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## Intractable & Rare Diseases Research

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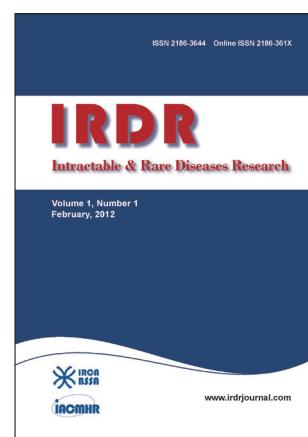
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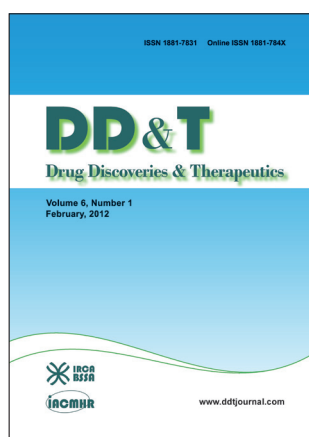
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# Intractable & Rare Diseases Research

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# A compilation of national plans, policies and government actions for rare diseases in 23 countries

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| Summary | Previous studies have focused on the comparison of specific laws among multiple countries and regions; for example, laws related to facilitating treatments with orphan drugs or laws seeking to address the multiple needs of patients with rare diseases. The purpose of this scoping review is to examine and compare published reports on national plans, polices and legislation related to all rare diseases in different countries. We also examine strategies or programs that countries may have for these diseases. Articles were obtained from journals and books published between January 1, 2000, through December 15, 2017. Reports from the grey literature (documents issued by government and private organizations) were included if they were available on the internet. The databases used were Google and Google Scholar, PubMed, and the websites of Orphanet and the National Organization for Rare Disorders (NORD). We obtained information on 23 countries. Among these countries, the way in which rare diseases were defined varied from having similar definitions to no definition. Multinational programs supported by common or similar laws are likely to have a greater impact on rare diseases than single country programs. |
|---|---|

*Keywords:* Rare disease legislation, program, strategy

## 1. Introduction

In the United States (US), a rare disease is one affecting fewer than 200,000 individuals at a given time. Despite their individual low frequencies, the large number of rare diseases (estimated between 6,000 and 8,000) makes them collectively common (*1,2*). Worldwide, rare diseases affect approximately 400 million people (*3*). About 80% of these diseases have a genetic origin and their severity ranges from minor to life threatening (*3,4*). Because of their genetic or congenital origin, numerous rare diseases have a serious impact on health starting at birth or early childhood and represent a major challenge for patients, caregivers, physicians, healthcare providers, and society in general. The financial impact

of rare diseases is also significant (*3,5,6*). For instance, the medical expenses for adults with spina bifida could be three to six times as high as that for adults without the disease (*7*). In 2014, the average annual cost in the US for orphan drugs developed for rare diseases was $137,782 per patient (*8*). This financial impact noticeably increases the overall cost of managing a rare disease for which orphan drugs are prescribed. Furthermore, the quality of life for patients with a rare disease may decline with age (*6*). A Swedish study showed that, in a ten-year period, the proportion of individuals with adult forms of muscular dystrophy able to walk without assistive devices decreased from 91% to 52% (*9*). The age range of this group was 16 to 65 years (*9*).

Rare diseases also affect physicians and the healthcare system in general. The scarcity of knowledge, guidelines, and training on rare diseases makes the diagnosis and management of these diseases difficult (*3,5*). For example, a survey conducted among caregivers of patients with a rare disease in the US and the United Kingdom (UK) found that these patients had often received a misdiagnosis from multiple physicians. It was not until approximately three years after the first misdiagnosis that patients with a rare disease received a

correct diagnosis (*6*). This delay in diagnosis adds to the costs of the disease incurred by patients, their caregivers, and the healthcare system. Further, patients with a rare disease may not only need to see multiple physicians to get a correct diagnosis, they may also need care from multiple practitioners. This can also present a challenge. A majority of physicians in the US (76%) and in the UK (88%) reported having difficulties coordinating care with other providers who are managing the same patient with a rare condition (*6*).

In addition to the challenges of accurately diagnosing rare diseases and assessing the impact these diseases have on patients and their caregivers, there is the challenge of how to reach a patient population that is few in number and widely scattered geographically. The typical public health approach, designed for diseases that are either common or tend to cluster, might not be relevant for rare diseases. Hence, a frequently used strategy to support public health approaches aimed at reducing the burden of rare diseases is by government action; for example, the US has used this approach extensively: in 1983 the US Congress passed the Orphan Drug Act, its first major federal statute dedicated to rare diseases. This law encouraged the production of orphan drugs (drugs developed to treat rare diseases) by providing financial incentives to pharmaceutical industries to offset the potential losses of marketing drugs to such a small market (*10*). Further, in 2001, the US Congress passed the "Muscular Dystrophy Community Assistance, Research and Education Amendments of 2001" (MD CARE Act) (*11*), which promoted surveillance and research, improved screening techniques, fostered collaboration among muscular dystrophy centers, and stimulated the development of educational programs for all types of muscular dystrophy. Research that resulted from the MD CARE Act included early detection, diagnosis, prevention, and treatment for muscular dystrophy (*12*).

Finally, a government may create laws aimed at reducing the occurrence of rare diseases that have a preventable cause, such as neural tube defects (NTDs). In 1998, a regulation issued by the US Food and Drug Administration (FDA) went into effect that mandated the addition of folic acid to cereal grain products labeled as enriched in the US (*13*). The objective of this regulation was to provide women with an avenue for increasing dietary intake of folic acid, which can help prevent NTDs. As a result of this regulation, just over 1,300 more babies were born without a NTD each year from 1999 through 2011 (*14*). To date, mandatory folic acid fortification of grain cereals now exist in 86 countries (*15*).

The use of legal means to benefit patients with rare diseases has also been underway in the European Union (EU). The academic literature shows the EU has created a unified approach toward the management of rare diseases through the use of regulations, directives,

recommendations, and communications (*16-21*). This approach seeks to make rare diseases more visible by identifying and coding them; by encouraging the development of national plans to ensure equal access to health care for people with rare diseases; and by promoting regional support to activities such as research, financial incentives, screening, and orphan drug development.

In sum, the literature demonstrates that law-based interventions aimed at rare diseases in the US and Europe have encouraged the production of orphan drugs, accommodated health care systems to the needs of patients with rare diseases, and promoted research to prevent and ameliorate the impact of these diseases on affected individuals and populations. The purpose of this study is a scoping review exploring the academic literature for the past 18 years, searching for publications in English that refer to national plans, policies and legislation on rare diseases from countries around the world. This review also includes strategies or programs for rare diseases from these countries. As a result, we will compile similarities and differences reported in the literature among law-based national approaches that focus on rare diseases.

## 2. Literature Search Strategy

First, we searched repositories of biomedical literature for articles and book chapters describing national plans, policies or laws for rare diseases in general. Second, we complemented the previous search by searching the same repositories for countries with specific strategies or programs for rare diseases that were national or multi-national in scope. Third, we searched the grey literature for reports about any of the items searched in the previous two steps. Our search was limited to publications and documents in English, or in other languages with complete abstracts in English, that were published from January 1, 2000 through December 15, 2017. In our search we used only keywords in English and we collected three types of documents: 1) all in English; 2) with a complete abstract in English but the rest of the document in another language; and 3) websites in English. The search engines we used were Google or Google Scholar, PubMed, Orphanet, and the database of the National Organization for Rare Disorders (NORD). The search terms, alone or combined, included "rare disorder," "rare disease," "national plan," "legislation," "policy," "strategy," and "program." We then examined abstracts, book chapters, websites, and reports resulting from the searches to assess their relevance to our objectives. We retrieved a document for inclusion in the study only if it described a plan, policy, legislation, strategy, or program for rare diseases at a national or multi-national scale. We excluded from this review documents that appeared to be duplicates, that had no publication date; that reported unpublished results

or personal communications, and conference abstracts. Figure 1 shows the flow diagram of our search. Finally, we organized the results of our review by country within large geographic regions and highlighted the characteristics of the national approaches to rare diseases that we found in the literature.

## 3. Results

Our exploratory search yielded 19,179 potentially relevant publications for the period under study. After applying the selection criteria (Figure 1), 56 publications qualified for our compilation: 37 from scholarly journals, 17 from the grey literature, and two books. The publications originated from four regions or their combinations as follows: 8 from Canada and the US, 19 from Europe, 13 from Asia-Pacific, 4 from Latin America, and 12 from multi-country regions (Figure 2).

Regarding these combined regions, two publications included Europe and Latin America, nine included Canada, the US, Europe and the Asia-Pacific region, and two included Canada, the US and Europe. Tables 1-4 present the rare disease definitions, plans, legislation, programs, and strategies by region and country. Of note, since the term *rare* refers to diseases of very low frequency in a population, the definitions of this term are based on the prevalence rather than the attributes of a disease. According to the literature, some countries use total number of cases in a population as a threshold to define a rare disease, while other countries use proportions.

### 3.1. *Canada and the US*

Table 1 reports the results for Canada and the US. Unlike the US, Canada defines rare diseases by the
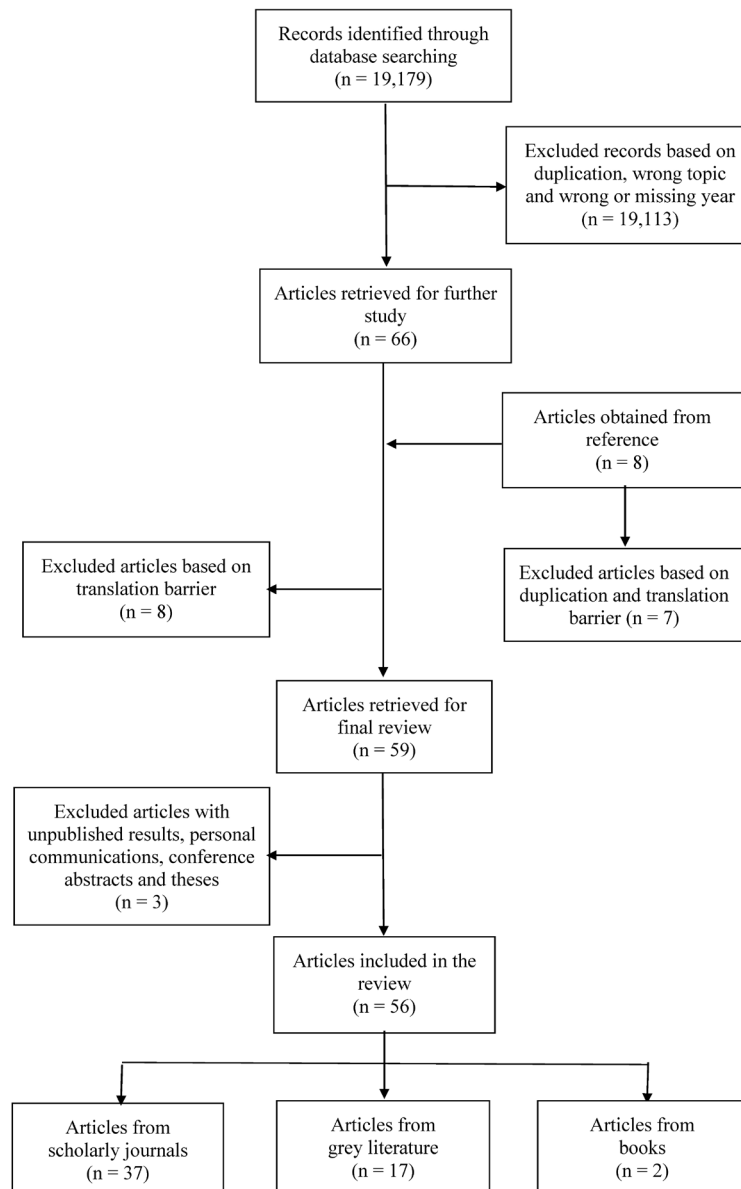


**Figure 1. Flow diagram of the search strategy.**
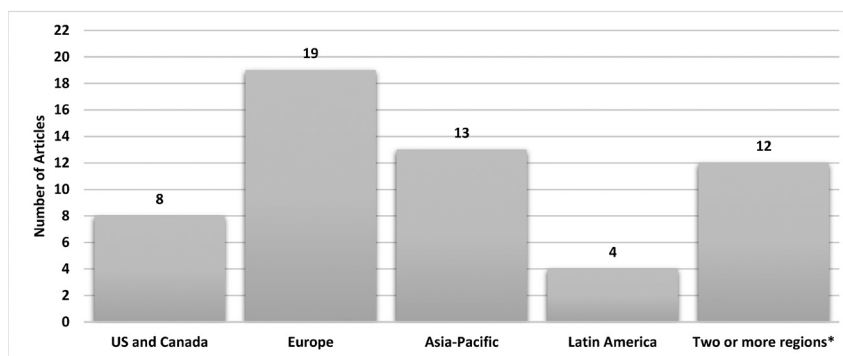
**Figure 2. Number of rare disease articles from different countries, by region.** \*indicates that the documents described a plan, policy, legislation, strategy, or program for multiple regions.

**Table 1. Rare disease plans, legislation, programs or strategies in Canada and the United States**

| Country | Definition of rare disease | National Plan | Legislation | Program or Strategy | Highlights |
|---|---|---|---|---|---|
| Canada (*1,2,10,22, 23,25*) | Fewer than 5 cases per 10,000 people (draft) | No | No | Yes | *Health Canada's Special Access Program*: Provides access to several orphan drugs for patients with rare diseases. *Orphan Drug Framework*: Promises to increase lifecycle of orphan drugs, work with other countries with an established orphan drug framework, and to improve access to orphan drugs and facilitate research. *The Canadian Organization for Rare Disorders (CORD) 5 Strategic Goals for Canada's Rare Disease Strategy*: Detection and prevention, timely, equitable and evidence-informed care, community support, access to promising therapies and research. |
| United States (*1-3,5,8,10,25, 35,39,49-57*) | Fewer than 200,000 cases | No | Yes | Yes | *Orphan Drug Act (1983)*: Regulatory fee waivers, 50% tax credit in clinical expenditure, grants for clinical research, development of medical devices and medical food, protocol assistance, accelerated review if indication permits, and 7 year market exclusivity. *Rare Disease Act (2002)*: Established the Office of Rare Diseases. Increased federal funding for the development of treatments for rare diseases. *National Institute of Health Research Programs*: Translational research on rare diseases and emphasis on diagnostics and interventions (*e.g.*: Genetic and Rare Disease Information Center). |

proportion of cases in a population (*22*). The reports that we found indicate that Canada has no national plan or specific legislation on rare diseases (*10,22*), the Minister of Health put forward a draft for an orphan drug regulatory framework in 2012 (*22,23*). According to a previous report, this framework would rely on existing Canadian laws that regulate health products and food, including the regulation of labelling and packaging, clinical trials, and manufacturing and marketing of these products; however, this framework has yet to be implemented (*24*). Also, the Canadian Organization for Rare Disorders (CORD) has provided five strategic goals for a Canadian Rare Disease Strategy (*23*).

About 35 years ago, the US passed its first major law on orphan drugs, known as the Orphan Drug Act, aimed at stimulating the production of medicines for rare diseases by offering pharmaceutical companies research grants, tax credits, fee waivers, and a seven-year market exclusivity for approved medications (*10*). Other pieces of legislation in the US include the Rare Disease Act, which established the Office of Rare Diseases at the National Institutes of Health (NIH) (*10*) and legislation for single rare disease entities, such as the MD Care Act (*11*).

### 3.2. Europe

For Europe, our exploratory search resulted in a handful of publications from the subset of countries that form the EU. Multiple articles, spanning a number of years, demonstrate that Europe has an integrated, multi-country approach to rare diseases (Table 2). The publications found report that all 28 countries in this union have a common definition of rare disease, based on the proportion of cases in the population, and their rare disease activities operate under multinational legislation called the Orphan Medicinal Product Regulation (EC) No. 141/2000. This regulation seeks to stimulate research and to promote the development of orphan drugs to treat patients with rare diseases (*17-20*). Europe also has developed the European Project

**Table 2. Rare disease plans, legislation, programs or strategies in Europe**

Within Europe, the European Union (EU) has adopted a common definition of a rare disease (Fewer than 5 cases per 10,000 people) and has a common legislation [Regulation (EC) No. 141/2000 (1999): Regulatory fees reduced or waived, access to centralized procedures, and protocol assistance. Member states implemented measures to encourage the development of orphan medicinal products. Tax credits are managed by member states. Market exclusivity for 10 years; 6 years if drug criteria not met.] Each member state has additional legislation (1-5,8,10,18-21,39,51,57,59,60).

| Country | National Plan | Legislation | Program or Strategy | Highlights |
|---|---|---|---|---|
| Bulgaria (*1,16,17,25-27,66*) | Yes | Yes | Yes | *National Plan (2009-2013):* To deliver prevention, diagnostics, treatment and rehabilitation to rare disease patients. |
| France (*1,3,16,17,25-28, 53,61*) | Yes | Yes | Yes | *First National Plan (2005-2008)*: To increase knowledge on the epidemiology of rare diseases, recognize the specificity of rare diseases, develop information on rare diseases for patients, healthcare professionals and the general public, train healthcare professionals for better identification of this disease, organize screening and access to diagnostic tests, continue efforts in favor of orphan drugs, meet the specific requirements for social services for patients with rare diseases, promote research on rare disease, and develop national and European partnership. *Second National Plan (2011-2014)*: Increase quality of patient care with the use of reference centers and telemedicine, develop research on rare disease such as translational clinics and therapeutic research, and increase European and global cooperation |
| Germany (*1,16,17,25-27,63*) | Yes | Yes | Yes | *"Nationales Aktionsbündnis für Menschen mit Seltenen Erkrankungen" (NAMSE)*: There were 52 policy proposals for action. Action fields for these proposals include care/centers/networks, research, diagnostics, information management, patient orientation, registries, and implementation and future development. |
| Greece (*1,16,17*) | Yes | Yes | Yes | *National Plan (2008-2012)*: Includes early diagnosis, medical treatment, prevention, research, education, and partnership and co-operation strategies. |
| Italy (*1,16,17,26-28, 58,64*) | Yes (Draft) | Yes | Yes | *Ministerial Decree n. 279/2001:* Provides a price exemption for care on certain rare diseases; contemplates setting up a network of centers for rare disease patients identified through their experience, activities and services for these patients. The decree also contains regulations for a national registry. |
| Portugal (*1,16,17,26,27*) | Yes | Yes | Yes | *National Plan (2008-2015)*: To determine the needs of rare disease patients and their families and to improve the quality and equity of healthcare services. |
| Spain (*1,16,17,26,27,65*) | Yes | Yes | Yes | *National Plan (2010)*: Includes information on rare disease (resources, registers and coding plus classification), prevention and early detection, healthcare, therapies, integrated health and social care, research and training. Centers of expertise provide services and focus on the needs of rare disease patients. Reference centers promise access to healthcare for these patients. |
| United Kingdom (UK) (*1,17,25-28,62*) | Yes (Plan approved for 4 UK states) | Yes | Yes | *UK strategy for rare disease*: Examines patients and their families. Includes empowering rare disease patients, identifying and preventing these diseases (screening and carrier testing), diagnosis and early intervention, coordination of care (specialist centers) and research. Strategy will continue to work with other countries with rare disease. |

for Rare Disease National Plan (EUROPLAN) to facilitate the creation of national plans in the region (*3,16,19-21,25*). These plans are defined as official public health strategies accepted by a government with explicit priorities, actions, a timetable and a budget (*16*). Research on rare diseases in Europe is supported by The Seventh Framework Program (FP7), which is a program that funds medium- to large-sized collaborative research projects (*19,20,25*). In addition, free, short-term access to orphan products is accomplished through Compassionate Use Programs (CUPs) in Member States (*26-28*), with some exceptions such as Greece (*26,27*). For the period of our search, eight countries from the EU issued publications related to their national plans, legislation, and programs or strategies for rare diseases. These results are summarized in Table 2.

