	dbscSNV	-
SPIDEX	-0.0467	SpliceAI	F(0.04)	ClinPred	0.99968004		

(D)

(E)

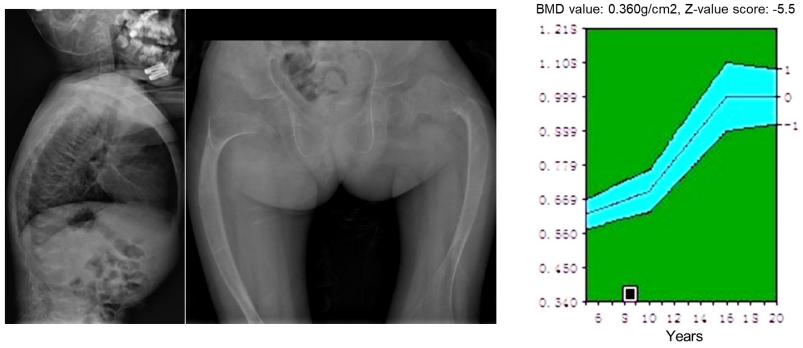


Figure 1. Clinical phenotype and Sanger sequencing results for the proband. **(A)** The proband was 8.5 years old, had a weight of 8.5 kg, a height of 75 cm, a brain circumference of 50 cm, and physical asymmetry (left side length: 94 cm, right side length: 97.5 cm, left lower limb: 47.5 cm, right lower limb: 49 cm). **(B)** The proband had a complex heterozygous variation of *WNT1* gene c.677C>T (p.S226L) and c.620G>A (p.R207H) from her father and mother respectively. **(C)** The two sites were predicted to be harmful according to REVEL, which is a bioinformatics program that compiles scores from multiple tools to predict the pathogenicity of missense variants. The mutations were predicted to be harmful according to SIFT, possibly harmful according to PolyPhen_2, harmful according to MutationTaster, and the sites were predicted to be conserved according to GERP+. **(D)** A plain CT scan of the temporal bone revealed bilateral inner ear dysplasia. **(E)** An X-ray bone density meter revealed a significant decrease in bone mineral density (BMD: 0.360 g/cm^2, Z-value score: -5.5). The X-ray revealed flattening of thoracic 4 to lumbar 5 vertebrae in a double concave shape, enlargement of the bilateral upper femur, deformity of the diaphysis, decreased bone density, sparse trabecular bone, thin bone cortex, and sequelae.

A temporal bone CT scan indicated bilateral inner ear dysplasia (Figure 1D). An X-ray bone density meter revealed a considerable reduction in bone mineral density (BMD: 0.360 g/cm^2, Z-value score: -5.5). The X-ray revealed a twofold concave flattening of the thoracic 4 to lumbar 5 vertebrae, expansion of the bilateral upper femur, deformity of the diaphysis, reduced bone density, scant trabecular bone, thin bone cortex, and sequelae (Figure 1E).

An eye examination revealed no substantial abnormalities, and a brain MRI revealed no significant abnormalities in the parenchyma.

Calcium serum levels were normal at 2.50 mmol/L, phosphorus levels were normal at 1.47 mmol/L, alkaline phosphatase levels were normal at 220 U/L, vitamin D levels were normal at 30.2 ng/mL, calcitonin levels were normal at 2.95 pg/mL, and parathyroid hormone levels were normal at 31.30 pg/mL.

The proband in this case inherited a complex heterozygous mutation of the *WNT1* gene from both parents, c.677C>T (p.S226L) and c.620G>A (p.R207H). REVEL, which is a comprehensive bioinformatics program that compiles scores from multiple tools to predict the pathogenicity of missense variants, predicted that these two mutations were detrimental, while SIFT predicted that they were harmful, PolyPhen 2 predicted that they were probably harmful, MutationTaster predicted that they were dangerous, and GERP+ predicted that the sites were conserved (Figure 1C). Her mother had a heterozygous *WNT1* mutation (c.620G >A (p.R207H)) while her father had a *WNT1* mutation (c.677C >T) (p.S226L). Her father is 33 years old (height: 171 cm, weight: 75 kg), and her mother is 30 years old (height:144 cm, weight: 45.5 kg), and neither has clinical symptoms of OI.

This study was approved by the ethics committee of the First People's Hospital of Yunnan Province, and written informed consent was obtained from a parent prior to this study.

2. Experience and insights

Type XV OI is uncommon; as the number of patients increases, new phenotypes will appear, and the phenotype-genotype relationship will be explored in greater depth. Type XV OI is an autosomal-recessive disorder in which individuals have either a *WNT1* homozygous mutation or a complex heterozygous mutation. The most prevalent mutation in the *WNT1* gene is c.677C >T (p.S226L). The clinical features of a type XV OI patient from a non-related family with a compound heterozygous missense *WNT1* mutation, c.620G>A(p.R207H) and c.677C >T, have been described here. The pathogenic variant c.677C >T (p.S226L), which changes amino acid 226 from serine to leucine, is a missense variant. According to the Human Gene Mutation Database (HGMD), it may result in the development of type XV OI (*8,12*). Another known missense mutation is c.620G>A (p.R207H), which results from a change in amino acid 207 from arginine to histidine. This missense mutation is sporadic in the population and has not been documented in the HGMD. REVEL, a sophisticated bioinformatics program that compiles scores from multiple tools to predict the pathogenicity of missense variants, indicated that these two mutations were detrimental. However, the proband's parents did not exhibit symptoms, indicating recessive inheritance. The pathogenicity of the c.677C>T (p.S226L) mutation has been well established in previous studies, but the pathogenicity of the c.620G>A (p.R207H) mutation has not been documented. The proband had apparent clinical signs of OI, and the gene mutation satisfied the genetic co-separation requirement. c.620G>A (p.R207H) is a unique *WNT1* gene mutation with new clinical signs. However, its precise mechanism needs to be investigated further in additional patients.