### 3.3. *Asia-Pacific*

Our search found documents that show evidence of legislative activity on rare diseases in seven countries of the Asia-Pacific region (Table 3). In the articles reviewed, the definition of a rare disease varies widely among these countries. Only Singapore and South Korea share a definition. Six of the countries are reported to have legislation addressing rare diseases, yet none appear to have a national plan for these diseases. All seven countries were shown to have programs or strategies aimed at rare diseases. Our review found that Australia has at least two regulatory programs, derived from healthcare laws enacted by the parliament: the Orphan Drug Program, which provides financial and marketing incentives for drug makers

**Table 3. Rare disease plans, legislation, programs or strategies in Asia-Pacific Countries**

| Country | Definition of rare disease | National Plan | Legislation | Program or Strategy | Highlights |
|---|---|---|---|---|---|
| Australia *(1-3,10,29,32, 35,39,51)* | Fewer than 2,000 cases. | No | Yes | Yes | *Orphan Drug Program (1998)*: Regulatory fee waivers, no grants or tax credits, protocol assistance, and priority review. 5 year market exclusivity. |
| China *(1,25,30-32, 35,71)* | Prevalence lower than 1/500,000 (disorder) or incidence lower than 1/10,000 (newborns). Definition not agreed by patient organizations. | No | No | Yes | *China pilot project*: to provide and use guidelines, create registries and encourage molecular testing for rare diseases. This project seeks to build relationships with collaborative networks, clinicians and patient organizations. *"Rare Disease Clinical Cohort Study"*: Research program will establish the unified National Registry System of China in 2020 and large cohort study will examine classification, diagnosis, treatment and prognosis for rare diseases. |
| Japan *(1-3,8,10,32, 35,39,51,57, 67-69)* | Fewer than 50,000 cases. | No | Yes | Yes | *Pharmaceutical Affairs Law (1993)*: Regulatory fee waivers, clinical and non-clinical study grants, 15% tax credits and up to 14% tax reduction (6% tax reductions for preclinical research). Protocol assistance and fast track approval. 10 year market exclusivity. *"Revision of Measures to Combat Intractable Diseases"*: Strategies stressed treatment and improve care for those affected, tools to reimburse medical costs, and promote participation. *Specified Disease Treatment Program*: Focused on funding to encourage research and orphan drug development |
| Philippines *(32,33,70)* | 1 case per 20,000 people. | No | Yes | Yes | *Rare Disease Act of the Philippines (2016)*: Focuses on several elements of rare disease management (diagnosis, clinical management, genetic counseling and drug research development), registry, research, and newborn screenings. |
| Singapore *(1,2,32,34)* | Fewer than 20,000 cases. | No | Yes | Yes | *Medicines Act (Chapter 176, Section 9) (1991)*: Focuses on managing and encouraging the use of orphan drugs for rare disease patients. Physicians and dentist should prescribe orphan drugs for these patients if there were no substitute medications available. 10 year market exclusivity. |
| South Korea *(32,35,36, 67)* | Fewer than 20,000 cases. | No | Yes | Yes | *Orphan Drugs Guideline (2003)*: Medical reimbursement and research. 6 year market exclusivity. *Research Center for Rare Diseases*: To do research as a single center or collaborate in clinical research networks. *Korean Biobank Project*: The project would manage and collect specimens from rare disease patients, and launch a network of healthcare providers working with these patients disease or would gather bio-resources from research projects. |
| Taiwan *(1,2,25,32,35, 37,57,67)* | No more than 1 case per 10,000 people. | No | Yes | Yes | *Rare Disease and Orphan Drug Act (2000)*: Grants, copay can be waived, fast track approval, protocol assistance, and medical reimbursement. 10 year market exclusivity. |

to produce medicines for patients with rare diseases (*10,29*); and the Life Saving Drugs Program, which subsidizes expensive and life-saving drugs for patients with serious, rare medical conditions (*29*). A China pilot project was implemented in 2013 (*30,31*) and the Rare Disease Clinical Cohort Study was implemented in 2016 for rare disease patients (*31*). The objectives reported for this pilot are to develop and apply guidelines and clinical pathways for rare diseases, to establish patient registries and data repositories and to promote molecular testing for rare diseases (*30*). We identified reports stating that Japan has policies promoting orphan drug research and development (*32*). For instance, Japan's Pharmaceutical Affairs Law (PAL)

encourages research that examines orphan drugs (*3*). The Rare Disease Act of the Philippines, approved in 2016, covers topics such as rare disease management, registry, research, and newborn screening (*33*). In 1991, Singapore implemented the Medicines Act (Chapter 176, Section 9) which focuses on managing, and encouraging the use of, orphan drugs (*34*). In 2003, South Korea created the Orphan Drugs Guideline (*35*) and, in 2013, the Korea Biobank Project (KBP) for rare disease (*36*). In Taiwan, the Rare Disease and Orphan Drug Act provides support for rare disease patients by encouraging rare disease research, and increasing awareness of rare disease (*25,37*). Furthermore, this act also facilitates access to orphan drugs (*35,37*).

**Table 4. Rare disease plans, legislation, programs or strategies in Latin American Countries**

| Country | Definition of rare disease | National Plan | Legislation | Program or Strategy | Highlights |
|---|---|---|---|---|---|
| Argentina (*25,38,39*) | EU Definition | No | Yes | Yes | *Law 26.689 (2011)*: Intended to help rare disease patients and their caregivers by promoting the development of patient registries and screening programs, and educational and social support activities. |
| Brazil (*25,39,40*) | No more than 65 cases per 100,000 people. | No | Yes | Yes | *"National Policy for Rare Diseases"(2014):* Includes equal healthcare services, create care guidelines for these patients at every stage of a Unified Health System care, offers comprehensive care in the Health Care Network, improves universal and regulated access for rare disease patients, ensures access to care, and quality healthcare. |
| Chile (*39*) | EU Definition | No | Yes | N/A | The Ricarte Soto law aims at providing funding for care of rare disease patients. It assigned a grant of 200 billion pesos for 4 years. |
| Colombia (*39,41,72*) | 1 case per 5,000 people. | No | Yes | N/A | *Law 1392 (2010)*: Identifies the lack of orphan drugs as a health issue impacting the healthcare system. Considers social protection policies. It contemplates creating a registry for rare disease patients and collaborating globally for research. |
| Mexico (*25,39*) | EU Definition | No | Yes | No | *Article 224 revision (2012)*: Recognizes orphan drugs and their treatments. Ministry of Health may enforce market authorization, no rules for market exclusivity. |
| Peru (*38,39*) | No Definition | No | Yes | N/A | *Law 29698 (2011)*: Includes diagnosis, surveillance, prevention, care and rehabilitation. |

3.4. *Latin America*

As seen in Table 4, our scoping review yielded publications from six countries in the Latin America region. The literature shows that Argentina, Chile and Mexico have adopted the proportion of cases in a population to define rare diseases. Brazil and Colombia have their own definitions, and Peru does not have a definition of rare disease in the document found for this review. None of the publications retrieved indicated whether or not any of the six Latin American countries had a national plan (explicit priorities, timetable, and a budget) for rare diseases. However, the literature revealed a number of legal approaches in Central and South America. Argentina implemented legislation in 2011 to aid patients with rare diseases and their caregivers (*38,39*). Brazil created the "National Policy for Rare Diseases" in 2014 aimed at decreasing morbidity and mortality and increasing quality of life for patients with rare diseases (*39,40*). Also, this policy called for the establishment of reference treatment centers that would provide genetic testing and counseling (*25,40*). Recently, Chile approved a law to provide funding for the care of patients with rare diseases (*39*), and Colombia approved a law in 2010 that identified rare disease as a public health issue (*39,41*). Mexico's legislation for rare disease was a revision to the general health law that authorized Seguro Popular, a national health insurance institution, to provide health insurance coverage and access to orphan drugs for Mexican patients (*39*). Finally, Peru passed legislation for rare diseases in 2011. This law covers from diagnosis to rehabilitation of patients with rare diseases (*38,39*).

**4. Discussion**

For this review, we searched the academic literature for articles about national plans, polices, legislation, strategies and programs for rare diseases from countries around the world. For the study period (2000-2017), we found 56 eligible publications on these subjects from 23 countries organized into four large geographic regions (Canada and the US, Europe, Asian-Pacific countries, and Latin American countries). Of these four regions, the publications reviewed suggest that the countries from the EU presented the most unified legislative approach to rare diseases. For example, the reports for the eight European countries indicate that all of them have adopted a common definition of rare diseases and have developed national plans, laws, and programs or strategies for these diseases. Reports reviewed for the countries within the other three regions indicate that none of these countries has developed a national plan for rare diseases with explicit objectives, a timeline and a budget; however, most countries in these regions appear to have laws and programs or strategies in place for these diseases. Further, according to the literature reviewed, even within a region, the definition of a rare disease varies widely from country to country. In general, our scoping review demonstrates that the legislative approach from all 23 countries seeks to promote the development of, and access to, orphan drugs to facilitate research on rare diseases, to stimulate the development of programs for screening, diagnosis, and registries, and to foster international

collaborations.

Previously, we mentioned studies highlighting the coherent approach of the EU to rare diseases (*16,19,20*), including countries from Eastern Europe (*42*). Other studies have focused on the comparison of specific laws among multiple countries and regions; for example, laws related to facilitating treatments with orphan drugs (*1*) or laws seeking to address the multiple needs of patients with rare diseases (*25*). Our scoping review was specifically designed to identify publications in the academic literature, for the past 18 years, about countries with existing or contemplated national plans, laws, or programs/strategies related to rare diseases. Together, the studies we found indicate that legislation is a widespread approach to addressing the care of patients with rare diseases in populations. Nevertheless, for the period of our research, we found no evidence published in English of the use of this approach in vast areas of the world, such as Africa, India, and Russia.

The enactment of legislation to address the collective needs of patients with rare diseases can vary from country to country and follow a complex path. It requires that a variety of elements are in place not only to promote the passing of a law but to follow through with its implementation. For example, the US Orphan Drug Act of 1983 and its implementation resulted from the combined efforts of patient advocacy groups, medical researchers, healthcare providers, medical associations, government agencies, legislators, and the pharmaceutical industry (*43*). With the help of political and economic treaties, which have facilitated the creation of a common system of laws, the countries of the EU have taken government action for rare diseases one step further by including not only treatments and drugs but also timely diagnosis, access to care, and social support for patients with rare diseases in a multinational setting (*25*). Our compilation also identified multi-country regions with a less cohesive approach to rare diseases than the EU approach. Among the publications that we found, one discusses Latin American as one example of such regions (*44*).

Our compilation highlighted wide variation in government policy approaches to rare diseases around the world, from incipient to comprehensive. This variation has also been noted in another publication (*1*) but here we have included the definition of rare diseases found in the literature as a key feature for comparison. We found that these definitions vary widely among countries, even among countries with well-established plans and strategies. This variation, which is much wider than the differences in definitions described in this report (*45*), could be an obstacle to the integration of national plans into larger international plans. Researchers on rare diseases have emphasized the need for the integration of rare disease plans and studies into international consortiums. This would replace the fragmented approach currently in place with a more coordinated effort that would include larger numbers of patients and

caregivers (*46-48*).

Because we only used English keywords and a limited period for the search, the key limitations of this study are that our results were restricted to documents and journal articles published in the last 18 years that were either completely written in English or had informative summaries written in English; therefore, we captured only a sample of all existing national laws or national law-based approaches to rare diseases. Other limitations include that we did not examine the actual pieces of legislation, our scoping review is based only on documents and articles that describe these pieces of legislation for the scientific community or for the general public. Finally, our search did not distinguish between proposed and enacted legislation; although, most of the publications selected for this exploratory review were about laws already in place, some of them for decades.

In conclusion, the specific creation of laws on rare diseases appears to be a common approach to providing care and support to individuals affected by these diseases, who are few and scattered over large national or international regions. The obstacles to this approach are many, but some countries and regions have made great advances in integrating into their legal system the view that rare diseases collectively deserve public health attention. Future research should bring attention to the evaluation of national or multinational enacted laws on rare diseases, in particular the ones enacted decades ago, to formally ascertain the extent to which the intention of these laws has been fulfilled.

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## Mini-Review

# A basic understanding of Turner syndrome: Incidence, complications, diagnosis, and treatment

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| Summary | Turner syndrome (TS), also known as Congenital ovarian hypoplasia syndrome, occurs when the X chromosome is partially or completely missing in females. Its main clinical manifestations include growth disorders, reproductive system abnormalities, cardiovascular abnormalities, and autoimmune diseases. TS is highly prevalent in China. Timely diagnosis is crucial, and non-invasive prenatal DNA testing can identify TS and other diseases. Treatment of TS mainly involves administration of growth hormone combined with very low doses of estrogen to increase the patient's height. This article describes the incidence, complications, diagnosis, and treatment of TS. |
|---|---|

*Keywords:* Turner syndrome, clinical features, diagnosis, treatment, complication

## 1. Introduction

Turner syndrome (TS) occurs when the X chromosome is completely or partially missing in females. This is the only monomer syndrome that humans can survive. TS is a relatively common type of human chromosomal aberration (*1*) that occurs in 1:2,500 female live births. The features of TS were first described by Turner in 1938, pathogenicity X chromosome monosomy was identified in 1959 (*2*). Monosomy 45,X is present in about 45% of cases, the remaining TS patients show a variety of chimeras and structural abnormalitie (*3*). The main phenotypic characteristic of patients with TS is a short stature, which is common to all patients. Other characteristics include a short neck, a broad chest, genu valgum, and nail dysplasia. The overall mortality rate for patients with TS is higher than that for normal people because of the higher incidence of cardiovascular disease and autoimmune diseases. This article mainly describes the epidemiology, diagnosis, treatment, and complications of TS.

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## 2. Epidemiology of TS

TS is a disease that affects females. The genetic background of the phenotype is highly variable, and analysis of the karyotype can improve understanding of the disease. The "classic" karyotype for TS is 45,X. In a recent study, the classic karyotype was only found in 45% of patients; the remaining patients had a mosaic karyotype (*i.e.* 45,X/46,XX or 45,X/47,XXX), a karyotype with an X chromosome structural abnormality (*e.g.* i(Xq) or i(Xp)), or a karyotype that included the Y chromosome or fragments of the Y chromosome (*4*). A karyotype analysis of 67 patients with TS in Suzhou, China identified the 45,X karyotype in 44.7%, a mosaic karyotype in 17.9%, a karyotype with a chromosomal structural abnormality in 31.4%, and a karyotype that included the Y chromosome or fragments of the Y chromosome in 6.0% (*5*). A karyotype analysis of 62 patients with TS in Linyi identified the 45,X karyotype in 40.3%, a mosaic karyotype in 8.1%, a karyotype with a structural abnormality in 43.5%, and a karyotype that included the Y chromosome or fragments of the Y chromosome in 8.1% (*6*). The 45,X karyotype was the main karyotype in those areas. Karyotypes of patients with TS in several Chinese cities are listed in Table 1.

Based on a number of cytogenetic studies, the incidence of TS is estimated to range from 25 to 210

**Table 1. Karyotypes in patients with Turner syndrome in several Chinese cities**

| Karyotype<br>Area | Monosomy<br>(45,X) | | Mosaic<br>(45,X/46,XX;45,<br>X/47,XXX) | | X chromosome structural abnormality<br>(isochromosome of the long arm, ring,<br>or translocation) | | Includes the Y chromosome<br>or fragments the Y<br>chromosome | | *Ref.* |
|---|---|---|---|---|---|---|---|---|---|
| | total | % | total | % | total | % | total | % | |
| Suzhou | 30/67 | 44.7 | 12/67 | 17.9 | 21/67 | 31.4 | 4/67 | 6 | (5) |
| Linyi | 25/62 | 40.3 | 5/62 | 8.1 | 27/62 | 43.5 | 5/62 | 8.1 | (6) |
| Zhenghou | 8/18 | 44.4 | 5/18 | 27.8 | 5/18 | 27.8 | / | / | (7) |
| Fuzhou | 13/58 | 22.4 | 10/58 | 17.3 | 30/58 | 51.7 | 5/58 | 8.6 | (8) |
| Ningbo | 23/44 | 52.0 | 22/44 | 48 | / | / | / | / | (9) |
| Dalian | 32/78 | 41.0 | 16/78 | 20.5 | 30/78 | 38.5 | / | / | (10) |
| Beihai | 20/73 | 27.4 | 22/73 | 30.1 | 18/73 | 24.7 | 13/73 | 17.8 | (11) |
| Liuzhou | 16/31 | 51.6 | 6/31 | 19.4 | 8/31 | 25.8 | 1/31 | 3.2 | (12) |

per 100,000 women (13). According to study from 1999 to 2004, the incidence of TS in 119,158 births was 1/1,180 or 0.85% (14). The incidence rate of Chinese (0.90‰ or 1/1,111) is higher than that of Malays (0.72‰ or 1/1,389) and India (0.38‰ or 1/2,632). The incidence of TS has increased according to a study in Denmark (15), and the known number of surviving patients with TS steadily increased during that study. Mortality due to TS has also increased. In a UK cohort study, the relative risk of death increased to 4.2 due to an increased risk of diseases of the nervous system, digestive system, cardiovascular system, respiratory system, or genitourinary system (16).

## 3. Complications of TS

### 3.1. *Cardiovascular abnormalities*

An epidemiological study indicated that the overall mortality rate for patients with TS was 3 times that for the normal population (17). Cardiovascular events are a major risk factor and occur in 41% of patients. Patients with TS have congenital cardiovascular abnormalities more often than normal people. Heart valve disease is a prevalent abnormality, and patients with TS have a significantly higher incidence of aortic bicuspid deformity. Patients with TS have a risk of dying mainly from an aortic dissection aneurysm, young people with TS have a significantly smaller aortic diameter than the general population, and aortic surgery is indicated for patients with TS over the age of 18 with an ascending aortic size index > 2.5 cm/m$^2$ to prevent aortic dissection (18). Due to the limited number of patients and ethnic differences, the exact incidence of cardiovascular disease in patients with TS is unclear and needs to be studied further.

### 3.2. *Autoimmune diseasess*

Secondary autoimmune disease is one of the most prominent features of TS due to aneuploidy of the X chromosome (19). TS causes a variety of autoimmune diseases such as thyroiditis, colitis, celiac disease, type 1 diabetes, and psoriasis, though the most common is autoimmune thyroiditis (20). Follow-up studies have indicated that the incidence of autoimmune thyroiditis in patients with TS is 3.2% (21). Chinese (Han) patients with TS are prone to Hashimoto's thyroiditis (22); the prevalence of Hashimoto's thyroiditis in the general population in China is about 0.4-1.5%. The incidence of Hashimoto's thyroiditis in children with TS is significantly higher than that in other regions (23). Compared to the general population, patients with TS have an increased incidence of celiac disease; depending on the number of patients studied, its prevalence varies from 2.2 to 8.1%. Celiac disease may aggravate the manifestation of short stature, hypogonadism, and osteoporosis (24,25). The incidence of other autoimmune disease impacts the lives of patients with TS to an extent.

### 3.3. *Skeletal abnormalities*

Fractures are considered to be one of the major complications of TS. However, there is currently no evidence of an increased risk of fracture in children and adolescents with TS, but there is evidence that women with TS have about a 25% increased risk of fracture, mainly in the form of forearm fractures (26). However, tomographic data from patients with TS are disputed, and especially those from studies of elderly patients who have never received estrogen or who have received delayed and suboptimal therapy, and the prevalence of fractures may be overestimated (15). Landin-Wilhelmsen *et al.* found that osteoporosis and fractures are related to age in patients with TS; of 70 patients with TS, 16% had suffered a fracture and 50% were over the age of 45 (27). Timely diagnosis and treatment can help to keep bone healthy in patients.