Described here is a patient with type XV OI with heterozygous mutations in *WNT1* genes (c.620G >A (p.R207H) and c.677C >T (p.S226L) from her father and mother, respectively. The child's symptoms include hearing loss, cranial softness, and repeated fractures. The most notable clinical feature of the case is inner ear dysplasia. It is uncommon in people with this type of OI. Gradual hearing loss has been described in some individuals with classic OI caused by the *COL1A1* or *COL1A2* genes. The processes, onset, and severity of these two types of hearing impairment vary. There are no reports of hearing loss in people with the c.677C >T (p.S226L) homozygous mutation. More research is required to determine if the proband's hearing impairment was caused by c.620G>A(p.R207H) alone or by interaction of c.620G>A(p.R207H) and c.677C>T(p.S226L). At the age of two months, the proband was diagnosed with inner ear dysplasia. Following treatment with a calcium supplement, there was no substantial progress until she received a hearing aid at 8 months. Thus, her language development is delayed for her age.

In conclusion, novel mutations in *WNT1* locus c.620G>A (p.R207H) or the interaction of c.620G>A (p.R207H) and c.677C>T (p.S226L) may be responsible for the new clinical symptoms of type XV OI described here, and early intervention is required to prevent and manage language and intellectual disabilities. Addition of the *WNT1* locus c.620G>A (p.R207H) broadens the genetic spectrum of OI and offers grounds for genetic testing of mothers and a medical consultation to estimate the risk of fetal illness.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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**Address correspondence to:*
Chunhui Tang, Department of Pediatrics, The First People's Hospital of Yunnan Province, Kunming 650021, Yunnan, China.
E-mail: ynkmtch@163.com

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Correspondence

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Re-evaluation of the symptoms of Hirayama disease through anatomical perspective

Bahar Tekin[1,2,*], Gamze Ansen[1,2], Tugrul Ormeci[3], Nesrin Helvaci Yilmaz[4], Bayram Ufuk Sakul[1]

[1] Department of Anatomy, Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkey;
[2] Department of Anatomy, School of Medicine, Institute of Health Sciences, Istanbul Medipol University, Istanbul, Turkey;
[3] Department of Radiology, Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkey;
[4] Department of Neurology, Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkey.

SUMMARY Hirayama disease is a rare disease of the anterior horn motor neuron caused by compression of the cervical spinal cord when the neck is flexed. Cervical myelopathy may accompany the disease. It is characterized by symmetrical or asymmetrical muscle weakness and atrophy of muscles innervated by lower cervical and upper thoracic motor neurons. We recorded two male cases of Hirayama disease between the ages of 15 and 21 based on magnetic resonance imaging (MRI) features obtained from the cervical neutral state and from the flexion position which appeared in the right upper extremity. Loss of strength and atrophy in the right upper extremities was existent in clinical findings of these patients. When MRI was taken in the flexion position, there were dilated veins as hypointense signal void on T2 weighted series in posterior epidural area. The contrast enhancement was seen on these veins. It was observed that the posterior dura was displaced anteriorly and the anterior subarachnoid space was narrow. In cases which show clinical findings such as atrophy and loss of strength, having normal MRI results obtained in the neutral position makes it difficult to diagnose Hirayama Disease. In case of a suspicion of Hirayama disease the diagnosis can be made more easily by MRI taken in the flexion position. These case reports aim to bring Hirayama disease to mind and optimize the management of affected individuals

Keywords cervical myelopathy, Hirayama, Juvenile spinal muscular atrophy, Monomelic amyotrophy, cervical MRI

Hirayama disease was first reported in 1959 in Japan by Keizo Hirayama, *et al.* as "juvenile muscular atrophy of the unilateral upper extremity" (*1*). It also takes place in the literature as "juvenile asymmetric segmental spinal muscular atrophy, benign focal amyotrophy, oblique atrophy due to brachioradial muscle involvement, and monomenic amyotrophy" (*2*).

Although it is seen more often in Asian countries such as Japan, India and Taiwan (*3*) similar cases have been reported from different countries as well. The MRI signal abnormalities and spinal cord atrophy are the characteristic radiological features of the disease. While C5-7 levels are especially affected in patients from western countries, it has been reported in the literature that C7-T1 levels are affected more in patients from Asian countries (*4*).

This disease is seen more often among young men between the ages of 15 and 25, and is characterized by asymmetric muscle weakness and atrophy in related muscles by affecting C7, C8 and T1 myotomes. We diagnosed Hirayama disease after examining the clinical findings and the affected anatomical structures supporting these findigs in the MRI taken in neutral and in flexion position of two male cases, aged 15 and 21, who visited our clinic with the complaint of muscle weakness in the right upper extremities.

1. Clinical manifestation of a rare disease

Case 1 A 15-year-old male patient presented with complaints of progressive weakness in the right forearm and hand for 3 months. The case applied to the clinic in 2021. Patient's anamnesis showed no family history of neuromuscular disease, no comorbidities and no cervical trauma.

clinical evaluation showed that the strength of the right hand interosseal muscles was 3/5, the right thumb abductor muscles, the right wrist and the extensor muscle strength of the 2nd and 5th fingers were evaluated as 4/5 according to Medical Research Council Scale (*5*).