## 4. Diagnosis of TS

Prenatal counseling is important, and in some countries a fetus diagnosed with TS is electively aborted. An increasing number of patients are diagnosed with TS during a prenatal examination. Some babies are

diagnosed with TS in the womb or at birth based on the results of an ultrasound examination or signs of lymphedema or congenital heart disease (such as aortic coarctation) (*28,29*). Next-generation sequencing technologies (such as genomes, whole exomes, and gene panel sequencing) are likely to identify more diseases during newborn screening than other methods (*30,31*). However, errors do occur during prenatal examinations, so a complete karyotype analysis needs to be performed to verify those results. The gold standard for diagnosis is karyotype analysis (*32*). Real-time polymerase chain reaction (PCR) gene quantification can be used to diagnose TS. CpG methylation sites specific to X-chromosome inactivation that are widely distributed on the X chromosome may be a marker of TS (*33*).

Attention should also be paid to other signs of TS: *i*) conductive and sensorineural deafness; regular hearing tests should be conducted every 1-3 years; *ii*) hyperopia; a regular eye examination should be performed at age 1.0-1.5; *iii*) strabismus, a normal eye examination should be performed at 4 months to 5 years of age; *iv*) abnormal kidney or liver function; a renal ultrasound should be performed, and at the age of 10 or so urea and creatinine levels, liver function, and the total blood cell count should be measured; *v*) hip dislocation and feeding difficulties; these manifestations should be monitored until infancy; *vi*) otitis media and delayed adolescence; these manifestations should be monitored throughout childhood; *vii*) scoliosis/kyphosis; these manifestations should be monitored during adolescence; and *viii*) dysplasia; this manifestation should be monitored during the entire growth process (*34*). Methods of diagnosing TS are listed in Table 2.

In short, timely diagnosis is very important. In addition to genetic testing, manifestations of TS should be monitored during the entire developmental process so that TS can be treated in a timely manner.

## 5. Treatment of TS

### 5.1. *Growth hormone therapy*

A study has indicated that growth hormone therapy can increase the adult height of patients with TS (*35*). A study administered growth hormone to 16 girls with TS in India over a prolonged period; the patients' height SD score and body mass index indicated that patients with TS did benefit from growth hormone (*36*). A large number of studies have indicated that administration of high doses of biosynthetic human growth hormone can significantly increase the lifelong height of children with TS, so growth hormone therapy is currently the treatment of choice. The sensitivity of an individual to recombinant human growth hormone (r-hGH) is known to vary (*37*); it causes significantly accelerated growth in the first year, but the response gradually diminishes over time (*38,39*). The patient's lifelong height is related to the age at treatment, time, and dose (*1*) and the administration of growth hormone (*40*). Various combination therapies are better than therapy with growth hormone alone. Long-term growth hormone therapy has a positive effect on craniofacial development in girls with TS, and its greatest impact is on posterior facial height and the height of the mandibular ramus (*41*).

### 5.2. *Estrogen therapy*

Retarded adolescent growth is related to a deficiency of estrogen in patients with TS, so estrogen is administered (*42*). In the past, estrogen replacement therapy started when the patient was 15 years old to avoid premature closure of the epiphysis, thus affecting the patient's lifelong height. The general recommendation is that patients be started on small doses of estrogen at age 12, enabling the patient to begin developing secondary sexual characteristics and the uterus and to improve liver function, cognitive function, and quality of life (*43*). A recent trial administered r-hGH and low-dose estrogen to patients with TS for 20 years (*44*). Results clearly indicated that administering very low doses of estradiol and r-hGH in adolescence produced estrogen levels close to those of healthy girls in puberty; as adolescent girls with TS mature, increasing the dose of estradiol greatly increases their final adult height. Many forms of estrogen can be used to treat patients,

**Table 2. Several methods for diagnosing Turner syndrome**

| Method of diagnosis | Characteristics | *Ref.* |
|---|---|---|
| Next-generation sequencing technologies (such as genomes, whole exomes, and gene panel sequencing) | Noninvasive prenatal test; allows the identification of more diseases. | (*30,31*) |
| Karyotype (gold standard) | Labor intensive, unrealistic for large-scale population or high-throughput testing. | (*32*) |
| Molecular methods (Southern blotting, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), fluorescent PCR genotyping, GeneScan-based genotyping, and real-time PCR) | Cannot effectively detect individuals with mosaic or partial X chromosome deletions. | (*33*) |
| Developmental process (hearing, vision, liver function, kidney function, and spine) | Can help with diagnosis. | (*34*) |

**Table 3. Treatment of Turner syndrome**

| Treatment of Turner syndrome | Characteristics | *Ref.* |
|---|---|---|
| Growth hormone therapy | Individual sensitivity is variable. Lifelong height is related to age, time, and dose of treatment. | *(1,37)* |
| Estrogen therapy | The general recommendation is to start a low dose of estrogen replacement therapy at the age of 12. Increasing the dose of estradiol given to girls with TS will greatly promote final adult height. | *(43,44)* |
| Oxymetholone treatment | Marked effect when used in combination with growth hormone. Adverse reactions may occur, so this treatment should be used with caution. | *(45,46)* |
| Other methods | Traditional Chinese medicine treatment; vitamin D supplementation; active lifestyle. | *(27,48-50)* |

the most common of which is oral estrogen followed by transdermal patches. However, whether young patients with TS should take oral estrogen or use estradiol transdermal patches needs to be verified further (*43*).

### 5.3. Oxandrolone therapy

In 1986, a trial administered r-hGH alone or in combination with androgen for the first time; once the trial was complete and patients with TS reached their final height, this combination therapy significantly increased growth and final adult height (*45*). However, the possibility of adverse reactions (such as masculinization (*e.g.* an enlarged clitoris, deeper voice, hirsutism, and acne), a delay in breast development, and lower HDL cholesterol levels) has prompted caution in the clinical use of androgens (*46*). Currently, oxytocin is seldom used because hormone replacement has proven to be a more effective treatment when using estradiol in combination with r-hGH (*47*).

### 5.4. Other treatments

Liao *et al*. administered nandrolone phenylpropionate in the early stages of TS to promote the synthesis of protein, and they also administered a traditional Chinese medicine – Liuwei Dihuang pills – to aid the kidneys (*48*). This alleviated the lack of estrogen and it also prompted the patient's genital organs and secondary sexual characteristics to develop to an extent, resulting in limited menstruation. Fractures are one of the major complications of TS. The mechanism of bone injury in patients is not clear, but an estrogen deficiency and X chromosome abnormalities are key factors. Several studies have noted a low level of vitamin D in the serum of patients with TS, and this may lead to lower bone mineral density (*27,49*). Therefore, vitamin D supplementation and an active lifestyle including weight-bearing activities and regular sports are of great benefit to the health of bone in patients with TS (*50*). Forms of treatment are listed in Table 3.

### 6. Prospects for diagnosis and treatment of TS

TS is a rare disease in which all or part of the X chromosome is missing, and patients' growth and lives are heavily affected. Timely diagnosis and treatment is crucial. The incidence of cardiovascular diseases and bone abnormalities in TS is currently being studied. In addition to unusual physical phenotypes, patients with TS exhibit characteristic neurocognitive features that involve deficits in visual spatial processing. Cognitive deficits that have been found in TS seem to persist into adulthood. Whether this is caused by genetic mechanisms or only by hormones and other biological factors is unclear. Genetic and hormonal effects may need to be studied in the same patient. Further research is needed in this area to determine how genes, karyotypes, and the brain are linked to cognition (*51*). The latest structural and molecular biology techniques need to be used in post-mortem studies, modern genomic strategies need to be adopted, and medical histories need to be routinely reported (*52*).

Given trends in biomedical development, the next generation of treatment will be based on stem cells and regenerative medicine. Stem cell research has become an area of interest. Stem cells are cells that have the potential to proliferate, differentiate, and self-renew. Somatic cells are dedifferentiated into pluripotent stem cells by introducing foreign genes, and those stem cells are known as induced pluripotent stem (iPS) cells. The major advantage of iPS cells over embryonic stem (ES) cells is that iPS cells can be derived from a patient's own somatic cells, thus avoiding immunological rejection and ethical issues (*53*). For rare diseases such as TS, the somatic cells of patients can be extracted and their dedifferentiation into stem cells can be induced to create a model of the disease in order to study its pathogenesis and to develop new methods of studying and treating that disease.

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## Original Article

# Aging-associated latent herpes viral infection in normal Japanese individuals and patients with Werner syndrome

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**Summary**  **A series of our "inflammageing" study examining serum samples from a maximum of 217 healthy Japanese individuals aged between 1 and 100 years and mutation-proven 40 patients with Werner syndrome (WS) indicated normal aging-associated elevations of highly sensitive CRP (hsCRP) and matrix metalloproteinase-9 (MMP-9). To further study the contribution of environmental factors such as persistent herpes viral infection to inflammageing, IgG antibodies against varicella/zoster virus (VZV) and cytomegalovirus (CMV) were examined in the same serum samples as has been done for hsCRP and MMP-9 analyses. The mean levels of serum IgG viral antibodies were comparable between normal (mean ± SE: 31.0 ± 4.3 unit) and WS (38.6 ± 7.6) for CMV, and between normal (42.0 ± 12.2) and WS (29.8 ± 3.8) for VZV, respectively. Significant associations of aging with IgG anti-CMV antibody were in normal aging ($p = 0.023$) and WS ($p = 0.037$), but not with IgG VZV in both conditions. Aging-associated change of IgG anti-CMV antibody titer in WS increased significantly (1.32 times higher) compared with normal aging ($p = 0.037$). IgG anti-CMV level was significantly elevated in the male gender than female in both conditions ($p = 0.006$). Elevated hsCRP level was significantly associated with IgG anti-CMV ($p = 0.016$) and IgG anti-VZV ($p = 0.008$) antibodies in normal aging, but not in WS. Serum MMP-9 was significantly associated with IgG anti-CMV level ($p = 0.0002$) in normal aging, but not in WS. Persistent herpes viral infection may constitute a part of "inflammageing" in normal aging and WS.**

***Keywords:*** *Aging, CRP, Cytomegalovirus, Herpes virus, Inflammageing, MMP-9, Werner syndrome*

## 1. Introduction

Human aging is probably controlled by a combination of intrinsic/genetic factors including metabolism/catabolism functions (*1*), and extrinsic/environmental factors such as infections (*2*). The possible intrinsic/genetic factors include genetically-determined progeroid syndrome genes such as *WRN* for Werner syndrome (WS) (*3*). WS has been proposed as the representative human natural ageing model. Because patients with WS, transmitted autosomal-recessively by the homozygous mutation of *WRN* (RecQ3 DNA helicase), usually manifest their premature aging phenotypes immediately after adulthood. Typical aging signs and symptoms in WS include voice change, bilateral cataracts, gray hair/alopecia, skin atrophy, skin pigmentation, type II diabetes mellitus, central obesity, atherosclerosis, hyperlipidemia, skin ulcer, renal dysfunction, osteoporosis, and cancer/sarcoma. The

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average age of death either by myocardial infarction or malignancy is about 50 years old. So far, 1,300 cases have been recognized all over the world, in which roughly 75% of the patients are of Japanese origin (*3*).

Human aging is inevitably encountered by an increasing chance of environmental attack from infectious agents such as herpes viruses during daily living leading to a minor/latent inflammation that could be evaluated by highly sensitive CRP (hsCRP) and matrix metallo-proteinase-9 (MMP-9) ( *1,2,4,5*).

Chronic elevation of hsCRP in the normal elderly population may probably be associated with aging-related pathological conditions including diabetes mellitus (DM), sarcopenia, osteoporosis, cancer, atherosclerosis, cognitive decline and finally death (*6,7*). Aging-related persistent, systemic, minimal and asymptomatic inflammation, termed 'inflammageing', is tightly associated with an imbalance between an increase in pro- and a decrease in anti-inflammatory substances including cytokines/chemokines (*8- 11*). Inflammation is widely recognized as a patho-physiologically fundamental metabolism to generate energy with thermogenesis, leading to wound healing and tissue destruction during healthy development and aging (*6,12,13*).

We have reported possible biomarkers for aging in a series of inflammageing studies by using the same serum samples from normal Japanese individuals aged between 1 and 100 years old and mutation-proven progeroid patients with WS (*4,5,8,9*).

The aim of this study was to clarify the associations of serum level of latent herpes viral infections including varicella/zoster virus (VZV) and cytomegalovirus (CMV) with normal aging and the WS patients, and also with the aging-associated increase of hsCRP and MMP-9 by directly comparing the same serum samples used in the series of our inflammageing study.

## 2. Materials and Methods

### 2.1. *Study population*

All the samples studied in the present experiment were the same sera as were used in the previous hsCRP and MMP-9 studies (*4,5*). A total of 72 normal serum samples from both sexes (M = 32, F = 40) aged between 18 and 100 years were used for the study (Table 1). Normal individuals, enjoying the usual daily life at home or nursing home, had neither apparent inflammatory diseases including infection, cancer, lymphoproliferative disorders, DM, Alzheimer's disease, autoimmune diseases and arthritis at the time of serum sampling, nor history of cardio-/cerebro-vascular accidents. Exclusion protocol for elderly individuals met the SENIEUR criteria (*14*).

Serum samples were also obtained from 40 mutation-proven WS patients without any medication at the time of serum sampling (M = 23, F = 17; between 32 and 70 years old); a part of "Goto collection of Werner syndrome" (*http://cell.brc.riken.jp/ja/gmc.html*).

As indicated in Table 2, nine WS patients were free from skin ulcers (SU) [SU (-)], while 31 had SU [SU (+)]. 23 had DM [DM (+)], but 17 did not [DM (-)]. WS patients were sub-grouped into 1) (SU (+) DM (+) (*n* = 19), 2) SU (+) DM (-) (*n* = 12), 3) SU (-) DM (+) (*n* = 4) and 4) SU (-) DM (-) (*n* = 5).

All of the individuals provided written informed consent for this study, which was approved by the ethics committee of Toin University of Yokohama. All of the samples were stored at -80°C until use.

### 2.2. *Screening of latent herpes viral infection*

Serum levels of IgG anti- CMV antibody and IgG anti- VZV antibody were measured using a CYTOMEGALLO IgG (II)-EIA "SEIKEN" kit and a varicella-Zoster IgG EIA "SEIKEN" kit according to the manufacturer's manual, respectively (Denka Seiken Co., Ltd., Tokyo, Japan). Colorimetric measurements were taken with an automatic ELISA reader (Behringer ELISA processor III) using a 486 nm filter. Assays were carried out in duplicate and the antibody titer was read from a standard curve made with known concentrations of the respective viral antibody. Results are given in arbitrary units, as defined by the reference serum diluted 1:400 and 1:102,400.

### 2.3. *Determination of hsCRP and MMP9*

The data of hsCRP (ug/mL) used in this study was obtained in the previous experiment (*4*) by using a CircuLex high-sensitivity CRP ELISA kit (Cyclex Co., Nagano, Japan) according to the user's manual. The concentration of MMP-9 (ng/mL) in the sera was determined by specific sandwich ELISA using a Human MMP-9 ELISA kit (Fuji Chemical Industries, Toyama, Japan) as described before ( Table 1 and Table 2 ) (*5*).

### 2.4. *Data analysis and statistics*

We examined association of the respective serum IgG anti-CMV antibody or IgG anti-VZV antibody and the respective healthy aging individuals or WS patients by using linear regression model expressed as

$$Y = \alpha + \beta_1 * Group + \beta_2 * Age + \beta_3 * (Age * Group) + \beta_4 * Sex, \qquad (1)$$

where Y means IgG anti-CMV antibody titer or IgG anti-VZV antibody titer (unit/ml). And α means an estimated intercept. Furthermore, $\beta_1$ indicates estimated coefficient for Group, where Group = 0 in normal aging and Group = 1 in WS, $\beta_2$ indicates coefficient for

**Table 1. Herpes viral infection in normal Japanese individuals**

| Age | Sex | hsCRP[a](µg/mL) | MMP9[b](ng/mL) | anti-VZV[c] | anti-CMV[d] |
|-----|-----|-----------------|----------------|-------------|-------------|
| 18 | F | 0.23 | 93.4 | 5.1 | 22.5 |
| 25 | F | 0.18 | 327 | 24.2 | 2 |
| 25 | M | 0.76 | 55.4 | 26.3 | 2 |
| 26 | M | 0.29 | 247 | 3.3 | 10.5 |
| 27 | F | 0.23 | 191 | 15.8 | 2 |
| 28 | M | 0.67 | 154 | 11.8 | 6.1 |
| 30 | F | 0.23 | 126 | 12.4 | 30.1 |
| 31 | F | 0.20 | 0.8 | 16.2 | 11.1 |
| 32 | M | 0.29 | 4.7 | 16.9 | 12.4 |
| 32 | F | 2.30 | 348 | 7.5 | 2 |
| 33 | F | 0.08 | 134 | 55.3 | 2 |
| 35 | M | 0.83 | 79.2 | 51.5 | 3.9 |
| 35 | M | 0.33 | 145 | 33.8 | 7.9 |
| 36 | F | 0.61 | 206 | 13.3 | 21.8 |
| 36 | F | 0.31 | 302 | 149 | 28.4 |
| 37 | F | 0.42 | 58.8 | 15.2 | 19.7 |
| 38 | F | 0.57 | 84.5 | 27.5 | 41.1 |
| 43 | M | 0.13 | 26.8 | 24.5 | 2 |
| 46 | F | 8.88 | 155 | 18.8 | 75.5 |
| 47 | F | 0.20 | 198 | 15.1 | 58.1 |
| 48 | M | 0.30 | 51.4 | 3.6 | 4.6 |
| 49 | F | 1.14 | 91.2 | 32 | 28.4 |
| 53 | M | 0.35 | 105 | 9.5 | 23.6 |
| 55 | M | 0.22 | 149 | 15.4 | 8.4 |
| 57 | M | 0.76 | 40.4 | 9.3 | 24.5 |
| 57 | M | 1.98 | 94.1 | 51.2 | 9.8 |
| 60 | M | 0.17 | 54.3 | 2.9 | 18.7 |
| 60 | M | 0.93 | 49.7 | 20.2 | 12 |
| 60 | M | 0.08 | 62.0 | 23.5 | 2 |
| 60 | M | 2.79 | 70.3 | 24.7 | 49.7 |
| 60 | M | 0.35 | 36.8 | 64.5 | 25.4 |
| 61 | M | 0.97 | 4.8 | 7.6 | 23.2 |
| 62 | M | 7.02 | 98.9 | 18.8 | 14.6 |
| 62 | F | 0.28 | 26.3 | 18.8 | 14.6 |
| 66 | M | 1.24 | 92.2 | 26.4 | 14.2 |
| 67 | M | 0.37 | 78.8 | 9.8 | 10.3 |
| 68 | M | 0.23 | 26.8 | 18.4 | 26.4 |
| 68 | M | 2.83 | 24.9 | 68.4 | 14.2 |
| 71 | M | 0.50 | 56.0 | 22.8 | 22.9 |
| 73 | M | 0.76 | 101 | 13.3 | 36.7 |
| 74 | F | 0.55 | 240 | 9.2 | 15.5 |
| 74 | F | 3.06 | 52.3 | 9.6 | 38.1 |
| 75 | M | 0.11 | 212 | 39.6 | 16.5 |
| 75 | F | 1.94 | 40.4 | 22.3 | 19.4 |
| 77 | F | 0.60 | 122 | 3.7 | 11.4 |
| 78 | M | 0.28 | 354 | 5.7 | 19.6 |
| 81 | M | 29.6 | 784 | 64.7 | 34.6 |
| 81 | F | 19.1 | 531 | 18.9 | 97 |
| 81 | F | 0.84 | 341 | 64.7 | 34.6 |
| 82 | F | 2.26 | 284 | 33 | 39.9 |
| 82 | F | 0.30 | 123 | 4.9 | 17.2 |
| 83 | M | 2.90 | 29.0 | 182 | 28.2 |
| 83 | F | 0.34 | 276 | 95 | 182 |
| 84 | F | 0.54 | 62.2 | 16.4 | 95 |
| 86 | F | 17.7 | 228 | 11.1 | 59.6 |
| 88 | F | 4.36 | 156 | 37 | 24.1 |
| 88 | F | 3.48 | 145 | 42.2 | 27.2 |
| 89 | F | 10.9 | 10.2 | 116 | 27.5 |
| 89 | F | 11.9 | 4.9 | 13.2 | 70.6 |
| 91 | F | 0.11 | 47.5 | 19 | 21.8 |
| 91 | F | 18.2 | 172 | 57.2 | 25.8 |
| 92 | F | 0.19 | 160 | 9.7 | 28.3 |
| 94 | M | 4.75 | 317 | 15.3 | 14 |
| 94 | F | 16.5 | 508 | 5.2 | 23.4 |
| 94 | F | 2.67 | 356 | 11.9 | 14.8 |
| 96 | M | 3.06 | 435 | 53.3 | 222 |
| 96 | M | 2.81 | 128 | 19.6 | 15.2 |
| 96 | F | 23.6 | 509 | 26.9 | 34.5 |
| 96 | F | 0.65 | 186 | 56.5 | 42.5 |
| 98 | F | 0.75 | 295 | 6.6 | 112 |
| 99 | F | 1.40 | 106 | 14.4 | 44.2 |
| 100 | F | 23.2 | 295 | 54.2 | 29 |

[a]hsCRP: highly sensitive CRP, [b]MMP-9: matrix metalloproteinase-9, [c]anti-VZV: anti-varicella/ zoster virus, [d]anti-CMV: anti-cytomegalovirus.