On inspection, atrophy in the first dorsal interosseal muscle of the right hand, and moderate atrophy in the other intrinsic hand muscles and the flexor and extensor muscles of the wrist were observed. The patient did not have loss of sensation and deep tendon reflexes, fasciculation and pain, and tremor was observed only in the fingers. Left upper and lower extremity neurological findings were normal, and there was no sign of pyramidal tract involvement. The signs of Babinski reflex and Hoffman were negative. Laboratory findings were normal.

After the electromyography (EMG) was examined, low amplitude in the activation of the muscles innervated by the right ulnar nerve was discovered while the left ulnar nerve and median nerve activation on both sides were found normal. In summary, active denervation was found on the right side C7, C8 and T1 myotomes. In addition to these clinical findings, MRI examinations were also performed in this patient. Since that the MRI findings in the neutral position were normal, the patient's MRI was taken in the full neck flexion position (by touching mandible to the sternum). As a result of these examinations, posterior internal vertebral plexus (Batson's plexus) dilatation was observed due to the anterior displacement of the posterior dura mater. It was observed that the enlargement in the posterior epidural space disappeared when placed in the neutral position. As a result of these evaluations, the patient was diagnosed with Hirayama disease. The patient and his family were informed of these findings and a conservative treatment was recommended. Since first applied there has been no change in the clinical and neurological findings of the patient.

Case 2 A 21-year-old male patient presented with complaints of weakness in the right upper extremity distal region and hand in 2021. Patient's anamnesis showed no family history of neuromuscular disease, no comorbidities and no cervical trauma. The clinical evaluation showed atrophy in the right thenar region of the right hand, and the muscle strength of the ipsilateral forearm extensor muscles was evaluated as 3/5 according to Medical Research Council Scale (*5*). The patient did not have loss of sensation and deep tendon reflexes, fasciculation or pain. Left upper and lower extremity neurological findings were normal, and there was no sign of pyramidal tract involvement. The signs of Babinski reflex and Hoffman were negative. Laboratory findings were normal.

After EMG was examined, low amplitude in the activation of the muscles innervated by the right median nerve was discovered. In summary, active denervation was found in C5-T1 myotomes. In addition to the clinical findings, MRI examinations were also performed in this patient. In the MRI findings taken in the flexion position, there were dilated veins which contrast enhancement in postcontrast series and which were as hypointense signal void in T2 series in posterior epidural area along the vertebral column begining from the C5 vertebra level to the upper thoracic level. Based on anterior displacement of the posterior wall of the dura mater and narrowing of the subarachnoid space in the anterior, we concluded that this patient had Hirayama disease. The findings described in dynamic MRI were not observed in post-contrast sagittal series taken in neutral position after flexion, and venous engorgement was not detected in this position. Neither a significant atrophy in spinal cord nor signal changes consistent with myelopathy were observed. Since first applied there has been no change in the clinical and neurological findings of the patient.
The informed consent was obtained from the patients for all descriptions (Figure 1 and Figure 2).

2. Insight into Hirayama disease

Theories proposed in the literature for Hirayama disease usually involve the anterior horn of the spinal cord at the lower cervical and upper thoracic levels. Therefore, the disease which has not yet been fully explained is likely to

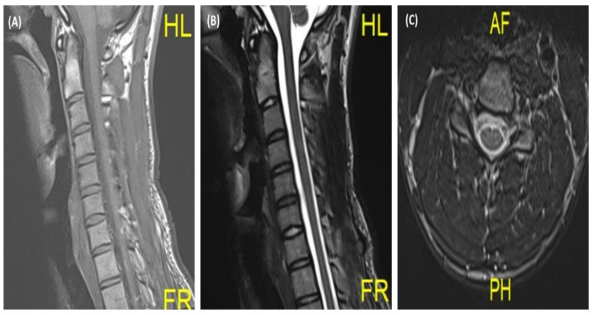

Figure 1. Sagittal turbo spin echo (TSE) T1 (A), sagittal turbo spin echo (TSE) T2 (B), axial turbo spin echo (TSE) T2 (C) images are seen as normal in standard cervical MRI protocol.

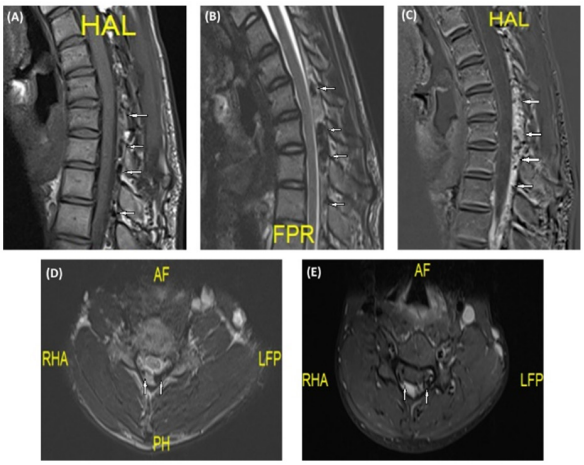

Figure 2. (A) Sagittal turbo spin echo (TSE) T1, sagittal turbo spin echo (TSE) T2 (B), sagittal post-contrast spectral presaturation with inversion recovery (SPIR) (C), axial turbo spin echo (TSE) T2 (D), axial post-contrast spectral presaturation with inversion recovery (SPIR) (E) images are taken in flexion position. In this position, venous engorgement and contrast enhancement (arrows) in posterior epidural space are evident.

be confused with other pathologies and the pathogenesis (*6,7*). The most widely accepted of these theories is cervical myelopathy due to neck flexion, which was introduced by Kikuchi *et al*. (*8*).

Anatomically the spinal dura mater is tightly attached to the dorsal periosteum along the vertebral canal surrounding the spinal nerves in healthy individuals whereas the dura mater in Hirayama patients is loosely attached to the posterior longitudinal ligament within the spinal canal. During cervical flexion, the cervical vertebral column can extend approximately 3 cm and the loose dura mater cannot adapt itself to this significant change in length. According to the theory of Kikuchi *et al*., in patients diagnosed with Hirayama disease, the dura mater remains relatively short and rigid compared to the vertebral canal and cannot compensate for the lengthening of the vertebral canal during neck flexion. During neck flexion, the spinal cord is exposed to compression between the vertebral corpuscles and the dura mater because of the anterior displacement of the posterior dura mater. As a result of repetitive neck flexions, the anterior part of the spinal cord and the vessels feeding the anterior horn (especially the anterior spinal arteries) are exposed to compression resulting in ischemia attacks and ultimately chronic traumas of the anterior horn. In the affected segment, denervation and atrophy occur in related muscles due to the anterior horn compression and the ischemia. The asymmetrical thinning of the lower cervical cord in MRI findings explains this myelopathy in the muscles (*9*) (Figures 3A and 3B).

In both cases where we analyzed the MRI findings, we observed that the posterior wall of the dura mater was displaced anteriorly in the flexion position, causing compression on the spinal cord and venous plexus. However, this pressure disappeared when the neck was put back in the neutral position. In addition, we did not observe significant atrophy of the spinal cord or signal changes consistent with myelopathy on MRI in these two patients. In our cases, as stated in the literature, especially lower cervical region involvement was dominant. The fact that the lower cervical vertebrae (C5-C6-C7) are more involved in the flexion position can explain anatomically the thinning of the spinal cord because of the compression that it was exposed to.

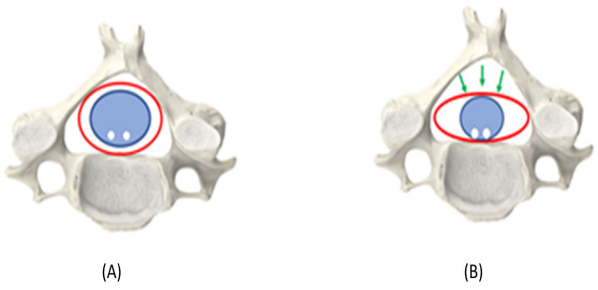

Figure 3. (A) Neutral position of cervical vertebrae. Red line: Spinal dura mater. Blue structure: Cervical spinal cord. The area between red and blue line: Dural sac. White structures: Anterior horn of spinal cord. **(B) Arrows show forward shifting of posterior dura mater on flexion and the compression on the anterior horn Red line: Spinal dura mater.** Blue structure: Cervical spinal cord. The area between red and blue line: Dural sac. White structures: Anterior horn of spinal cord.

There are neuroradiologists and spinal surgeons who claim that the dura mater alone can not be sufficient to explain the mechanism of Hirayama disease because of its rigid structure and its relativelty short stature compared to the bone structure of the vertebral column. Accordingly, it has been suggested that the part of the posterior dural structure adhering to the pedicles is loose even in the neutral position on MRI and the elastic and collagen fibers of the operatively resected dura mater show pathological abnormalities (*7*).

Myodural bridges which have the soft connective tissue connection between the fascia of the suboccipital muscles and the dura are thought to be structures that passively attach the dura mater and act as an active stabilizer for the spinal cord. In other words, these structures are seen as a dural tension monitoring system by restricting the movements of the dura mater (*10*). There are studies in which the etiology of cervicocephalic headache, cervicocephalic pain syndromes and dural pathologies are associated with myodural bridges (*11*). Some neuroanatomists think that the myodural bridges, which they define especially at C1-2 levels, allow anterior displacement of the dura mater in the lower cervical cord; thus cause the Hirayama disease (*7*).

Libin has stated that the cranial bones partially prevent the posterior part of the dura mater from moving forward during neck flexion. Since the dura mater is attached to the base of the cranium, it has been revealed that the sliding movement of the temporal bone, especially by turning backwards on the parietal bone, during cervical flexion prevents the dura mater from making pressure by moving away from the spinal cord (*12*). Therefore, in order not to prevent sliding, the joint type of the temporal bone and parietal bone in this region is squamous rather than sutura (*13*). If this joint were a suture like the other joints in the skull, almost all bones would have to participate in this movement. Based on the above facts, we hypothesize that one of the pathogenesis of Hirayama disease may be the relationship between cranial bone movements and dura mater. We conclude that in addition to the cervical region examination and MRI finding taken during the neck flexion and extension, examining whether there is a problem with the movements of the temporal and parietal bones in these cases as stated above may contribute to the understanding of Hirayama disease (*14*).

In addition to these stated mechanical causes, hyperIgEemia, immunological abnormalities in cytokine and chemokine amounts in serum and cerebrospinal fluid have been reported in patients with Hirayama disease (*7*).