**Table 2. Herpes viral infection in Werner syndrome patients**

| Subgroups | | | Age | Sex | hsCRP[a] (ug/mL) | MMP-9[b] (ng/mL) | anti-VZV[c] | anti-CMV[d] |
|---|---|---|---|---|---|---|---|---|
| | SU[e] | DM[f] ID | | | | | | |
| 1 | + | + WS12901 | 32 | F | 2.61 | 134 | 20.7 | 35.1 |
| 1 | + | + WS57201 | 37 | M | 22.8 | 351 | 8.9 | 9.4 |
| 1 | + | + WS56301 | 39 | M | 0.79 | 284 | 35.9 | 12.3 |
| 1 | + | + WS19201 | 38 | M | 7.03 | 82.2 | 19.2 | 28.9 |
| 1 | + | + WS5501 | 40 | F | 11.1 | 10.2 | 4.6 | 43.3 |
| 1 | + | + WS57801 | 41 | M | 1.04 | 122 | 45.5 | 2 |
| 1 | + | + WS51301 | 42 | M | 17.4 | 252 | 11.8 | 70.4 |
| 1 | + | + WS4705 | 45 | F | 42.4 | 224.3 | 33.9 | 33.8 |
| 1 | + | + WS6301 | 46 | M | 27.2 | 184 | 4.5 | 7.3 |
| 1 | + | + WS53601 | 46 | M | 16.3 | 24 | 37.2 | 28 |
| 1 | + | + WS0101 | 47 | M | 15 | 49.3 | 9.2 | 25.6 |
| 1 | + | + WS58501 | 51 | M | 4.02 | 414 | 2.7 | 22.3 |
| 1 | + | + WS58301 | 53 | M | 3.21 | 357 | 75 | 53.7 |
| 1 | + | + WS4704 | 54 | M | 3.42 | 32.1 | 53.4 | 41.4 |
| 1 | + | + WS17201 | 54 | F | 2.26 | 198.5 | 50.2 | 44.1 |
| 1 | + | + WS0801 | 55 | F | 8.99 | 167 | 44.1 | 50.1 |
| 1 | + | + WS54801 | 57 | M | 5.88 | 127 | 38.7 | 22.9 |
| 1 | + | + WS56201 | 70 | M | 10.3 | 85.8 | 12.7 | 10.3 |
| 1 | + | + WS1801 | 70 | M | 28.3 | 235.5 | 17.5 | 189 |
| 2 | + | - WS6103 | 32 | M | 1.62 | 2 | 3.7 | 2 |
| 2 | + | - WS6104 | 32 | M | 25 | 867.6 | 260 | 2 |
| 2 | + | - WS14501 | 35 | M | 4.55 | 129 | 40.8 | 25.5 |
| 2 | + | - WS53101 | 38 | F | 22.9 | 294 | 10.7 | 37.1 |
| 2 | + | - WS51601 | 36 | F | 0.98 | 222 | 28.8 | 2 |
| 2 | + | - WS53901 | 43 | F | 1.28 | 38.4 | 18.1 | 30.7 |
| 2 | + | - WS53801 | 46 | F | 0.98 | 103 | 44.8 | 172 |
| 2 | + | - WS2101 | 50 | F | 8.66 | 30.8 | 8.4 | 34.3 |
| 2 | + | - WS55801 | 53 | F | 18.2 | 45.4 | 21.3 | 66 |
| 2 | + | - WS52901 | 54 | F | 10.8 | 45.8 | 5.4 | 28.6 |
| 2 | + | - WS54001 | 57 | F | 7.04 | 70.3 | 6 | 5.9 |
| 2 | + | - WS4701 | 59 | F | 11.8 | 33.6 | 56.2 | 15.1 |
| 3 | - | + WS57701 | 38 | F | 2.07 | 197 | 22.6 | 82.8 |
| 3 | - | + WS58701 | 35 | M | 2.93 | 108 | 15.4 | 2 |
| 3 | - | + WS57401 | 41 | M | 26.9 | 0 | 23 | 2 |
| 3 | - | + WS4401 | 41 | M | 24.9 | 30 | 13.3 | 16.6 |
| 4 | - | - WS5801 | 43 | M | 1.28 | 2.9 | 5.7 | 8.2 |
| 4 | - | - WS10501 | 52 | F | 3.86 | 16.7 | 72.7 | 150 |
| 4 | - | - WS0402 | 47 | M | 1.15 | 19.5 | 488 | 2 |
| 4 | - | - WS7501 | 48 | M | 22.3 | 66.7 | 8.3 | 28.2 |
| 4 | - | - WS0401 | 49 | F | 4.76 | 52.9 | 2 | 2 |

[a]hsCRP: highly sensitive CRP, [b]MMP-9: metalloproteinase-9, [c]anti-VZV: anti-varicella/ zoster virus, [d]anti-CMV: anti-cytomegalovirus, [e]SU: skin ulcer, [f]DM: diabetes mellitus.

continuous age, $\beta_3$ indicates a coefficient of interaction for Age and Group, and $\beta_4$ indicates coefficient for Sex, where Sex = 1 for male and Sex = 0 for female. In order to show a reliability of mean, we show its standard error of the mean. Furthermore, we used Pearson correlation coefficients to show a statistical relationship between two variables. *P*-values < 0.05 are considered to be statistically significant.

## 3. Results

### 3.1. *Latent herpes viral infection in normal aging and WS*

The serum levels of IgG viral antibodies were comparable between normal aging (mean ± SE: 31.0 ± 4.3 unit) and WS (38.6 ± 7.6) for CMV, and between normal aging (42.0 ± 12.2) and WS (29.8 ± 3.8) for VZV, respectively (Table 1 and Table 2).

In WS, IgG anti-VZV antibody levels in respective subgroups were 1) 27.7 ± 4.7 (mean ± SE), 2) 42.0 ± 20.4, 3) 18.6 ± 2.5, and 4) 115.3 ± 94.1. The mean ± SE of IgG anti-VZV antibody was not significantly different between any combination of subgroups. IgG anti-CMV antibody levels in respective subgroups of WS were 1) 38.4 ± 9.3, 2) 43.4 ± 17.4, 3) 25.9 ± 19.3 and 4) 38.1 ± 28.4. The mean ± SE of IgG anti-CMV antibody was not significantly different between any combination of subgroups in WS.

Furthermore, we did not observe any significant

**Table 3. Association of anti-CMV antibody, age and sex in normal aging and WS**

| Variables | Estimated regression coefficient | SE | *p* value |
|---|---|---|---|
| Intercept | - 10.303 | 12.17 | 0.399 |
| Group(1, 0) | - 38.812 | 30.648 | 0.208 |
| Age | 0.404 | 0.175 | 0.023 |
| Age*Group | 1.318 | 0.624 | 0.037 |
| SEX(1, 0) | 19.988 | 7.141 | 0.006 |

Linear regression model used in this study was expressed as $Y = \alpha + \beta_1*Group + \beta_2*Age + \beta_3*(Age*Group) + \beta_4*Sex$, where Y means IgG anti-CMV antibody titer (unit/mL). And $\alpha$ means an estimated intercept. Furthermore, $\beta_1$ indicates estimated coefficient for Group, where Group = 0 in normal aging and Group = 1 in WS, $\beta_2$ indicates coefficient for continuous age, $\beta_3$ indicates a coefficient of interaction for Age and Group, and $\beta_4$ indicates coefficient for Sex, where Sex = 1 for male and Sex = 0 for female. Estimated regression coefficients are indicated in the left column and *p* values in the right column. IgG anti-cytomegallovirus (CMV) antibody titer is expressed as an arbitrary unit/mL.



**Figure 1. Age-associated increase in IgG anti-CMV antibody in normal individuals and Werner syndrome.** Linear regression model for female described in Table 3 was used. WS: solid line, normal aging: dotted line. Regression lines for male (bold line) in both groups were higher than the fine line of female by a value of 20 as indicated in Table 3.

relationship of the titers of IgG anti-VZV antibody/ IgG anti-CMV antibody and patients' calendar age, sex, hsCRP, and MMP-9 in the respective subgroups of WS by linear regression analysis.

### 3.2. *Aging-associated changes of latent herpes viral infection*

Simple Pearson correlation of IgG anti-CMV antibody and normal aging was 0.39 (*p* = 0.0008) for normal group and 0.35 (*p* = 0.027) for WS. The results of the multiple linear regression model (1) for IgG anti-CMV antibody titer and age with covariates for group and sex are indicated in Table 3. Estimated regression lines for WS and normal aging are shown in Figure 1. Here, solid line indicates WS (male: bold line and, female: fine line) and dotted line normal aging (male: bold and female: fine).

Aging-associated change of IgG anti-CMV antibody titer in WS increased significantly (1.32 times higher) compared with that in normal aging (*p* = 0.037). IgG anti-CMV level was significantly elevated in male gender compared to female in both conditions (*p* = 0.006).

IgG anti-VZV antibody titer neither correlated with calendar aging in normal aging nor WS.

### 3.3. *hsCRP-associated changes of latent herpes viral infection*

Inflammation monitored by the serum level of hsCRP was significantly associated with healthy aging as shown in the previous report. No significant gender difference was observed concerning to the age-associated increase in hsCRP level (*4*).

IgG anti-CMV antibody was significantly correlated with hsCRP (*p* = 0.016) in normal aging, if age and sex were adjusted.

IgG anti-VZV antibody was significantly (*p* = 0.008)

correlated with serum level of hsCRP in normal aging, if sex was adjusted, but not, if age was adjusted.

In WS, neither IgG anti-CMV antibody nor IgG anti-VZV antibody was correlated with hsCRP.

### 3.4. *MMP-9-associated changes of latent herpes viral infection*

IgG anti-CMV antibody significantly (*p* = 0.0002) correlated with serum level of MMP-9 in normal aging, if age and sex were adjusted, but not in WS.

IgG anti-VZV antibody significantly correlated with sex (*p* = 0.019) in normal aging, if MMP-9, age and sex were adjusted, but not in WS.

### 4. Discussion

We have for the first time reported in the present study that calendar aging-associated anti-CMV antibody titer in WS significantly increased compared with that in the healthy aging Japanese population living under a similar environment. As aging-associated increase in IgG anti-CMV antibody in healthy aging has been well documented (*15-19*), the persistent viral infections during aging may attribute to a mild but significant decline in immune function with normal aging and WS followed by tissue-destructive chronic inflammation (inflammageing) after maturation stage (*12,13*).

We have already reported the increasing level of serum hsCRP and MMP-9 in accordance with healthy calendar aging and also significantly more elevation of hsCRP compared with healthy aging controls in patients with a genetically-determined progeroid syndrome such as WS (*4,5*).

The pathogenesis of aging-associated inflammation,

probably driven by a combination of environmental factors including viral infections and genetic factors, is not well studied (*20*).

Possible environmental factors, which can produce inflammatory cytokines may include beta-herpes virus such as CMV (*21,22*). Human CMV causes many infection-related birth defects, and may trigger a variety of age-associated inflammatory diseases such as vascular diseases, autoimmune diseases, hepatitis, interstitial pneumonitis, gastrointestinal diseases and atherosclerosis (*23,24*). The CMV-infected mononuclear cells produce a variety of cytokines such as IL-2, IL-6 and TNF-α through the MAPK/ERK pathway (*21*). Human herpes virus 6, the same subfamily of CMV, has recently been shown to have ATPase, helicase, exonuclease and DNA-binding activities and can integrate into telomeres of the human chromosome (*22*).

CRP, induced by IL-6 can act as pro-inflammatory by inducing the expression of TNF-α and IL-1β, and is the prototypical acute-phase reactant in man (*25*). Serum hsCRP has been proposed as a marker of atherosclerosis-associated diseases including coronary heart disease and cerebro-vascular accidents, and also inflammageing (*7,26*).

CRP can also function as a component of the innate immune system by activating the classical pathway of the complement system (*27*), enhancing phagocytosis (*28*). CRP may act as a protective machinery against a variety of inflammatory conditions and autoimmunity by interacting with many anti-inflammatory mediators such as IL-10, transforming growth factor-β and IL-12 (*25-29*). So, CRP has an antagonistically pleiotropic activity and the elevates inflammation associated with healthy aging and WS may not be the direct result of one-way traffic destruction of tissues, but the sum result of ongoing tissue degradation and repair by MMPs and cytokine/chemokine circuit-driven inflammation and regeneration (*6*).

Inflammation has been believed to be an energy supply mechanism to proceed to normal repair mechanisms during a whole life and normal development before maturation followed by tissue destruction after a senescent stage, though the precise mechanism of inflammageing is still uncovered (*20*).

The possible pathogenetical contribution of VZV and CMV to SU and DM either in natural aging and WS has never been reported and we did not find a relationship between SU/DM and VZV/CMV in WS, although the incidence of VZV and CMV infection and the frequency of SU and DM are generally age related.

The reason why there is more inflammation with an elevated level of anti-CMV antibody in WS than healthy counterparts is a complete mystery.

As the recent WS gene (WRN) knock-out mice study suggested an induction of immune dysfunction followed by chronic inflammation (*30*), we would like to speculate that the loss-of-function mutation of WRN

may lead to some immune dysfunction leading to more susceptibility to CMV infection in WS than normal aging populations. Because natural killer cell function declined significantly in WS and WS patients produced more auto-antibodies compared with the normal aging population as we already reported (*4,5,8,9,31-35*). Obviously, this is a highly speculative idea and further study may be needed to clarify the pathogenesis of mild inflammation: inflammageing in healthy aging and also in WS.

## Acknowledgements

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## Original Article

# Uptake and radiological findings of screening cerebral magnetic resonance scans in patients with hereditary haemorrhagic telangiectasia

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**Summary**     Hereditary haemorrhagic telangiectasia (HHT) results in arteriovenous malformations (AVMs), most commonly in the lungs, liver and brain. Discussion of cerebral vascular malformations is an important element of patient management. The current study objectives were to examine uptake and results of screening cerebral magnetic resonance (MR) scans, excluding symptomatic patients requiring neurological investigations. The remaining non-symptomatic individuals received formal pretest counselling that differed according to family history. For the 603 patients with no neurological symptoms of concern, screening scan uptake was higher after publication of the ARUBA trial. Patients with a family history of cerebral haemorrhage were 4 to 14-fold more likely to have a screening scan than patients with no such family history. For patients without neurological symptoms suggesting cerebral AVMs, none of the 59 screening scans performed at our institution demonstrated a cerebral AVM. Four scans (6.8%) demonstrated small aneurysms. The most common abnormality was cerebral infarction (20/59, 33.9%), predominantly identified in patients with pulmonary AVMs. Of 29 pulmonary AVM patients with no previous history of clinical stroke, 16 (55.2%) had between one and five silent infarcts. For HHT patients with pulmonary AVMs, the most frequently affected sites were the cerebellum (40%) and thalamus (14.3%), and the age-adjusted odds ratio for an infarct was 21.6 (95% confidence intervals 3.7, 126), $p = 0.001$. We concluded that for cerebral screening programmes in HHT, the findings support informed patient choice incorporating understanding that cerebral AVMs are rare in non-symptomatic HHT patients, but that screening scans commonly detect silent cerebral infarction due to pulmonary AVMs.

***Keywords:*** Cerebral infarction, cardioembolic stroke, counselling, magnetic resonance imaging, paradoxical emboli, arteriovenous fistulas, stroke, pulmonary arteriovenous malformations

## 1. Introduction

Hereditary haemorrhagic telangiectasia (HHT) is a vascular dysplasia that is inherited as an autosomal trait (*1*). Epidemiological studies in a variety of countries suggest HHT affects approximately 1 in 6,000 individuals

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(*2-4*) although there are regional differences, and higher prevalences in isolated communities due to founder effects (*3,5*). HHT results in mucocutanous and visceral vascular malformations with nosebleeds (epistaxis) the hallmark of HHT. Arteriovenous malformations (AVMs) are most frequent in the lungs and liver, with pulmonary and hepatic AVMs each affecting approximately 50% of HHT patients (*6,7*). Latest data suggest cerebral AVMs affect between 7.7-12.8% of HHT patients (*8-10*). Cerebral AVMs and fistulae raise concern due to the possibility of cerebral haemorrhage, and it is the risk of undiagnosed cerebral AVMs that seems to cause most concern to HHT communities (*11,12*).

Risk benefit considerations strongly support the screening of asymptomatic individuals with HHT to identify pulmonary AVMs, and if identified, to treat in order to reduce the risks of paradoxical ischaemic stroke and cerebral abscess, and improve low blood oxygen levels (hypoxaemia) (*13*). Asymptomatic screening programmes have been in place for decades, with treatments (usually by embolization) demonstrated to reduce the risk of ischaemic stroke and brain abscess (*14*). Patients who have had pulmonary AVMs embolized are often left with smaller, untreatable AVMs (*15,16*) and remain at lower (*16,17*) but not negligible risks of complications.

Although the risk-benefit considerations for cerebral AVM screening have always been less clear cut than for pulmonary AVMs, HHT patients commonly undergo screening cerebral magnetic resonance imaging (MRI) scans for the purposes of identifying cerebral vascular malformations (*8-10,18*). We, like many other groups, initially considered the risk of cerebral haemorrhage to be sufficient to support asymptomatic screening (*19*). International Guidelines generated in 2006 (*18*) recommended screening of "adult patients with possible or definite HHT for cerebral vascular malformations" although at marginal levels of evidence and agreement (level of evidence III; strength of recommendation: weak; agreement: 77%). Taken together with the non-negligible risks of treatments, the practice of screening remained controversial in many countries, and was not universally adopted. Publication of the ARUBA trial (*20*), demonstrating that for unruptured cerebral AVMs in the general population, stroke risk was higher following treatments used at that time, and data that suggest haemorrhage risk may be lower for cerebral AVMs in patients with HHT than in the general population (*21,22*), have heightened the controversy (*23*).

Good clinical practice advocates involving patients in important decisions regarding their care, and this is particularly relevant where the question concerns an area for which evidence to support a particular investigation or treatment is less than compelling. For this reason, we offer pretest counselling prior to arranging any screening cerebral MR scan. Further, to support the screening pathway, in 2010 we adopted the formal categorisation proposed by the late Pr. Pierre Lasjaunias (*24*): Patients are assigned to Group 1 if they have neurological symptoms potentially of concern, and a cerebral MR scan is organised to investigate the symptoms, alongside formal neurological review. The remaining non-symptomatic group receive formal pretest counselling that differs according to their family history. Patients are assigned to Group 2 if they have no personal symptoms that raise concern regarding cerebrovascular malformations, but where at least one family member has had a cerebral haemorrhage (this was an adaptation of general population recommendations regarding familial Berry aneurysms (*24*)). For patients assigned to Group 2 due to their family history in the absence of personal symptoms of concern, we suggest they may wish to have a cerebral MR scan to rule out high haemorrhagic-risk lesions such as aneurysms or arteriovenous fistulae. Patients with no symptoms and no family history of cerebral haemorrhage are assigned to Group 3. We inform them that they are in our lowest risk category, do not recommend a scan, though say that a scan can be arranged if they wish.