Hirayama disease is likely to be confused with neuromuscular diseases such as spinal muscular atrophy,

amyotrophic lateral sclerosis or structural cervical cord lesions (syringomyoli) due to similar clinical symptoms. Therefore, keeping in mind Hirayama disease can help when conventional examinations result in no diagnosis. In addition to MRI in the neutral position, MRI in neck flexion may be very useful in patients with otherwise normal clinical findings. In MRI results taken in the flexion position in our cases, it was observed that the posterior dura mater was not only displaced anteriorly, but also a band-shaped lesion was formed in the posterior epidural space of the lower cervical canal. This band-shaped structure is related to congestion of the posterior internal vertebral venous plexus rather than vascular venous malformation. The internal vertebral venous plexus is a structure that runs along the entire vertebral canal and is responsible for the venous drainage of the structures therein. This plexus is an alternative route for circulation when the jugular veins are exposed to compression or when there is obstruction of the inferior vena cava. It also acts as a protective cushion and thermoregulation factor for important structures within the vertebral canal (*6*). Because of the fact that the anterior displacement of the dura mater would expose the anterior part of this venous plexus to compression as a result of the compression in Hirayama disease, congestion occurs in the posterior of this plexus. Congestion should not be observed in this anatomical structure in healthy people in the flexion position, but even if this situation in Hirayama patients does not cause a permanent problem in the veins, it is critical for diagnosis as it will cause the appearance of congestion (*15*). In our cases, as stated in the literature, we observed a dilatation in this plexus caused by the dura mater compression on the venous plexus in the flexion position. In MRI, we observed that the vascular structures reverted to their normal anatomical appearance when the neck was placed in neutral position.

There are other useful criteria for the distinctive diagnosis of Hirayama disease. The distinctive features of the disease include muscle weakness and atrophy, especially in distal muscles of upper extremity forearm and hand, unilateral involvement of the upper extremity, and absence of lower extremity involvement. This disease occurs widely among young adult males, progresses in the first few years and remains stable thereafter, having no loss of sensation and abnormal tendon reflexes. In addition, the elimination of other pathologies such as motor neuron diseases, spinal cord tumors, brachial plexopathy, and cervical vertebra abnormalities after examinations should bring Hirayama disease to mind (*16*). In the cases we encountered, loss of strength and atrophy in hand and in the unilateral upper extremity distal region, negative pathological reflexes and the fact that the patients were young adult males suggested Hirayama disease.

Another method of examination to guide the diagnosis process is EMG. There are studies suggesting chronic denervation in the muscles of the same extremity depending on the lesion level in the EMG results of the affected extremity (*1*). In the EMG results of our cases, denervation was observed in the ulnar and median nerve myotomes depending on the level of pressure and lesion.

In addition to the loss of strength, the most common complaint of the patients, irregular tremor was observed in the fingers of the affected upper extremity in the literature (*17*). Our first case where tremor was observed in the patient lends support to this observation.

Right-sided asymmetrical involvement is more common in Hirayama disease and the underlying mechanism of this condition is still unknown. Shinomiya *et al*. explained this with the "posterior epidural ligament factor". Anatomically, the epidural ligaments (Hofmann's ligaments) are connective tissue structures that extend from the dura mater to the vertebral canal. The posterior part of these structures is located between the posterior wall of the dura mater and the ligamentum flavum in the cervical spine. These ligaments prevent the anterior displacement of the dura meter by acting as a tent for it. The 'posterior epidural ligament factor' theory proposes that the posterior wall of the dura mater is displaced anteriorly because of the absence or the unequal distribution from right to left of posterior epidural ligaments and thereby causes an asymmetrical atrophy by creating a pressure in spinal cord. Generally, the more frequent occurrence of Hirayama disease on the right side is attributed to the anatomically abnormal development of the posterior epidural ligaments on this side (*18-21*). Consistent with the literature, the right upper extremities of our cases were also affected.

3. The importance of awareness on Hirayama disease

Hirayama disease is very likely to be overlooked during diagnosis because of similarities in clinical findings with other diseases. Due to the fact that clinical examination and MRI taken in neutral position may be insufficient to support the diagnosis process, it will be useful to further examine the anatomical structures with MRI in flexion position. Therefore, we believe that even if it is seen rarely, awareness of Hirayama disease will lead to correct diagnosis and thus appropriate and early treatment. In addition, this study is unique in that it reveals different anatomical theories about the pathophysiology of Hirayama Disease.

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Address correspondence to:
Bahar Tekin, Department of Anatomy, Faculty of Medicine, Istanbul Medipol University, Kavacık, Göztepe Mah, Atatürk Cd. No:40, 34810 Beykoz/Istanbul, Turkey
E-mail: bahar.tkn@gmail.com

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Correspondence

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Dent disease manifesting as nephrotic syndrome

Qiaoping Chen, Yongzhen Li, Xiaochuan Wu*

Department of Pediatrics, the Second Xiangya Hospital, Central South University, Changsha, Hunan, China.

SUMMARY Dent disease is an X-linked recessive renal tubular disorder, which is mainly caused by mutations of the *CLCN5* gene and *OCRL* gene. It is characterized by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis or nephrolithiasis, and progressive renal failure. Nephrotic syndrome is a glomerular disorder characterized by massive proteinuria, hypoalbuminemia, edema, and hyperlipidemia. In this study, we report two cases of Dent disease manifesting as nephrotic syndrome. Two patients were initially diagnosed with nephrotic syndrome due to edema, nephrotic range proteinuria, hypoalbuminemia, and hyperlipidemia, and responded to prednisone and tacrolimus therapy. Genetic testing revealed mutations in the *OCRL* and *CLCN5* genes. They were eventually diagnosed with Dent disease. Nephrotic syndrome is a rare and insidious phenotype of Dent disease, and its pathogenesis is not fully understood. Patients with nephrotic syndrome are recommended to routinely undergo urinary protein classification and urinary calcium testing, especially those with frequently recurrent nephrotic syndrome and poor response to steroid and immunosuppressive therapy. To date, there is no effective drug treatment for Dent disease. About 30% to 80% of patients progress to end-stage renal disease at the age of 30-50.