There do not appear to have been any studies on pre-test counselling for HHT patients prior to cerebral AVM screening scans, or patient choices that result after such counselling. The aim of this study were to evaluate the uptake of screening opportunities, and the outcomes of the cerebral screening scans, in order to add to the information provided to patients considering screening for cerebral AVMs.

## 2. Materials and Methods

### 2.1. *Ethical approvals*

Ethics approval was from the Hammersmith and Queen Charlottes Local Research Ethics Committee (LREC 2000/5764: "Case Notes Review: Hammersmith Hospital patients with pulmonary arteriovenous malformations and hereditary haemorrhagic telangiectasia (HHT).") The ethics committee approved the review of the case notes for research purposes without seeking individual consents.

### 2.2. *Cerebral AVM screens: Informed choice*

As part of routine management, at our HHT reference centre that receives HHT referrals from across the UK, all patients with HHT are informed of an approximate 10% risk of cerebral AVMs. Discussions have evolved, with 3 key boundaries to time intervals (Figure 1). Two are universally applicable - the 2008 acceptance of HHT International Guidelines (*18*) which recommended screening MR scans for all HHT patients (although at marginal levels of evidence and agreement (level of evidence III; strength of recommendation: weak; agreement: 77%)), and the 2014 publication of the ARUBA trial that demonstrated for unruptured AVMs, stroke risk was higher following current treatments (*20*). The third time boundary was specific to our unit-the local formal categorisation of individual patients according to symptoms and family history (*24*). This was instituted as a formal clinic guidance sheet in early 2010, in order to assist patients to make an informed choice as to whether to have a screening cerebral MRI scan or not (Supplementary Figure S1, *http://www.irdrjournal.com/ action/getSupplementalData.php?ID=31*). The ARUBA data have been referred to in pre-test counselling, but as they refer to general population data, have not been added to the HHT-specific cerebral AVM screening sheet
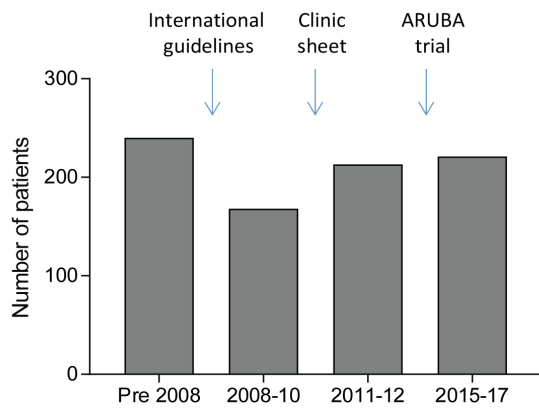
**Figure 1. Audit time periods and boundaries.** Number of patients refers to number of HHT cases audited across the 4 time periods. Bar charts indicate the % (mean) and 95% confidence intervals of the mean.

(Supplementary Figure S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=31*).

To evaluate the evolution of cerebral AVM screening recommendations, two audits of HHT patient notes were performed. The first in 2012 categorised patients into 3 periods of relatively comparable numbers: pre-2008 (pre-international guidelines recommending scan); 2008-2009 (pre-formal categorisation sheet), and 2010- February 2012. A fourth group was added in 2018, of new HHT patients first reviewed consecutively in 2015-2017 for whom the ARUBA trial results (*20*) were consistently mentioned in clinic discussions.

To evaluate clinical features present at the time of the MR scans, the original handwritten patient paper notes including clinic letters were reviewed. Since reported scan results influenced subsequent management (*e.g.* re-review of symptoms knowing the site of an abnormality; referral to neurology *etc*), only features documented prior to the scan date were analysed, though note was made of subsequent clinical progress.

### 2.3. *Cerebral MR Scans and evaluations*

To optimise consistency of scan methodologies and comparisons, scan results focussed purely on those performed at our institution. For the purposes of this study, all MR screening scans were evaluated, including 12 performed between March 2012-2014 when the uptake rates were not formally audited because the advice to patients was inconsistent during this period. Two emergency scans performed early in the series (and of inferior quality to the remainder) were excluded.

The screening MR scans had been performed between 18/08/2000 and 08/03/2018, all using a 1.5 Tesla (T) device, though there were evolutions of scan protocols with time: A T2-weighted image was performed in all patients. 59 (98%) had T1-weighted imaging; 58 (98%) had fluid-attenuated inversion

recovery imaging (FLAIR); 58 (98%) had diffusion weighted imaging (DWI); 14 (24%) had susceptibility weighted imaging (SWI) or gradient echo T2*; 50 (85%) had gadolinium (contrast) enhanced imaging; and 58 (98%) had time-of-flight angiography (TOF-MRA). From 2017 onwards, 67% of patients had SWI in keeping with an update to the standard imaging sequence protocol. All scans were analysed using visual diagnosis by two independent neuroradiologists, blinded to patient demographics and PAVM status. Standardisation was ensured with the addition of a further Radiologist opinion should there be a discrepancy between reports, after which a consensus agreement was reached.

### 2.4. *Clinical corollaries*

Scan data were supplied in Excel charts to a third researcher who unblinded the data, added clinical demographics and performed statistical analyses. All patients who had MRIs had already had at least one thoracic computerised tomography (CT) scan to screen for pulmonary AVMs.

### 2.5. *Data Analyses*

For data analysis and to generate graphs, Excel chart data were uploaded to STATA IC v15 (Statacorp, Texas) and Graph Pad Prism 7.03. Summary statistics were generated. For comparison of groups, patients were categorised by time-period of screening, family history of haemorrhage (Group 2 present, versus Group 3, none), or by presence/absence of pulmonary AVMs. For three or more groups, p values were calculated using Kruskal Wallis with Dunn's post test correction used. For two groups, p values were calculated by Mann Whitney, or for categorical analyses, using Fisher's exact test. STATA IC v15 was also used to perform multiple regression analyses.

### 3. Results

#### 3.1. *Overview of screening scan uptake cohort*

Over the study period, case records of 864 patients with HHT were audited. Data on cerebral screening were available for 842. 188/842 had already had scans performed externally, predominantly for investigation of acute cerebrovascular events. For these scans arranged by other hospitals, over the period of study, there was a trend for the overall number of HHT patients having a cerebral MR scan to increase, with the proportion relatively unchanged since 2008 (Figure 2A). A further 51 patients had neurological symptoms of potential concern (Group 1) and the identification of cerebral vascular malformations in their scans is to be reported elsewhere.
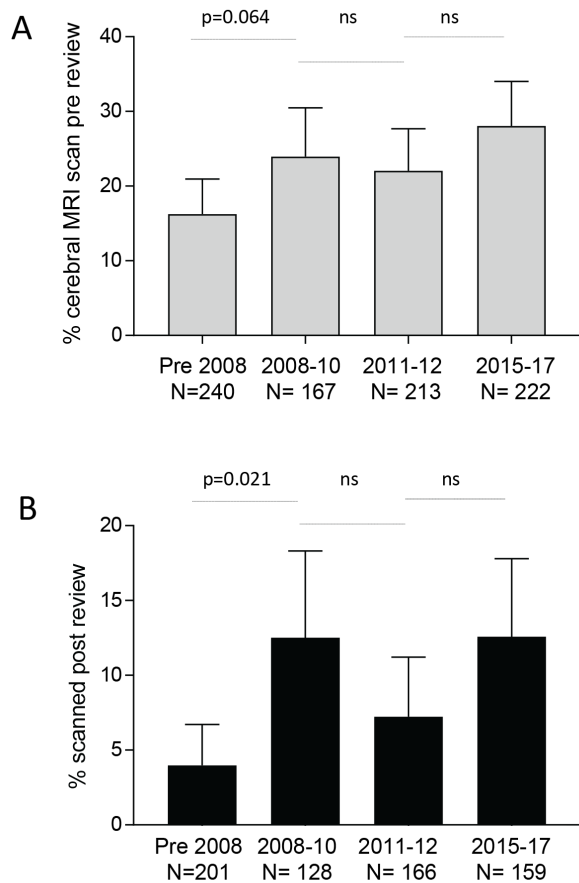
**Figure 2. Overall MR scans rates.** Percentage of 842 patients audited who had MR scans performed for any indication, across the 4 time periods as in **Figure 1**: **(A)** Prior to review by us (external scans), and **(B)** Following review by us (internal and external scans). Bar charts indicate the % (mean) and 95% confidence intervals of the mean. Displayed *p*-values calculated by Dunn's test after Kruskal Wallis (overall *p*-values A: *p* = 0.023; B: *p* = 0.0091).

The current manuscript presents uptake of screening scans in the remaining 603 "non-symptomatic" patients who were categorised by presence (Group 2) or absence (Group 3) of a family history of cerebral haemorrhage.

### 3.2. *Cerebral MR screening scans rates*

Figure 2B demonstrates the screening scan rates in the 603 HHT patients who had no previous cerebral scan, and no neurological symptoms. Scan rates were initially very low, and increased over the audit period (*p* = 0.0002). There were trends for the scan rates to increase after dissemination and online publication of the international guidelines, and to fall with the introduction of our formal clinic sheet categorising patients. The most notable change was an increase in scan rates after publication of the ARUBA trial (Figure 2B).

### 3.3. *Scan rates differed by family history of cerebral haemorrhage*

To evaluate the influence of our screening sheet
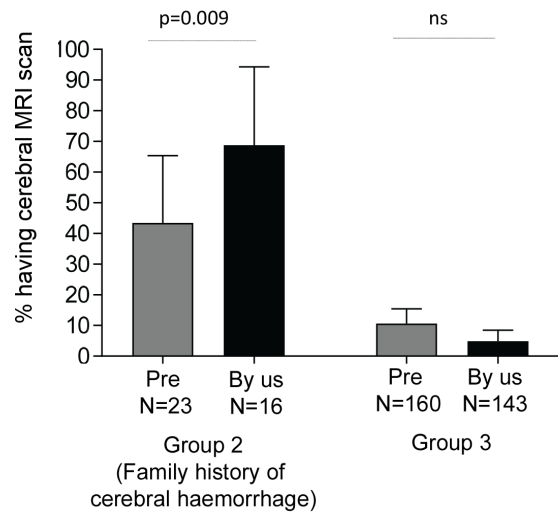


**Figure 3. Proportion of non-symptomatic cases undergoing screening scans in 2015-2017.** Patients are categorised by family history of haemorrhage (Group 2), and by whether the scans were performed by other institutions (grey bars, "Pre") or at our institution (black bars, "By us"). Bar charts indicate the % (mean) and 95% confidence intervals of the mean. Overall Kruskal-Wallis *p* < 0.0001, displayed *p*-values calculated by Dunn's test.

categorisations, we focused on scan uptake in the most recent period (2015-2017) when the results of the ARUBA trial were communicated. None of the patients had neurological symptoms of concern with respect to possible cerebral vascular malformations. (If such symptoms were present, patients were categorised into Group 1 and MR scans performed urgently, usually at their local institutions).

In this period there were 183 non symptomatic patients - 23 with a family history of cerebral haemorrhage (Group 2) and 160 with no family history of cerebral haemorrhage (Group 3). As shown in Figure 3, 10 (43.4%) Group 2 patients and 17 (10.6%) Group 3 patients had screening scans instituted prior to review by us. The overall screening rate was therefore 14.8%. This low rate in part reflected the lack of a formal HHT diagnosis prior to review, but notably was 4.1-fold higher for those with a family history of cerebral haemorrhage, compared to those with no such family history (Figure 3).

At our institution where all patients received a formal diagnosis of HHT and screening scans were only requested followed pre-test counselling and categorisation to Group 2 or Group 3, a more pronounced difference was observed in screening scan rates between the two categories (Figure 3). For patients with a family history of cerebral haemorrhage (Group 2), 11/16 (68.8%) of patients took up the opportunity to have a screening scan. In contrast only 7/143 (4.9%) of group 3 patients (no family history of haemorrhage), chose to have a scan (*p* < 0.0001).

### 3.4. *Cerebral screening MRI scan results*

Overall, at our institution between 20/04/2009 and

**Table 1. Abnormalities identified by screening MR scans**

| Items | All patients ($n$ = 59) | Group 2 ($n$ = 24) | Group 3 ($n$ = 35) | $P$ value |
|---|---|---|---|---|
| Age, ys | 16-74, median 41 | 21-66(median 40.9) | 17-74 (median 41) | 0.58 |
| Sex, N (%) female) | 35 (59.3) | 17 (70.8) | 18 (51.4) | 0.14 |
| Arteriovenous malformation (AVM) | 0 | 0 | 0 | > 0.99 |
| Arteriovenous fistula | 0 | 0 | 0 | > 0.99 |
| Evidence of prior haemorrhage | 0 | 0 | 1 (2.7%) | 0.41 |
| Aneurysm† | 4 (6.8) | 1 (4.3) | 3 (8.3%) | 0.51 |
| Telangiectasia* | 2 (3.4) | 1 (4.3%) | 1 (2.7%) | > 0.99 |
| Infarcts | 20 (33.9) | 9 (39.1) | 11 (30.6) | 0.63 |
| Posterior circulation infarcts | 15 (25.4) | 6 (26.1) | 9 (25) | 0.95 |
| Anterior circulation infarcts | 9 (15.3) | 5 (21.7) | 4 (11.1) | 0.33 |
| Microangiopathic changes | 9 (15.3) | 3 (13.0) | 6 (16.7) | 0.45 |
| Contusion | 2 (3.4) | 1 (4.3) | 1 (2.7) | > 0.99 |
| Meningioma | 1 (1.7) | 0 | 1 (2.7) | > 0.99 |

†One at right ophthalmic/left middle cerebral artery junction, one at left internal carotid artery. *one had left cerebellum, left parietal, and left frontal telangiectasia, the second, contrast enhancement suggestive of telangiectasia. *P*-value calculations: age by Mann Whitney, remainder by Fisher exact test.

05/04/2018, 59 screening MR scans were performed in non-symptomatic patients with a clinical or molecular diagnosis of HHT, aged 16-74 (median 41) years. 26 (44.1%) of the cases were males. 23 (39.0%) were patients from Group 2 families where 1-4 (median 1) relatives had suffered a cerebral haemorrhage, between the ages of 3 months and mid 50s (median 29 years). 36 patients who had a scan (61.0%) gave no family history of cerebral haemorrhage.

None of the 59 screening MR scans performed at our institution demonstrated a cerebral AVM or cerebral arteriovenous fistula. One provided evidence of a prior cerebral microhaemorrhage. Four scans (6.8%) demonstrated changes compatible with small aneurysms, and two (3.4%) demonstrated possible telangiectatic changes. In this cohort therefore, the overall proportion of cerebrovascular abnormalities was 6/59 (10.2%, Table 1). There was no significant difference in any finding between Groups 2 and 3.

Only 32/59 (54.2%) of scans were reported as normal. As noted in Table 1, the most commonly identified abnormality was cerebral infarction which affected 20/59 (33.9%) of patients. Three of these cases had a prior neurological event many years earlier- one clinical stroke, one cerebral abscess and one prior cerebral haemorrhage with normal subsequent angiography. None of the 59 cases had epilepsy, and none had received a formal diagnosis of transient ischaemic attacks.

### 3.5. Clinical corollaries with cerebral infarcts

To examine further, potential associations between the infarcts and clinical characteristics were examined. There was no discernible association between infarcts and neurological symptoms described by many patients such as migraines (*25-28*) or pre-syncopal type dizzy spells (*29*) (Supplementary Table S1, *http://www.irdrjournal.com/action/getSupplementalData.*

*php?ID=31*). The most notable clinical association was with pulmonary AVMs which had been present in 30 (50.9%) of the patients scanned. 22 patients had been treated previously (by embolization or surgery), and 3 went on to have their first treatment (embolization) within the subsequent 3 months.

Ages did not differ between pulmonary AVM and non- pulmonary AVM cohorts (median 40.5 (range 16-74) versus 42 (21-66) years respectively, $p$ = 0.94). A cerebral infarct was present in only 3/29 (10.3%) of patients without pulmonary AVMs, when scanned aged 41, 59 and 65 years, compared to 17/30 (56.7%) patients who had at least one pulmonary AVM ($p$ = 0.0002). There was also an excess of microangiopathic change in the pulmonary AVM cohort. As a result, while 22 (76%) of the 29 HHT patients without pulmonary AVMs had a normal scan, only 10 (33%) of the HHT cohort with pulmonary AVMs had a normal scan ($p$ = 0.0012).

Nine of the 30 patients with pulmonary AVMs (30%) had between 2 and 5 infarcts at median age 53 (range 39-65) years - none had a previous history of stroke. Of the 8 patients with pulmonary AVMs and a single cerebral infarct, one had a prior clinical diagnosis of stroke in the corresponding territory. Table 2 provides further detail of the infarcts present in pulmonary AVM patients and indicates that the excess of pulmonary AVM-associated cerebral infarcts occurred in posterior circulation territories.

For HHT patients with pulmonary AVMs, the age-adjusted odds ratios for any infarct was 21.6 (95% confidence intervals 3.7, 126, $p$ = 0.001). This reflected the higher number of posterior circulation territory infarcts (Table 3). The pulmonary AVM patients' age-adjusted odds ratios for any infarct or microangiopathic change (considered a marker of ischaemia) was 10.6 (95% confidence intervals 2.12, 53.0, $p$ = 0.004). As in the univariate analyses above, there was no association between MR-confirmed infarcts and patient-described

**Table 2. Infarct distribution in 30 HHT patients known to have had PAVMs**

| Items | Patients affected, total (%) | Left | Right | Bilateral | Total sites | Sites per patient (n = 30 PAVM) | Sites per patient (n = 17 infarct- affected) |
|---|---|---|---|---|---|---|---|
| **All infarcts** | **17 (56.7)** | **8** | **4*** | **7** | **31** | **1.13** | **2.13** |
| **Posterior circulation** | **14 (46.7)** | **5** | **0** | **8** | **26** | **0.83** | **1.56** |
| Cerebellar | 12 (40.0) | 4 | 2 | 6 | 18 | 0.60 | 1.06 |
| Thalamus | 4 (16.7) | 3 | 1 | | 4 | 0.13 | 0.24 |
| Temporal | 1 (4.2) | 0 | 1 | | 1 | 0.03 | 0.06 |
| Pons | 1 (4.2) | 1 | | | 1 | 0.03 | 0.06 |
| Midbrain | 1 (4.2) | 1 | | | 1 | 0.03 | 0.06 |
| Occipital | 1 (4.2) | 1 | | | 1 | 0.03 | 0.06 |
| **Anterior circulation** | **7 (23.3)** | **3** | **4** | **0** | **10** | **0.33** | **0.59** |
| Parietal | 2 (6.7) | | 1 | 1 | 3 | 0.10 | 0.18 |
| Frontal | 2 (8.3) | 1 | 1 | | 2 | 0.07 | 0.12 |
| Striatocapsular | 2 (6.7) | 2 | | | 2 | 0.07 | 0.12 |
| Caudate | 1 (4.2) | | 1 | | 1 | 0.03 | 0.06 |

*One corresponded to a known clinical stroke.

**Table 3. Association of pulmonary AVMs with cerebral infarction by multivariate regression**

| Items | Age-adjusted* odds ratio (95% CI) | Pseudo $R^2$ | p-value for PAVM |
|---|---|---|---|
| Any cerebral infarct | 21.6 (3.7, 126) | 0.38 | 0.001 |
| Anterior circulation infarct | 4.51 (0.70, 28.9) | 0.29 | 0.110 |
| Posterior circulation infarct | 28.0 (3.16, 249) | 0.32 | 0.003 |
| Any infarct or microangiopathic change | 10.6 (2.12, 53.0) | 0.38 | 0.004 |

*Similar findings in crude analyses without age-adjustment, data not shown.

## 4. Discussion

The aim of this study was to provide data that can add to the information provided as part of informed consent for screening for cerebral AVMs in non-symptomatic patients with HHT. The data indicate low scan uptake rates in patents with no family history of cerebral haemorrhage, and a low rate of cerebrovascular malformation detection where patients with potential neurological symptoms of concern have been separated out. However, the study demonstrated cerebral infarcts in more than 50% of "non-stroke" pulmonary AVM patients.