Keywords Dent disease, nephrotic syndrome, low molecular weight proteinuria, *CLCN5* gene, *OCRL* gene

1. Introduction

Dent disease (MIM 300009) is a rare genetic renal tubular disorder, with X-linked recessive inheritance. It is generally believed that the main clinical manifestations of Dent disease are low molecular weight proteinuria (LMWP), hypercalciuria, nephrocalcinosis, nephrolithiasis, and progressive renal failure (*1*). The *CLCN5* gene and *OCRL* gene are responsible for Dent disease 1 and Dent disease 2, respectively. About 25% of patients with the phenotype of Dent disease that the two genes cannot explain are classified as Dent disease 3 (*2*). The *CLCN5* gene is located at chromosome Xp11.23 and encodes 2-chloride (Cl-)/proton (H+) exchanger ClC-5, which is expressed in several tissues including brain, liver, renal and intestinal epithelia (*3*). In the kidney, as is known to all, ClC-5 is highly expressed in the distal nephron and proximal tubules (*4*). ClC-5 is indispensable for reabsorbing low molecular weight proteins through receptor- and non-receptor-mediated endocytosis in the proximal tubule (*5*). Additionally, studies have shown that ClC-5 is overexpressed in the glomeruli of proteinuric patients, and may have a role in protein endocytosis (*6*). The *OCRL* gene is located at chromosome Xq26.1 and encodes a phosphatidylinositol 4,5-bisphosphate-5-phosphatase (PIP2), widely expressed in the glomerulus and almost all of the tubular segments (*7*). Nephrotic syndrome (NS) is a glomerular disorder characterized by massive proteinuria, hypoalbuminemia, edema, and hyperlipidemia. It includes congenital nephrotic syndrome, primary nephrotic syndrome, and secondary nephrotic syndrome (*8*). NS is a rare and unstable phenotype of Dent disease, and its pathogenesis is still unclear (*9*).

Here, we present two cases of Dent disease presenting as NS. This study is consistent with the Declaration of Helsinki. Written consent was obtained from the patient's family. The Ethics Committee of the Second Xiangya Hospital of Central South University exempted the ethical approval of this study.

2. Clinical data

Patient 1 was diagnosed with NS for edema, proteinuria, hypoalbuminemia, and hyperlipidemia at the age of 4 years. After prednisone treatment, edema and proteinuria still frequently recurred. In 2021, at the age of 11, he received treatment at The Xiangya Second Hospital of Central South University. His weight was 24 kg and height was 125 cm. He was normotensive on admission (99/54 mmHg), with mild peripheral edema. The

laboratory findings were as follows: white blood cell count 7.41 × 10^9/L, hemoglobin 114 g/L, and platelets 402 × 10^9/L. Serum albumin 14.3 g/L, total protein 37 g/L. His urinalysis revealed 3+protein, and the quantity of 24h urinary protein was 3850.65 mg (160.44 mg/kg). Immunology tests showed low levels of IgG and high levels of IgE. IgA and IgM were within the normal range. C3 and C4 levels were within the normal range. Also, antinuclear antibodies, anti-ENA antibodies, and anti-dsDNA antibodies were all negative.

Renal ultrasound showed that both kidneys were of normal size, with no evidence of nephrocalcinosis or nephrolithiasis. Light microscopy revealed the glomeruli were swollen, mesangial cells and mesangial matrix proliferated, and the glomerular basement membrane was normal. Partial renal tubular epithelial cell vacuolar degeneration, renal interstitial focal edema, fibrosis, and inflammatory cell infiltration was observed. Obvious inflammatory changes could be seen in renal arterioles. Immunofluorescence revealed IgM++. Electron microscopy revealed extensive glomerular fusion of foot process and segmental sclerosis, considering focal segmental glomerulosclerosis (FSGS). The boy was tentatively diagnosed with IgM nephropathy. Genetic testing indicated a novel duplication of exons 7 to 8 of the *OCRL* gene. His mother was identified as a carrier for the same *OCRL* gene mutation. The patient was eventually diagnosed with Dent disease 2.

Patient 2 was diagnosed with NS for edema, proteinuria, hypoalbuminemia, and hyperlipidemia at the age of 5 years. His urine protein returned to normal and edema was relieved after receiving prednisone treatment. However, during the process of gradual reduction of prednisone, edema and proteinuria recurred frequently. In 2018, at the age of 8, the boy was referred to The Second Xiangya Hospital of Central South University. His weight was 49 kg and height was 125 cm. He was normotensive on admission (96/65 mmHg). The laboratory findings were as follows: white blood cell count 13.08 × 10^9/L, hemoglobin 131 g/L, platelets 360 × 10^9/L, serum albumin 10.5 g/L, and total protein 31.4 g/L. His urinalysis revealed 3+protein, the quantity of 24h urinary protein was 13,703.24 mg (279.66 mg/kg). Immunology tests showed low levels of IgG and IgA. IgM and IgE were within the normal range. C3 levels were below normal range, C4 levels were within the normal range. Also, antinuclear antibodies, anti-ENA antibodies, and anti-dsDNA antibodies were all negative.

Renal ultrasound showed that both kidneys were of normal size, with no evidence of nephrocalcinosis or nephrolithiasis. He did not undergo a kidney biopsy. The boy was tentatively diagnosed with NS. Genetic testing indicated a hemizygous mutation of the *CLCN5* gene c.796A>G (p.I266V), confirming the diagnosis of Dent disease 1. His mother was identified as a carrier for the same *CLCN5* gene mutation.