We are unaware of any previous evaluations of pre-test counselling for HHT patients prior to cerebral AVM screening scans, or patient choices that result after the counselling. Further, PubMed searches performed most recently on 27.08.2018 using the search terms ["HHT" "counselling" "cerebral" "screening"] or ["HHT" "choice" "cerebral" "screening"] did not identify any earlier studies examining HHT patient preference for cerebral MR screening scans. This study has clear limitations, particularly it was a single centre, retrospective study of two prospectively recorded datasets in adults only (patient preferences; MR scans). Study numbers for the MR scans were relatively small reflecting the "real-life" nature of ordering.

symptoms of migraines or dizzy spells, once adjusted for pulmonary AVMs (data not shown).

Nevertheless, we think the information valuable for patients and our fellow practitioners who are struggling with how to weight patient perceptions (*11,12*) and older recommendations (*18*) in the light of emerging (*20-23*) and potential future evidence.

For the past eight years we have counselled those HHT patients who do not have concerning neurological symptoms regarding additional investigation. This has given them the opportunity to embark on cerebral screening knowing the risk-benefits of treatment of the vascular abnormalities that may be detected, or to decline screening in an informed manner for what is a highly emotive issue in those with HHT. It is intriguing that our local screening scan rates rose after communication of the ARUBA trial results which may have been expected to reduce screening scan numbers. Our interpretation is that the extra reassurance offered in case an AVM was detected may have enabled some patients to take that risk when seeking a screening scan that could exclude more worrying intracerebral pathology. The pattern of enhanced uptake by patients with a family history of cerebral haemorrhage was also seen in patients reviewed by other clinicians, although the increase was more pronounced in our clinic using a formal clinic categorisation sheet (14-fold, compared to 4.1-fold).

Cerebral AVM detection rates were lower than reported in other HHT series (*8-10*) – none were identified in the 59 screening scans in this series. While there are a number of possible explanations, including

chance given the study size, the most obvious is the removal of patients with potentially relevant symptoms to a higher risk group, and exclusion from the "non-symptomatic screening cohorts". It is important to note that Group 2 and 3 patients were not "asymptomatic" with respect to neurological symptoms. The majority of patients with pulmonary AVMs experience migraine with aura (*25-28*), and in addition, many have transient dizzy spells that cannot be assigned to postural haemodynamic changes (*29*). Neither symptom was considered suggestive of cerebral AVMs in the setting of pulmonary AVMs, and patients remained in Group 2 or 3. The absence of cerebral AVM detection supports this classification with respect to cerebrovascular malformation screening.

Four patients (6.8%) had findings compatible with very small cerebral aneurysms. Aneurysms have been described before in HHT, including 8/376 (2.1%) cases in (*10*), and 9/372 (2.4%) cases in (*9*). Cerebral aneurysms were the cause of cerebral haemorrhages in three HHT cohorts (*8,19,24*). However, a systematic meta-analysis in the general population estimated the prevalence of cerebral aneurysms as 3.2% (95% CI: 1.9-5.2) in a population without comorbidity, with a mean age of 50 years (*30*). The cerebral aneurysm rate in HHT is therefore comparable to the general population.

The study findings support the long-held clinical viewpoint that neurological manifestations of HHT are more commonly attributable to pulmonary AVMs than to cerebrovascular abnormalities (*31*). A seventeen year old study using computerised tomography demonstrated cerebral infarcts in 34/67 (51%) patients with pulmonary AVMs at median age 42 years (*25*), and a more recent study demonstrated silent brain infarcts in approximately 28% of patients with pulmonary AVMs and no clinical ischaemic history (*32*). Based on previous data (*27,29*), we had expected patients with migraines and dizzy spells would have more infarcts, but in crude, age, and pulmonary AVM-adjusted analyses, no relationships were identified. This could support the interpretation that a proportion of infarcts occurred at a prior symptomatic stage (*e.g.* prior to PAVM treatments). Further study is needed to address the extent to which cerebral infarction is ongoing after embolization of pulmonary AVMs.

The excess of posterior circulation territory infarcts in patients with pulmonary AVMs was unexpected. Since paradoxical emboli through PAVMs will traverse the left atrium and ventricle, the high rate of posterior circulation infarcts may simply be secondary to the natural consequence of cardioembolic sources of cerebral infarction, rather than any specific relation to pulmonary AVMs: In the wider population, approximately 20% of all infarcts are within the posterior circulation (*33*), which is a relatively small number. However, approximately 25% of all cerebral infarcts are caused by cardioembolic events (*34*) and 40% of posterior circulation infarcts are due to cardioembolic events (*35*) indicating a natural propensity for cardioembolic events to affect the posterior circulation. Importantly, PAVMs are currently not included in many lists of causes of cardioembolic infarcts (*33-35*), and they should be as they result in paradoxical emboli travelling through the left ventricle.

These data do not alter any guidance for management of children, symptomatic adults, or the management of a cerebral AVM once detected in HHT, but we think they can helpfully support decisions on screening of patients. For cerebral AVM screening in HHT, we suggest all patients are first categorized into whether or not they have neurological symptoms of concern (*e.g.* epilepsy, severe headaches that are non migrainous, focal neurological deficits), and that all patients with such symptoms are scanned. For the remainder, they may be advised that their risk of cerebral AVMs due to HHT is lower than previously thought, and additionally, advised that the ARUBA trial demonstrated that for unruptured cerebral AVMs, stroke risk was higher if cerebral AVMs were treated. It still seems appropriate to offer a screening scan in the setting of a family history of haemorrhage, as in the general population (*24*). But given the emotive and controversial nature of the discussions, we consider it appropriate to then let patients make a decision on whether or not to have a scan, recognizing that it may identify silent cerebral infarcts due to pulmonary AVMs, or cerebral aneurysms/other neurological pathologies at a comparable rate to the general population. These risks should be articulated prior to performing the screening scan, noting detection of an incidental pathology would then be managed as for detection in any other setting. Particular comment is required for patients who have had pulmonary AVMs, indicating that scans detect their lifetime of risks which would be different before and after treatment. An important question for the future is whether patients with pulmonary AVMs should have a cerebral MR following their final planned treatment, to use as a baseline against which to judge the occurrence of new infarcts, and whether such infarct-detection scans should be performed regularly, or only after symptomatic events (as would usually occur). This leads to a further difficult area, weighing risks and benefits of antiplatelet therapies for a patient population at higher risk of haemorrhage (*13,18,23,36,37*).

In conclusion, in non-symptomatic screened cohorts with HHT, there was a negligible detection of cerebral AVMs, and this may further modify rates of screening scan uptake in HHT populations offered informed choice. However, pulmonary AVMs place patients at greatly enhanced risk of cerebral infarction, and in our opinion, the possibility of detection of silent infarcts should be added to prescreening information.

## Acknowledgements

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# Identification of a rare homozygous *SZT2* variant due to uniparental disomy in a patient with a neurodevelopmental disorder

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**Summary**    **Because biallelic *SZT2* variants have been reported in patients with neurodevelopmental disorders associated with various degrees of developmental delay, intractable seizures, and distinctive features; this condition is recognized as an autosomal recessive disorder. Previously, eleven patients have been reported and most of them have compound heterozygous *SZT2* variants, leading to premature termination. In these patients, all reported variants were unique and there were no common pathogenic variants identified. In this study, we identified a paternal uniparental disomy of chromosome 1 in a patient with a neurodevelopmental disorder associated with severe intellectual disability, intractable epilepsy, autistic features, distinctive features, and transient macrocephaly. This resulted in homozygous patterns through chromosome 1. Among the variants in chromosome 1, a rare *SZT2* variant, NM_015284.3:c.6553C>T (p.Arg2185Trp), was selected as a powerful candidate variant in this patient. Although the clinical features of this patient are relatively milder than that reported previously, it may be derived from genetic heterogeneity. This is the first report of a homozygous missense *SZT2* variant.**

*Keywords:* Monosomy rescue, high forehead, loss-of-heterozygosity (LOH)

## 1. Introduction

Patients with neurodevelopmental disorders often show triad features with intellectual disability, autistic features, and epilepsy (*1-3*). Previous large-scale studies of patients with undiagnosed rare neurodevelopmental disorders showed the predominance of de novo mutations in genes that encode for molecules involving in neuronal functions (*4-6*). In such cases, haploinsufficiency and/or loss-of-function of the genes are suggested as the major mechanisms and only hetero-allelic involvement can cause the disorders. Compared to the prevalence of these cases, recessive disorders are rare because bi-allelic involvements are necessary for development of this condition (*7*).

Prevalent autosomal recessive disorders are often caused by homozygous alterations due to common variants within ethnic groups. Consanguinity can also result in a homozygous gene status. As rare cases, uniparental disomy can also cause homozygous patterns.

In this study, we identified a rare homozygous variant of the seizure threshold 2 gene (*SZT2*) in a patient with a severe neurodevelopmental disorder presenting with triad features. A microarray testing revealed that uniparental disomy (UPD) was the mechanism of disease in this patient. This is the first report of UPD associated with S*ZT2* involvement in a neurodevelopmental disorder.

## 2. Materials and Methods

### 2.1.*Methods*

This study was performed in accordance with the Declaration of Helsinki and approved by the Tokyo Women's Medical University ethics committee. Blood

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samples were obtained from the patient and his parents after receiving informed consent.

Genomic DNA was extracted using a QIAamp DNA extraction kit (QIAGEN, Hilden, Germany). Next generation sequencing (NGS) was performed using a TruSight One v1.0 sequencing panel (Illumina, San Diego, CA) and Agilent SureSelect v5 (Agilent Technologies, Santa Clara, CA) according to previously described methods (*8,9*). The extracted data was annotated and filtered by VariantStudio (Illumina) and SureCall v4 (Agilent Technologies) software, respectively. Chromosomal microarray testing was performed using an Agilent microarray CGH+SNP 180K (Agilent Technologies), according to previously described methods (*10*). Standard Sanger sequencing was performed using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a 3130 Genetic Analyzer (Applied Biosystems). The primer sets (forward; 5'-AGCATCCTTCCCAGACTCAG-3', reverse; 5'-GGGCAAAGGTACATATAGGGG-3') were designed using the UCSC genome browser (*https://genome.ucsc.edu/*).

### 2.2. Patient's descriptions

A 15-year-old Japanese boy was delivered at 39 weeks and 2 days of gestation by emergency caesarean section due to prolapse of the umbilical cord. The patient's parents are healthy and non-consanguineous. At the time of the patient's birth, the father and mother were 41 and 36 years old, respectively. There are two healthy brothers at 21 and 19 years of age. The patient's Apgar scores were 8 and 9 at 1 and 5 minutes, respectively. There is no remarkable family history of neurological diseases. His birth weight was 3,310 g (75~90th percentile), length was 52.0 cm (97th percentile), and occipitofrontal circumference (OFC) was 35.5 cm (90~97th percentile). Although his neonatal course was uneventful, he showed mildly delayed motor milestones with walking at 2 years and language development was notably delayed. At 4.5 years old, his OFC was 56.0 cm (> 97th percentile). This indicated post-natal macrocephaly. At 10 years, the first epileptic attack occurred. Although several anti-epileptic drugs have been prescribed, seizure attacks were noted several times per year, indicating intractable epilepsy. Electroencephalography showed multi-focal spikes or spikes and waves predominantly in the frontal lobes. Routine laboratory tests including a complete blood count, biochemical tests (including lactate, pyruvate and ammonia), and thyroid function test, were unremarkable. Brain magnetic resonance imaging showed no abnormalities. Conventional chromosomal G-banding showed normal male karyotype of 46,XY.

At present, his height is 173.5 cm (75~90th percentile), his weight is 100 kg (> 97th percentile),

and his OFC is 57.0 cm (50~75th percentile), indicating obesity due to over-eating but not macrocephaly. Toilet training is established and he can remove clothing; however, he cannot dress by himself. Because his motor skills are not strong enough to allow the use chopsticks, he eats by using a spoon or with his hands. The patient uses no meaningful words and he seldom uses gestures for communication. He is also irritable and often has unwarranted temper tantrums. Together, these observations were recognized as autistic features. There are no dysmorphic features, excluding high forehead. He still has epileptic attacks, which are classified as complex partial seizures.

## 3. Results

To identify the underlying genetic cause of the disorder, NGS using a TruSight One sequencing panel was initially performed using a trio of samples derived from the patient and his parents. Although no strong candidate variants were identified, most of the variants in chromosome 1 showed homozygous patterns. Microarray analysis showed no genomic copy number aberration. Alternatively, loss-of-heterozygosity (LOH) throughout chromosome 1 was identified (Figure 1A). Haplotypes in chromosome 1 were compared between the patient and his parents and it was confirmed that both copies of chromosome 1 in the patient were derived from his father, confirming paternal UPD.

The underlying homozygous variants in chromosome 1 were suspected as a mechanism of disease causation. For more detailed analysis, whole-exome sequencing was performed and the homozygous variants in chromosome 1 were analyzed in detail. Finally, variants were manually filtered by functional relevance to the clinical findings and a homozygous variant in *SZT2*, NM_015284.3(SZT2_v001):c.6553C>T (p.Arg2185Trp), was selected as a possible candidate. This variant has since been registered in dbSNP database (*https://www.ncbi.nlm.nih.gov/SNP/*) as rs765848129. However, the allele frequency is shown as 0.001% (1/121,308). The Exome Aggregation Consortium database (ExAC) also includes this variant with a frequency of 0.000008243. This variant is not observed in the Human Genetic Variation Database (HGVD; *http://www.hgvd.genome.med.kyoto-u.ac.jp/*), which contains genetic variants identified by exome sequencing of 1,208 Japanese individuals (*11*). These findings suggest that the incidence of this variant is extremely low. The functional consequences of the *SZT2* variant were annotated through wANNOVAR (*http://wannovar.wglab.org/*). As a result, CADD_phred score was 34, suggesting that this variant is deleterious (Supplemental Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=30*). Standard Sanger sequencing confirmed that this variant was derived
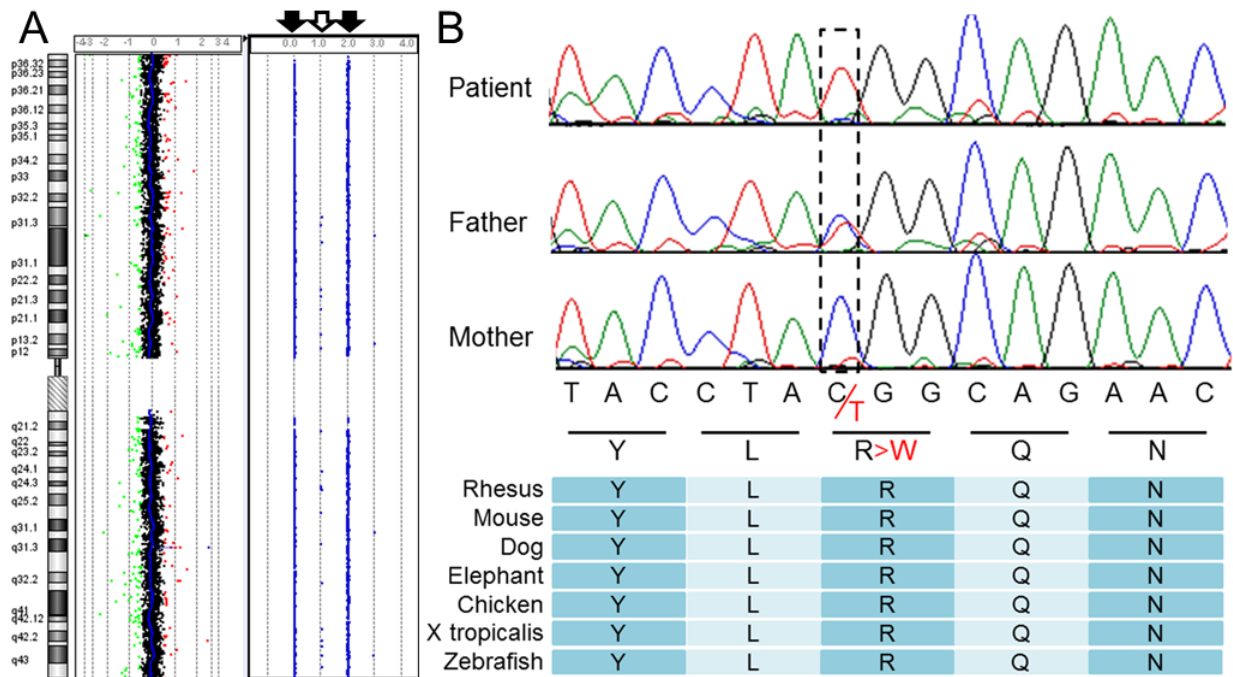
**Figure 1. Results of genomic analyses. (A)** Chromosomal microarray testing. The schematic representation of chromosome 1 data constructed by Agilent Genomic Workbench (Agilent Technologies) obtained through CGH+SNP array. In the CGH view (left), no genomic copy number aberration is shown. In the SNP view (right), almost all probes show homozygosity (black arrows) and very small numbers of probes show heterozygous patterns (a white arrow; these would be analytical errors). **(B)** Results of Sanger sequencing. Electropherograms of Sanger sequencing show the homozygous variant (indicated by a dotted rectangle) in the patient. This variant is identified in the patient's father as heterozygous but not in the mother. The affected amino acid is conserved among various species (lower panel).

from the patient's father who had this variant in the heterozygous state (Figure 1B).

## 4. Discussion

*SZT2* is highly expressed in the brain, primarily in the parietal-frontal cortex, hippocampus, and dorsal root ganglia (*12*). Bi-allelic *SZT2* variants were first identified in two independent patients with early-onset epileptic encephalopathies (*13*). Both unrelated patients showed common facial features, severe developmental delay with hypotonia, and refractory seizures associated with secondary generalization. Following this report, additional nine patients have been reported (*14-17*). The clinical features of all reported patients are summarized in Table 1. Neurodevelopmental delay associated with intractable epilepsy, distinctive features, and dysmorphic findings of the corpus callosum are all common features of these patients (Table 1). Although the patient discussed in this report showed no abnormality in the findings of brain MRI, he fulfilled most of the other described features including developmental delay, intractable epilepsy, and distinctive features such as a high forehead. Macrocephaly is often observed in patients with *SZT2* variants. The present patient showed no macrocephaly at the last examination; however, it was transiently

observed at 4.5 years of age. Thus, macrocephaly may be typically observed in these patients during childhood.

Motor developmental delay observed in this patient was milder than that reported in previous patients. Autistic features, which have never been reported previously, are additional characteristics for this patient. These characteristics may be related to the type of *SZT2* substitutions. Previously, 16 types of *SZT2* variants have been reported. Amongst these variants, 10 are related to premature termination. This indicates that loss-of-function would be the major mechanism and patients with *SZT2* loss-of-function mutations exhibited severe neurological symptoms. Compared to a loss-of-function variant, familial cases with in-frame *SZT2* mutations showed milder manifestations (*14*). The variant identified in this study contains a single nucleotide alteration leading to a missense substitution and is already registered in the dbSNP database. However, the frequency is extremely low. Because *SZT2* related phenotypes could be caused by bi-allelic involvement, the theoretical incidence of bi-allelic *SZT2* involvements will be low. Therefore, we concluded that the homozygous state of this rare *SZT2* variant would be disease causing.

The nine previously reported patients showed unique variants and there was no recurrent variant

**Table 1. Summary of the clinical information of the present patient and previously reported patients with SZT2 involvements**

| Items | Present patient | Nakamura et al. 2018 | | Tsuchida et al. 2018 | | Vanderver et al. 2016 | Venkatesan et al. 2016 | Falcone et al. 2013 | Basel-Vanagaite et al. 2013 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age | 13y | 4y | 4y | 2y | 5y | 7y | 3y | 18y, 10y, 7y | 10y | 9y |
| Gender | Male | Female | Female | Male | Femal | Female | Male | Males | Female | Male |
| Genotype | Homozygous | Compound heterozygous | Compound heterozygous | Compound heterozygous | Compound heterozygous | Compound heterozygous | Compound heterozygous | Homozygous | Homozygous | Compound heterozygous |
| 1st | c.6553C>T (p.R2185W) | c.896dup (p.Y2866Lfs*42) | c.3700_3716del (p.N1234Afs*35) | c.3497dup (p.E1317Gfs*4) | c.7303C>T (p.R2435T) | c.5499del (p.F1834Sfs*47) | c.3509_3512del (p.T1170Rfs*22) | c.4202_4204del (p.F1401del) | c.73C>T (p.R25*) | c.1496G>T (p.S499I) |
| 2nd | | c.2930-17_2930-3 delinsCTCGTG | c.5482del | c.2929+1G>A | c.8162C>G | c.6916G>A | c.9703C>T | | | c.2092C>T |
| | | | (p.G1829Vfs*52) | (p.L939Dfs*19) | (p.S2721C) | (p.G2306R) | (p.R3235*) | | | (p.Q698*) |
| Epilepsy | Intractable epilepsy | + | EOEE | EOEE | EOEE | Refractory epileptic encephalopathy | EOEE | None | EOEE | EOEE |
| Motor development | No delay | Walk unassisted | Bedridden | Bedridden | Bedridden | NA | Walk | NA | Sit with support | Bedridden |
| Language development | A few words | No meaningful word | No speech | No speech | No speech | NA | No speech | Speech delay | No speech | No speech |
| Cognition | | Recognize speaking | | | | NA | NA | | | No social interaction |
| Distinctive features | | | | | | | | | | |
| High forehead | + | + | + | + | - | NA | + | - | + | + |
| Hypertelorism | - | + | - | - | - | NA | + | - | NA | - |
| Macrocephaly | - | + | - | - | | + | + | + | + | - |
| Brain MRI | - | Thick and short CC | Thick and short CC | Thick and short CC | Volume loss of CC | Myelination deficit/mild cerebeller atrophy | Heterotopia/abnormal gryal formation | - | Thick and short CC | Thick and short CC |

y, years; EOEE, Early-onset epileptic encephalopathy; NA, not available; MRI, magnetic resonance imaging; CC, corpus callosum.
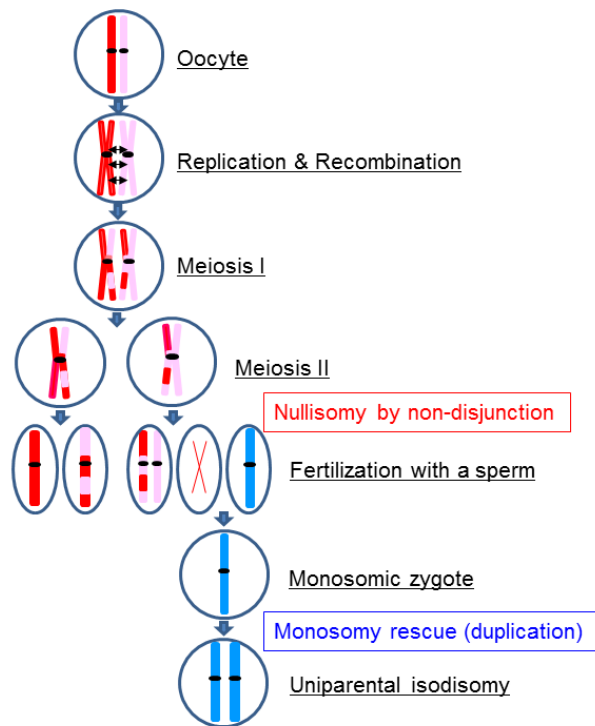
**Figure 2. Representation of the suspected mechanism of monosomy rescue.** Monosomic zygote is created by the fertilization of a nullisomic oocyte derived from meiotic non-disjunction in maternal meiosis II. As a monosomic embryo cannot survive, the paternally derived chromosome will be duplicated for compensation.

amongst them. Furthermore, only two families showed homozygosity. This indicates that bi-allelic involvements in the patients are incidental. *SZT2* is located on 1p34.2 and the observed homozygosity was caused by paternal UPD of chromosome 1 in the present patient. Homozygous variants induced by UPD are rare, but there are many cases of UPD induced neurological disorders (*10,18*).

From the genotypes and results of the CGH+SNP microarray for chromosome 1, we determined that the present patient did not show heterozygous region in chromosome 1. This finding suggested UPD, which describes disomy where both chromosomes are inherited from a single parent. UPD causes autosomal recessive disorders when the indicated parent carries pathogenic variants. UPD can be divided into two subtypes, the first is hetero-UPD (hUPD). In this subtype, two different homologous chromosomes are inherited from a single parent. The second is iso-UPD (iUPD), in which a single homologous chromosome is duplicated from a single parent. If the UPD was caused by trisomy rescue, heterozygous regions will be observed as evidence of homologous recombination through meiosis (Figure 2). However, the CGH+SNP array showed iUPD of chromosome 1 with LOH in the all regions. Thus, complete homozygosity throughout chromosome 1 indicates that monosomy rescue would be the mechanism (Figure 2). When a nullisomic oocyte

that arose from meiotic non-disjunction in maternal meiosis II is fertilized with a monosomic sperm, the zygote becames monosomic. Because the monosomic embryo cannot survive, the paternally derived chromosome will be duplicated for compensation. The mother of the patient was relatively old (36 years) at the time of the patient's delivery. Thus, monosomy 1 may have been caused by chromosomal non-disjunction in the oocyte.

In conclusion, we identified UPD of chromosome 1 in a patient with neurological disorder. Owing to that, the rare missense *SZT2* variant, located in 1p34.2, was identified as a homozygous pattern. This is the first report of bi-allelic involvement of *SZT2* by a missense substitution and this may be related to milder phenotype of *SZT2*-related neurodevelopmental disorder.

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# Original Article

# Microglia express gamma-interferon-inducible lysosomal thiol reductase in the brains of Alzheimer's disease and Nasu-Hakola disease

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| Summary | Gamma-interferon-inducible lysosomal thiol reductase (GILT), expressed in antigen-presenting cells (APCs), facilitates the reduction of disulfide bonds of endocytosed proteins in the endocytic pathway and they are further processed for presentation of immunogenic peptides loaded on major histocompatibility complex (MHC) class II. Although the constitutive and IFNγ-inducible expression of GILT was observed in various APCs, such as dendritic cells, monocytes/macrophages, and B cells, GILT-expressing cell types remain unknown in the human central nervous system (CNS). Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder characterized by sclerosing leukoencephalopathy and multifocal bone cysts, caused by a loss-of-function mutation of either *TYROBP* (*DAP12*) or *TREM2*, both of which are expressed on microglia. A rare heterozygous variant of the *TREM2* gene encoding p.Arg47His causes a 3-fold increase in the risk for late-onset Alzheimer's disease (LOAD), suggesting that both NHD and AD are induced by dysfunction of the microglial TREM2 signaling pathway in the brains. We studied by immunohistochemistry GILT expression in NHD and AD brains. GILT was expressed on amoeboid microglia with the highest levels of expression in AD brains, compared with those in non-neurological control (NC) brains and in NHD brains. In AD brains, the clusters of amoeboid microglia surrounding amyloid-beta (Aβ) deposition strongly expressed GILT. Furthermore, a human microglial cell line expressed GILT in response to IFNγ. These results indicate that microglia, expressing constitutively high levels of GILT, act as a principal cell type of APCs in AD brains, in contrast to baseline levels of GILT expression in NHD brains. |
|---|---|

*Keywords:* Alzheimer's disease, GILT, IFI30, microglia, Nasu-Hakola disease

## 1. Introduction

Gamma-interferon-inducible lysosomal thiol reductase (GILT), alternatively named IFI30, lysosomal thiol reductase, is the only known enzyme to catalyze disulfide bond reduction in the endocytic pathway (*1-4*). GILT enhances the major histocompatibility complex (MHC) class II-restricted presentation of a subset of epitopes derived from disulfide bond-containing antigens. GILT-dependent epitopes tend to be buried and they require reduction to be exposed for MHC class II binding. GILT also plays a role in the cross-presentation of exogenous viral antigens on MHC class I. In GILT$^{-/-}$ mice, antigen-presenting cells (APCs) displayed impaired MHC class II-restricted presentation and MHC class I-restricted cross-presentation (*5,6*).

Following delivery into the endosomal/lysosomal system by the mannose 6-phosphate receptor, N-terminal and C-terminal propeptides of the precursor 35-kDa GILT are cleaved in the early endosome, and the mature 28-kDa protein resides in late endosomes and lysosomes, where it is optimally active at acidic pH (*1,7*). Furthermore, an enzymatically active dimer of GILT precursors is secreted by activated macrophages/

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monocytes in response to bacterial stimuli (*1,8*). GILT catalyzes the reduction of disulfide bonds through the active site consisting of a thioredoxin-like CXXC motif, corresponding to Cys-46 and Cys-49 in human GILT (*1,3*). GILT also regulates the cellular redox state. In GILT$^{-/-}$ cells, there is a shift from the reduced to the oxidized form of glutathione, resulting in reduced mitochondrial membrane potential, increased mitochondrial autophagy, decreased superoxide dismutase 2, and elevated superoxide levels (*4,9*). The absence of GILT in fibroblasts and T cells results in increased phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and increased cellular proliferation (*4*). GILT maintains the proteolytic activity of cathepsin S in phagosomes in alternatively activated macrophages, while GILT's reductase activity facilitates the degradation of cathepsin S in B cells (*10,11*).

GILT is constitutively expressed in most antigen presenting cells (APCs), such as dendritic cells, macrophages, and activated B cells, and induced by exposure to IFNγ in many cell types with maximal protein expression at 48 h (*1,4*). Signal transducer and activator of transcription 1 (STAT1) but not class II transactivator (CIITA) is responsible for IFNγ-inducible GILT expression, and it negatively regulates constitutive GILT expression (*12*).

Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder characterized by sclerosing leukoencephalopathy and multifocal bone cysts, caused by a loss-of-function mutation of TREM2 or DAP12, both of which are expressed as a receptor-adaptor complex exclusively on the microglia in the central nervous system (CNS) (*13*). Pathologically, NHD brains exhibit extensive demyelination, astrogliosis, an accumulation of axonal spheroids, and activation of microglia predominantly in the white matter of frontal and temporal lobes (*14*). Alzheimer's disease (AD) is characterized by the hallmark pathology comprised of widespread amyloid-β (Aβ) deposition, neurofibrillary tangle (NFT) formation, extensive neurodegeneration, and profound activation of microglia in the brain (*15*). A single nucleotide polymorphism (SNP) p.Arg47His (R47H) of TREM2 elevates approximately three-fold the risk of AD, suggesting that the partial loss of function of TREM2 in microglia causes the AD pathology (*16,17*). Microglia is postulated to be the principal cell type of APCs (*18,19*), although it remains unknown whether microglia express GILT in NHD and AD brains. In the present study, we characterized GILT expression in NHD and AD brains by immunohistochemistry.

## 2. Materials and Methods

### 2.1. *Human brain tissues*

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

For immunohistochemical studies, serial sections of the frontal cortex and the hippocampus were prepared from four subjects who died of non-neurological causes (NC), composed of a 63-year-old man who died of prostate cancer and acute myocardial infarction (NC1), a 67-year-old man who died of dissecting aortic aneurysm (NC2), a 57-year-old man who died of alcoholic liver cirrhosis (NC3), and a 61-year-old man who died of rheumatoid arthritis with interstitial pneumonia (NC4), ten AD patients, composed of a 68-year-old woman (AD1), a 70-year-old woman (AD2), a 68-year-old woman (AD3), a 56-year-old man (AD4), a 59-year-old man (AD5), an 81-year-old man (AD6), a 68-year-old woman (AD7), an 80-year-old man (AD8), a 72 year-old man (AD9), and a 77-year-old woman (AD11), and five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5). The homozygous mutation of a single base deletion of 141G (c.141delG) in exon 3 of DAP12 was identified in NHD1, NHD2, and NHD5, while the genetic analysis was not performed in NHD3 or NHD4. All AD cases were satisfied with the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for diagnosis of definite AD (*20*). They were categorized into the stage C of amyloid deposition and the stage VI of neurofibrillary degeneration, following the Braak's staging (*21*).

### 2.2. *Immunohistochemistry*

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 110℃ for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Pacheco, CA, USA). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. They were then incubated with phosphate-buffered saline (PBS) containing 10% normal rabbit or goat serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4℃ overnight with goat polyclonal anti-GILT antibody (T-18, sc-

21827, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-Iba1 antibody (Wako Pure Chemical, Tokyo, Japan) for a marker specific for microglia. The specificity of anti-GILT antibody was validated by western blot analysis of recombinant human GILT protein expressed in HEK293 cells, which were transfected with the pcDNA4/HisMax TOPO vector (Thermo Fisher Scientific, Carlsbad, CA, USA) containing the mature GILT sequence. After washing with PBS, tissue sections were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated anti-goat or anti-rabbit secondary antibody (Nichirei, Tokyo, Japan), followed by incubation with diaminobenzidine tetrahydrochloride (DAB) substrate (Vector, Burlingame, CA, USA). They were processed for a counterstain with hematoxylin. Negative controls underwent all the steps except for exposure to primary antibody. In limited experiments, double immunolabeling was performed using T18 followed by incubation with HRP-conjugated anti-goat secondary antibody, and rabbit anti-Iba1 antibody (Wako), or mouse monoclonal antibody against MHC class II (TAL.1B5; HLA-DR, Dako, Tokyo, Japan) or amyloid-β peptide (12B2; Immunobiological Laboratories, Gunma, Japan), followed by incubation with alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody (Nichirei) and exposure to DAB substrate and Warp Red chromogen (Biocare Medical).

### 2.3. Quantification of GILT immunoreactivity

To quantify immunolabeled areas, the images derived from three fields of the frontal cortex per each section were captured at a 200 X magnification on the Olympus BX51 universal microscope. They were then processed for quantification by using ImageJ software (National Institute of Health, Bethesda, MD, USA). The GILT-immunolabeled area was calibrated by the Iba1-immunolabeled area. The differences in the GILT/Iba1 ratio among NC, AD, and NHD subjects were evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test.

### 2.4. Quantitative RT-PCR analysis and western blot analysis

To investigate the effects of inflammatory mediators on GILT expression, v-*myc*-immortalized human microglial cells named HMO6 (*22*), incubated in 10% fetal bovine serum (FBS)-containing Dulbecco's Modified Eagle's Medium (DMEM), were exposed for 24 hours to 1 μg/mL lipopolysaccharide (LPS; Sigma, St. Louis, MO, USA), recombinant human IFNγ, IL-4, IL-13 or TGFβ1 (50 ng/mL each; Peprotech, London, UK), followed by extraction of total cellular RNA for RT-PCR analysis or total cellular protein for western

blot analysis. For quantitative RT-PCR (qPCR) analysis, cDNA was amplified by PCR on LightCycler 96 (Roche Diagnostics, Tokyo, Japan) with SYBR Green I and a primer set composed of 5'agtgtggagaccatcaaggaagac3' and 5'cagttcagccatcacttggatgag3' for detection of an 156 bp product of the *GILT* gene (NM_006332.4). The expression levels of GILT were standardized against the levels of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) detected in the corresponding cDNA samples. All the assays were performed in triplicate.

### 3. Results

First, we validated the specificity of anti-GILT antibody T-18 by western blot of an Xpress-tagged recombinant mature GILT protein expressed in HEK293 cells (Figure 1A). Next, by qPCR and western blot, we found that human immortalized microglial cells HMO6 express high levels of GILT after stimulation for 24 hours with IFNγ but not with LPS, IL-4, IL-13, or TGFβ (Figures 1B and 1C). Then, by immunohistochemistry, we found that GILT is intensely expressed predominantly in Iba1-immunoreactive amoeboid microglia and occasionally in perivascular macrophages in the frontal cortex and the hippocampus of AD brains (Figure 2, panels a, c, d), while the much smaller number of microglia was labelled with GILT in both NHD and NC brains (Figure 3, panels a, c). In NC brains, most of the GILT-positive cells were perivascular macrophages. Since GILT is a marker of APCs, Iba-1 immunoreactive microglia and perivascular macrophages expressing GILT might represent a subset of professional APCs. The levels of GILT immunoreactivity were significantly elevated in AD brains, compared with NC brains ($p = 0.0396$) and NHD brains ($p = 0.0057$) (Figure 4). The levels of GILT expression were not elevated in NHD brains and not significantly different between NC and NHD brains ($p = 0.8183$) (Figure 4). In AD brains, clusters of amoeboid microglia strongly expressing GILT were accumulated in and around of amyloid plaques and these cells coexpressed HLA-DR, a MHC class II molecule and Iba1 (Figure 5a-5d).

### 4. Discussion

GILT is a lysosomal thiol reductase optimally active at acidic pH capable of catalyzing reduction of inter- and intrachain disulfide bonds (*1-4*). GILT is the only known enzyme to catalyze disulfide bond reduction in the endocytic pathway. GILT plays a key role in MHC class II-restricted antigen processing (*5*). GILT facilitates the presentation of a subset of epitopes from disulfide bond-containing antigens. Enhanced presentation of MHC class II-restricted epitopes alters immunological tolerance and modulates CD4^{+} T-cell mediated autoimmunity (*2,3*).