The clinical manifestation and laboratory tests are summarized in Table 1.

Table 1. Clinical manifestation and laboratory tests of our patients

Items	Patient 1	Patient 2
Gender	male	male
Age of onset (years)	4	3
Current age (years)	11	12
Blood pressure (mmHg)	99/54	96/65
Chief complaint	edema and proteinuria	edema and proteinuria
Urine investigations		
Urine protein	3+	3+
24 h protein (mg/kg)	160.44	279.66
Microalbumin (mg/L)	10	-
Alpha1-microglobulin (mg/L)	-	-
Beta 2-microglobulin (mg/L)	-	-
Retinol-binding protein (mg/L)	-	-
Urine calcium/ urine creatinine (mg/mg)	-	-
24 h urine calcium (mg/kg)	-	-
Nephrocalcinosis/nephrolithiasis	No	No
Kidney biopsies		
Light microscopy	IgM nephropathy	-
Electron microscopy	FSGS	-
Blood investigations		
Serum albumin (g/L)	14.3	10.5
Serum sodium (mmol/L)	138.0	127.9
Serum potassium (mmol/L)	3.26	3.11
Serum chlorine (mmol/L)	105.8	94.8
Serum calcium (mmol/L)	1.51	1.69
Serum phosphate (mmol/L)	1.24	0.97
Serum creatinine (μmol/L)	31	28.1
eGFR (mL/min/1.73 m^2)	195.97	216.19
Uric acid (μmol/L)	439.0	727.6
Urea (mmol/L)	4.90	6.32
IgG (g/L)	2.11	0.62
IgA (g/L)	1.08	2.13
IgM (g/L)	1.88	1.96
IgE (ng/mL)	1694.00	440.20
Serum C3 (g/L)	1.28	0.62
Serum C4 (g/L)	0.34	0.19

3. Discussion

In this study, we present two cases of Dent disease manifesting as NS. Two patients all clinically featured recurrent edema, nephrotic range proteinuria, hypoalbuminemia, and hyperlipidemia, and were initially diagnosed with NS. Genetic testing revealed mutations of the *OCRL* gene and *CLCN5* gene, confirming the diagnosis of Dent disease 2 and Dent disease 1, respectively. The genetic testing results of our patients are showed in Table 2. To our knowledge, the mutations of the *OCRL* gene (duplication of exons 7 to 8) and *CLCN5* gene c.796A>G (p.I266V) have not been reported so far (*1,2*).

Gianesello L, *et al.* conducted a literature review and found that 55 Dent disease 1 patients and 20 Dent disease 2 had nephrotic range proteinuria (37% and 48% respectively of the collected literature). These patients were found to have varying degrees of glomerular dysfunction, though Dent disease is widely accepted as

Table 2. Genetic testing results of our patients

Items	Patient 1	Patient 2
Gene mutation	*OCRL1*	*CLCN5*
Nucleotide change	exon 7-8 repeats	c.796A>G
Amino acid change	-	p.I266V
Type of mutation	-	missense mutation
Mutation source	mother	mother

a proximal tubulopathy disorder (*2*). Guohua He *et al.* reported that the urinary α1-microglobulin/albumin ratio was considered to be a useful parameter to differentiate LMWP caused by Dent disease from albuminuria caused mainly by NS (*10*). Makino S, *et al*. reported that the urine β2-MG/urine protein can be used to monitor the relapse of NS in patients with Dent disease, as it is consistent with the progression of NS (*10*).

Dent disease is an inherited renal tubular disorder. It is characterized by LMWP, hypercalciuria, nephrocalcinosis, nephrolithiasis, and progressive renal failure. A large Dent disease study analyzed data from 109 male patients with *CLCN5* mutations and 9 patients with mutations of the *OCRL1* gene. They observed that the phenotype of Dent disease 1 and Dent disease 2 was similar, and with age, the phenotype evolves from mainly proximal nephropathy to a mixed proximal/distal salt-losing tubulopathy (*11*). Proteinuria apparently worsened with age, while hypercalciuria gradually resolved as renal function worsened. Also, estimated GFR has been shown to correlate with the degree of interstitial fibrosis.

The urine protein of patients with Dent disease is mostly mild to moderate, NS is a rare and insidious phenotype of Dent disease. The traditional view is that if NS patients do not respond to hormones and immunosuppressant therapy, the possibility of hereditary kidney disease should be considered. While in this research, two patients were initially diagnosed with NS and responded to prednisone and tacrolimus therapy. This means that the classification of urine protein and the detection of urinary calcium needs to be carried out routinely. LMWP is a characteristic clinical phenotype of Dent disease, and there is no significant difference between Dent disease 1 and Dent disease 2 (*9*). Long-term proteinuria may be an important factor in renal function injury in patients. Are patients with Dent disease manifesting as NS more likely to develop renal insufficiency? The specific pathogenesis of Dent disease manifesting as NS needs further study.

The relationship between genotype and phenotype of Dent disease remains unestablished. Patients with the same gene mutation site can induce different clinical phenotypes, even in the same family. In 2008, two brothers were reported with identical variants of the *CLCN5* gene (c.473G>A, p.Gly158Asp) in Denmark. The patient had nephrocalcinosis, proteinuria, hypercalciuria, and was diagnosed with Dent disease, whereas the patient's brother was asymptomatic with laboratory findings all within the normal range (*12*). In 2017, two Chinese brothers were reported to both carry the hemizygous mutation c.731C>T (p.S244L) in exon 7 of the *CLCN5* gene, but they had different phenotypes (*13*). The elder brother presented with nephrotic range LMWP, aminoaciduria, polydipsia, polyuria, nephrocalcinosis, hypophosphatemia, hypokalemia, acidosis, hyposthenuria, and rickets but without hypercalciuria. He was finally diagnosed with Fanconi syndrome. However, the younger brother presented with nephrotic-range LMWP, hypercalciuria, and aminoaciduria, and was diagnosed with Dent disease.

To date, there is no effective treatment for Dent disease. The main therapeutic goals are to reduce hypercalciuria, prevent renal calcification, and delay the progression of renal failure (*1*). A high amount of drinking water, a low calcium diet, and thiazide drugs are still the most effective supportive treatments for Dent disease. Patients diagnosed with Dent disease should be treated with low-dose thiazines and citrate as soon as possible. Approximately 30% to 80% of patients with Dent disease progress to end-stage renal disease at the age of 30–50 (*14*). It is rare for Dent disease to develop into chronic kidney disease in childhood and only a few cases have been reported so far (*15-18*).

4. Conclusion

NS is a relatively rare phenotype of Dent disease. The phenotypic heterogeneity of Dent disease prevents its prompt diagnosis. Both our patients were initially diagnosed with NS and responded to prednisone and tacrolimus therapy. This reminds us that in patients presented as having NS, who respond to steroid and immunosuppressive therapy, the possibility of Dent disease also needs to be considered. Therefore, for patients with proteinuria, urine protein classification and urinary calcium detection should be routinely performed, which is a more economical and convenient method than genetic testing. The pathogenesis of Dent disease manifesting as NS and the mechanism of response to hormones and immunosuppressant therapy needs to be further studied.

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**Address correspondence to:*
Xiaochuan Wu, Department of Pediatrics, the Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China.
E-mail: xiaochuanwu@csu.edu.cn

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IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

Intractable & Rare Diseases Research (Print ISSN 2186-3644, Online ISSN 2186-361X) is an international peer-reviewed journal. *Intractable & Rare Diseases Research* devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables. Supplementary Data are permitted but should be limited to information that is not essential to the general understanding of the research presented in the main text, such as unaltered blots and source data as well as other file types.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of a maximum of 10 figures and/or tables and 100 references. Mini reviews are also accepted, which should not exceed 4,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 50 references.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in *Intractable & Rare Diseases Research* in the last 6 months or issues of general interest. Summaries of research results and sharing of experiences in clinical practice and basic research (findings based on case reports, clinical pictures, *etc*.) can also be published as Letters. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

3. Editorial Policies

For publishing and ethical standards, *Intractable & Rare Diseases Research* follows the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals issued by the International Committee of Medical Journal Editors (ICMJE, *https://icmje.org/recommendations*), and the Principles of Transparency and Best Practice in Scholarly Publishing jointly issued by the Committee on Publication Ethics (COPE, *https://publicationethics.org/resources/guidelines-new/principles-transparency-and-best-practice-scholarly-publishing*), the Directory of Open Access Journals (DOAJ, *https://doaj.org/apply/transparency*), the Open Access Scholarly Publishers Association (OASPA, *https://oaspa.org/principles-of-transparency-and-best-practice-in-scholarly-publishing-4*), and the World Association of Medical Editors (WAME, *https://wame.org/principles-of-transparency-and-best-practice-in-scholarly-publishing*).

Intractable & Rare Diseases Research will perform an especially prompt review to encourage innovative work. All original research will be subjected to a rigorous standard of peer review and will be edited by experienced copy editors to the highest standards.

Ethical Approval of Studies and Informed Consent: *F*or all manuscripts reporting data from studies involving human participants or animals, formal review and approval, or formal review and waiver, by an appropriate institutional review board or ethics committee is required and should be described in the Methods section. When your manuscript contains any case details, personal information and/or images of patients or other individuals, authors must obtain appropriate written consent, permission and release in order to comply with all applicable laws and regulations concerning privacy and/or security of personal information. The consent form needs to comply with the relevant legal requirements of your particular jurisdiction, and please do not send signed consent form to *Intractable & Rare Diseases Research* to respect your patient's and any other individual's privacy. Please instead describe the information clearly in the Methods (patient consent) section of your manuscript while retaining copies of the signed forms in the event they should be needed. Authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013, *https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki*). When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

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Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

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The manuscript must be accompanied by a cover letter prepared by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For an example of Cover Letter, please visit: (Download Centre: *https://www.irdrjournal.com/downcentre*)

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6. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with

the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *https://www.ICMJE.org*.

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g*. DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; 5) author contribution statements to specify the individual contributions of all authors to this manuscript, and 6) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose").

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For articles that are Original Articles, Brief Reports, Reviews, or Policy Forum articles, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For Communications, Editorials, News, or Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. For articles reporting clinical trials, the trial registration number should be stated at the end of the Abstract. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Three to six key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should provide sufficient background information to make the article intelligible to readers in other disciplines and sufficient context clarifying the significance of the experimental findings.

Materials/Patients and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All clinical investigations must have been conducted in accordance with the Declaration of Helsinki (as revised in 2013, *https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki*). All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

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Acknowledgments: All funding sources (including grant identification) should be credited in the Acknowledgments section. Authors should also describe the role of the study sponsor(s), if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. If the funding source had no such involvement, the authors should so state.

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References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. The EndNote Style of *Intractable & Rare Diseases Research* could be downloaded at **EndNote** (*https://www.irdrjournal.com/examples/Intractable_Rare_Diseases_Research.ens*).

Examples are given below:

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Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):

Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

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Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2022).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

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Intractable & Rare Diseases Research
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@irdrjournal.com