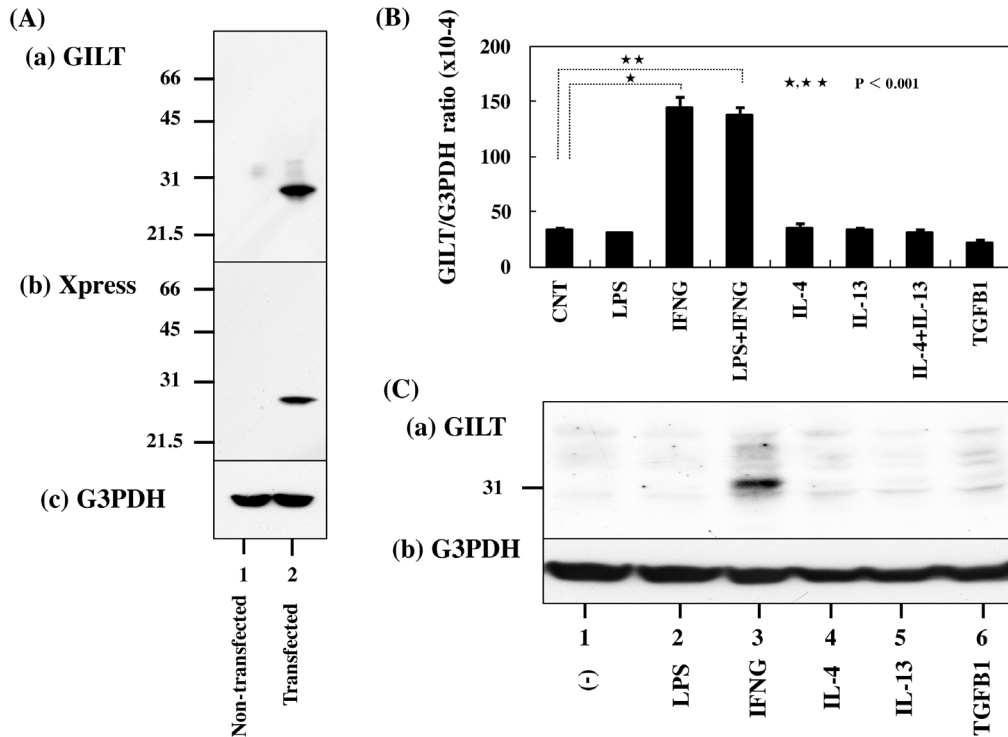We found that microglia express GILT in AD

**Figure 1. GILT expression in human microglial cells. Panel A. The specificity of GILT antibody.** Western blot of non-transfected HEK293 cells **(lane 1)** and the cells transfected with the vector containing the mature GILT sequence **(lane 2)**. **(a)** GILT, **(b)** Xpress tag, and **(c)** G3PDH as a loading control. **Panel B. Quantitative RT-PCR analysis of GILT expression in HMO6 microglia in culture.** HMO6 cells were exposed for 24 hours to 1 μg/mL lipopolysaccharide (LPS), recombinant human IFNγ(IFNG), IL-4, IL-13 or TGFβ1 (TGFB1) (50 ng/mL each), followed by extraction of total cellular RNA that is processed for qRT-PCR. The expression levels of GILT were standardized against the levels of G3PDH. **Panel C. Western blot analysis.** HMO6 is exposed for 24 hours to 1 μg/mL LPS, recombinant human IFNγ(IFNG), IL-4, IL-13 or TGFβ1 (TGFB1) (50 ng/mL each). Then, total cellular protein was processed for western blot analysis.
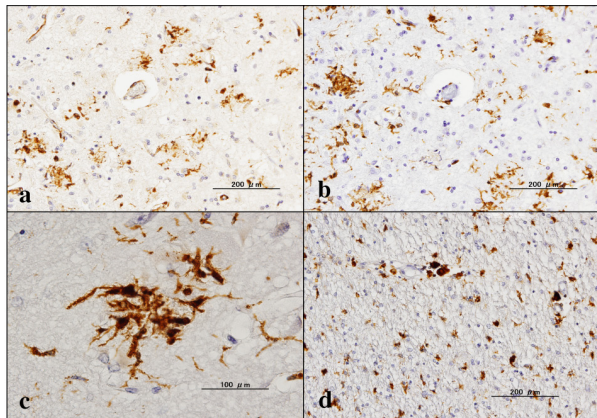


**Figure 2. Expression of GILT in AD brains. (a)** the frontal cortex, GILT, **(b)** the same area of **(a)**, Iba1, **(c)** the hippocampus, GILT, and **(d)** the hippocampus white matter, GILT.
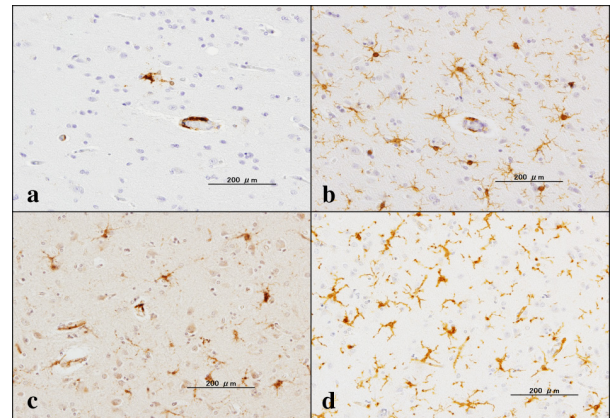


**Figure 3. Expression of GILT in NC and NHD brains. (a)** the frontal cortex of NC, GILT, **(b)** the same area of **(a)**, Iba1, **(c)** the frontal cortex of NHD, GILT, and **(d)** the same area of **(c)**, Iba1.

and NHD brains. Both NHD and AD are induced by dysfunction of the microglial TREM2 signaling pathway in the brains. GILT immunoreactivity was upregulated on microglia in AD brains compared with NHD brains. This is attributable to a difference in demand for disulfide bond reduction by microglia between AD and NHD brains. Microglia, resident

myeloid cells in the CNS, play a pivotal role in maintenance of brain homeostasis, along with progression of neurodegenerative disease (*23*). Microglia originate from erythromyeloid progenitor cells in the yolk sac and populate the CNS during early embryonic development (*24*). Microglia actively survey the surrounding microenvironment with dynamic
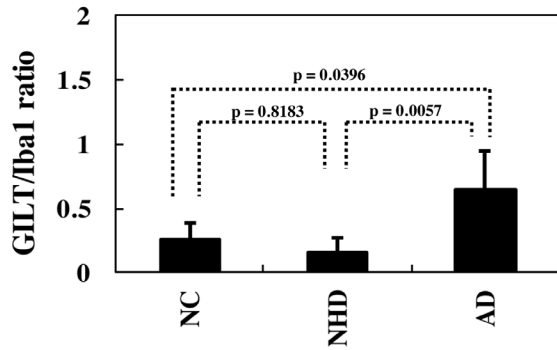
**Figure 4. Quantitative analysis of GILT expression on microglia in NC, NHD, and AD brains.** The GILT-immunolabeled area/Iba1-immunolabelled area ratio in the frontal cortex is shown.



**Figure 5. Expression of GILT, Iba1, HLA-DR and Aβ in AD brains. (a)** the hippocampus, GILT (dark brown), Iba1 (red), **(b)** the hippocampus, GILT (dark brown), HLA-DR (red), **(c)** the hippocampus, GILT (dark brown), Aβ (red), and **(d)** the hippocampus, GILT (dark brown), Aβ (red).

processes. Microglial phagocytosis utilizes different types of receptors to initiate function, such as Toll-like receptors (TLRs) for foreign microbial pathogens and TREM2 for apoptotic cellular substrates (*25*). Microglia adopt two distinc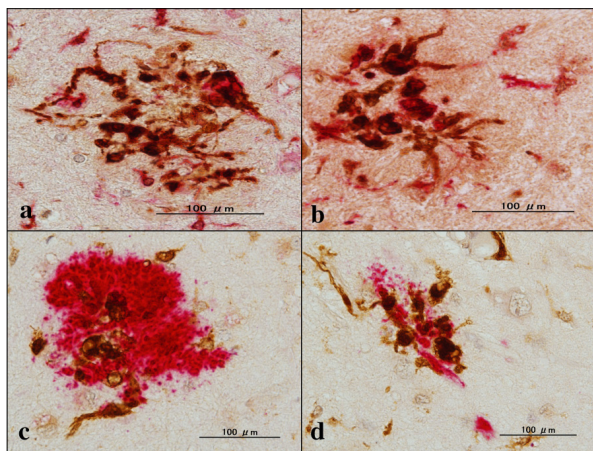t activation phenotypes, composed of a proinflammatory and neurotoxic "classical" activation (M1) phenotype by exposure to IFNγ or LPS and an anti-inflammatory and neuroprotective "alternative" activation (M2) phenotype following treatment with IL4 or IL-13 (*26*). However, at present, a definite marker capable of clearly separating M1 and M2 phenotypes in human microglia remains largely unidentified (*27*). HMO6 human microglial cells expressed high levels of GILT after stimulation with IFNγ but not with LPS, IL-4, IL-13, or TGFβ, suggesting that GILT serves as a valid marker for detection of IFNγ-activated M1 microglia. A previous study showed that CD4^{+} and CD8^{+} T cells are accumulated in the hippocampal parenchyma of AD brains (*28*). We previously reported a discernible infiltration of CD3^{+} T cells in NHD brains

(*14*). Therefore, the possibility exists that primed T cells in AD and NHD brains are locally reactivated by recognizing antigens presented by APCs in the CNS. Although the precise antigens presented by microglia in the brain for CD4^{+} and CD8^{+} T cells remain totally unknown, the adaptive immune response mediated by T cells plays a key role in exacerbation of the existing inflammation in AD, to a lesser extent, NHD brains (*29*). Both CD4^{+} T and CD8^{+} T cells produce large amount of IFNγ, a potent inducer of the M1 phenotype. IFNγ also increases the expression of MHC class II, CD86, CD40, and ICAM-1 on the cell surface of microglia that enhance their APC function. These results suggest that GILT-expressing M1 microglia observed in close proximity to amyloid plaques of AD brains are capable of efficiently presenting certain antigens enriched with disulfide bonds derived from the plaques in a MHC class II-restricted manner. Furthermore, GILT might regulate the cellular redox state in these cells under Aβ-induced oxidative stress conditions in AD brains (*30*).

Disulfide bonds are the most common covalent link between amino acids in proteins. Disulfide bonds are present in 15% of the human proteome (*31*). Disulfide bonds stabilize proteins to confine conformational changes. They exist in 55% of the proteins involved in pathological amyloid formation. They play an important role in the kinetics of aggregation and the structure and toxicity of the formed aggregates. Several proteins related to AD pathogenesis have disulfide bonds. Beta-secretase 1 (BACE1), a type I transmembrane aspartic protease responsible for the β-secretase cleavage of amyloid beta precursor protein (APP) has three intramolecular disulfide bonds in the catalytic domain that regulate protein maturation (*32*). APP has three intramolecular disulfide bonds in the copper-binding domain (CuBD) (*33*). Tau, whose phosphorylation induces neurofibrillary tangle (NFT) formation, exists as six different isoforms and the longest isoform of Tau has two cysteine residues that form both intramolecular and intermolecular disulfide bonds (*34*). Intermolecular disulfide bonds promote Tau aggregation, while intramolecular disulfide bonds prevent Tau aggregation. A detergent-insoluble disulfide-linked form of G3PDH is found in brain tissues of AD patients (*35*). Aβ42 and oxidative stress promote the nuclear and cytoplasmic accumulation of insoluble aggregates of disulfide-bonded G3PDH that cause neuronal cell death. It remains unknown whether disulfide bonds of these proteins are reduced by GILT in the endocytic pathway for antigen presentation on the MHC class II (*36*).

In conclusion, GILT, serving as a M1 marker for IFNγ-activated microglia, is intensely expressed by a subset of microglia and perivascular macrophages, although GILT expression levels are significantly increased in AD brains, compared with NC and NHD brains. We found a coexpression of GILT and MHC class II antigen in microglia. The class II-MHC-

expressing microglia are increased in AD brains (*37*). GILT facilitates the MHC class II-restricted presentation of epitopes derived from disulfide bond-rich antigens in the endocytic pathway (*1-4*). These results suggest a pivotal role of GILT in antigen presentation by M1 microglia in AD brains, and to a lessor extent, NHD brains. The precise antigens presented by GILT-expressing microglia in AD brains and NHD brains remain to be investigated.

## Acknowledgements

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# Spectrum and hematological profile of hereditary anemia in North Indians: SGPGI experience

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| | |
|---|---|
| **Summary** | **Thalassemia and other hemoglobinopathies together with red cell enzymopathies are a common cause of anemia, which can be prevented by population screening and genetic counseling. This study was designed to screen the anemic patients for thalassemia, structural hemoglobin variants and red cell enzymopathies. A total of 17047 cases were evaluated from 2009 to 2018 for thalassemia, hemoglobin variants, glucose 6 phosphate dehydrogenase deficiency, pyruvate kinase deficiency and hereditary spherocytosis. Patients' records were entered in a Microsoft excel sheet and a spectrum of disorders was evaluated. Year wise spectrum was also analyzed to see the difference in incidence at different time periods. Incidence of beta thalassemia trait and thalassemia major was found in 11.0% and 3.4% respectively, whereas other hemoglobinopathies were observed in 3.2% of the cases. G6PD deficient cases were 0.2 % and 0.4% had hereditary spherocytosis. No significant difference was observed in incidence of thalassemia and other hemoglobinopathies at different time points. This study provided a health burden and detailed spectrum and prevalence of hemoglobinopathies in North Indians high risk population which contribute toward the development of prevention strategies for better management of hemoglobinopathies. In view of high incidence of thalassemia a routine hematological screening at a primary health center may be introduced as a prospective premarital screening under a thalassemia control program. Moreover rapid and easy quantification of hemoglobin variants (Hb variants) make Cation exchange – High Performance Liquid Chromatography (CE-HPLC) a suitable diagnostic test for the routine investigation of genetic causes of anemia.** |

*Keywords:* Thalassemia, Hb variants, G6PD deficiency, Premarital Screening, HPLC

## 1. Introduction

The inherited disorders of hemoglobin synthesis are the most common monogenic disorders worldwide. Hemoglobinopathies are a group of inherited disorders of hemoglobin synthesis and a major cause of anemia. Hemoglobinopathies responsible for hemolytic anemia may be divided into two groups. The first one corresponds to thalassemia and the second to the presence of structurally abnormal hemoglobin. Though, there are many variants of thalassemia, β-thalassemia is the most common variant found in the Indian sub-continent. Since thalassemia is difficult to cure, it becomes a priority to prevent this disorder. The clinical spectrum of these disorders varies from asymptomatic conditions (beta-thalassemia minor) to serious disorders such as thalassemia major that require regular blood transfusions and extensive medical care. The only cure for affected children is bone marrow transplantation, which is expensive, risky and difficult to perform with discouraging success rates. The other treatment to sustain life is regular blood transfusion with iron chelation but this is very painful. As anemia is considered a major public health problem in India, so early detection of anemia in these disorders allows timely intervention and prevention from serious consequences.

The most common effective approach for developing countries like India is preventing disorders associated with β-globin gene cluster and major efforts need to be directed at applying a simple and well defined strategy to control these disorders by carrier detection, genetic

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counseling and prenatal diagnosis (*1*). However, accurate and timely detection of various known and unknown Hb variants can prevent occurrence of serious Hb disorders such as thalassemia major in newborns.

Mohanty *et al.* in 2013 (*2*) conducted a multicenter study and after analyzing 56,780 samples from 6 different centers; they reported overall a 2.78% prevalence of thalassemia trait in the studied cohort. They highlight that the prevalence of β-thalassemia varied from 0 to 10.5 % among the different caste/ethnic groups of India (*2*).

Since there is lot of complexity in the Indian population structures and due to high demand for resources; large migration of communities occured in the past from one geographical area to another, and therefore there is a need to evaluate the prevalence of hemoglobin disorders from time to time.

The present study was carried out to determine the prevalence of hemoglobinopathies and β-thalassemia among the North Indian populations from 2009-2018. In addition, our study also aimed to identify rare/unknown Hemoglobin variants by molecular analysis to provide accurate diagnosis and genetic counseling.

## 2. Materials and Methods

This cross-sectional descriptive study was conducted in Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow from 2009 to 2018. Individuals with suspected risk of thalassemia and other hemoglobinopathies, with known family history, premarital, preconception (postmarital screening before planning child birth) or antenatal subjects who apparently did not have any suspicion of risk of carrier state and individuals coming voluntarily for screening were included.

To evaluate the cause of anemia a battery of tests related to hematological and biochemical parameters such as Glucose-6-phosphate dehydrogenase deficiency (G6PD), Pyruvate kinase Enzyme Deficiency, Hereditary Spherocytosis and Iron deficiency Anemia (IDA) were carried out.

Four ml blood sample was collected from each individual in ethylene diamine tetra acetic acid (EDTA) and plain vials. Red Cell indices analysis was carried out on a Sysmex 21 automated blood cell counter for red cell indices.

Serum Iron and total iron binding capacities (TIBC) was estimated by the Ferrozine method (International Council for Standardization in Hematology 1990) and using IRON & TIBC kit (Crest Biosystems, India) on the same day of blood collection.

The samples were stored at 4°C and further analyzed in batches for CE-HPLC (Cation exchange – High Performance Liquid Chromatography). CE-HPLC was performed with each blood sample on a BIO-RAD Variant II using beta thalassemia short program pack.

DCIP test was performed for G6PD deficiency. Dichloro phenol indophenols decolorizing test (DCIP Bernstein test) is a screening dye test for G6PD deficiency diagnosis. Nicotinamide adenine dinucleotide phosphate (NADP) reduces the dye DCIP into a colorless state (DCIPH) through the action of G6PD. The rate and the degree of this decolorization are proportional to the G6PD activity in RBCs. Phenazonemethosulphate (PMS) is used as an electron carrier between NADPH and 2-6 DCIP in the test. 20 μL of whole blood followed by 400 μL of DCIP dye solution was mixed in a test tube containg 50 μL of PMS, 25 μL of G6P and 25 μL of NADP and 1 mL of distilled water and overlaid with liquid paraffin incubate at 37°C for 10 min. The mixture color changes from blue to red in presence of G6PD deficiency within 10 min.

Pyruvate kinase (PK) deficiency is due to defects in pyruvate kinase enzyme expression or activity. Pyruvate kinase enzyme catalyzes the last step of glycolysis, the transfer of a phosphate group from phospho(enol) pyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of adenosine triphosphate (ATP). In the pyruvate kinase deficiency test, PEP and ADP were catalyzed by PK to generate pyruvate and ATP. The generated pyruvate is oxidized to produce fluorescence (at 340 nm). The 1000 μL reaction mixture in a tube contained 25 mM potassium phosphate buffer pH 7.4 50 μL, 8 mM magnesium sulfate 100 μL, 0.15mM NADH 100 μL, 0.15 mM phosphoenolpyruvate 30 μL, 0.3 mM ADP 100 μL, and water 620 μL. RBC solution was prepared using packed red blood cells suspended in 20% normal saline. 200 μL reaction mixture was mixed with 20 μL RBC solution in a tube and the mixed solution was spotted on Whatman no 1 filter paper at zero time. Tubes were placed in water bath at 37°C for 10 minutes and again a drop from the mixture was spotted on filter paper after 20 and 30 minutes. In a normal sample disappearance was seen with in 30 min but in pyruvate deficient samples fluorescence takes a longer time to disappear. Since the increase in color or fluorescence intensity is proportional to the increase in pyruvate amount, the PK activity can be accurately measured.

Osmotic fragility is a test to measure the resistance to hemolysis of RBCs exposed to a series of hypotonic solutions. This test is performed to detect thalassemias, red cell membrane disorders and hereditary spherocytosis. In the osmotic fragility test, whole blood is added to varying concentrations of buffered sodium chloride solution and allowed to incubate at room temperature. The amount of hemolysis in each saline concentration is then determined by reading the supernatants on a spectrophotometer at 540 nm. The results of the test may then be graphed, with the percent hemolysis plotted on the vertical axis and the sodium chloride concentration on horizontal axis.

The flow diagram for sample processing is depicted

in Figure 1.

Statistical analysis: Data is presented as number and percentages for discrete variables and as mean ± standard deviation for the continuous variables. The chi square test for trend analysis was done to see the pattern of change of prevalence of different studied causes of hemolytic anemia.

## 3. Results

Out of 17,047 cases, 3,379 cases were found with normal red cell indices (Table 1). High value of mean corpuscular volume (MCV > 100fl) and mean corpuscular hemoglobin (MCH > 36pg) were found in 211 cases. These 211 cases were found to be Vitamin B12 deficient and excluded for further study. Low Mean corpuscular volume (< 76fl) and Low mean corpuscular hemoglobin (< 26pg) was found in 13,457 cases. Out of these 13,457 samples, 10,201 cases were identified as iron deficiency anemia on the basis of their Serum Iron and Total Iron Binding Capacity (TIBC) levels. G6PD deficiency was found in 37 cases where as hereditary spherocytosis in 75 cases was noted (Table 1). We did not find any case of pyruvate kinase deficiency during the study period.

The remaining samples of 2,607 individuals were further analyzed for thalassemia and other hemoglobinopathies. After doing HPLC 1,764 cases were identified with abnormal hemoglobin variants.



**Figure 1. Flow chart showing detail methodology.**

These 1,764 cases were further sub characterized on the basis of their clinical and biochemical evaluation like thalassemia major (579) and thalassemia trait (1,185).

Hemoglobin variant disorders found in the present study were HbE trait (204), HbE disease (68), E βthalassemia (51), HbS trait (187), HbS disease (136), Sβ thalassemia (85), HbD trait (85), HbS/HbE Double heterozygous state (17) and HbS/HbD double heterozygous state (34).

Red cell indices were carried out in all samples as this is a primary screening step to find out the cause of anemia. Since thalassemia is a disorder of anemia, hemoglobin values were found reduced in thalassemia along with the microcytic and hypochromic RBCs. Low MCV and low MCH values were recorded in cases as compared to normal. The mean hemoglobin levels, MCV and MCH values of all thalassemia groups are shown in Table 2. MCV and MCH values were lowest in EE group.

HPLC analysis was carried out in subjects with low MCV and MCH and without iron deficiency to find out the status of thalassemia and other hemoglobinopathies. Comparison of mean percentages of various hemoglobins in each thalassemia group are shown in Table 3.

Year wise comparison was done to see whether the prevalence of thalassemia was increasing or decreasing by time and we observed that there was no trend in prevalence of thalassemia and other hemoglobinopathies across different time intervals ($p = 0.826$) (Table 4).

In this study, molecular characterization of beta globin gene sequencing identified a rare HB Köln (HBB:c.295 G>A) and a novel single nucleotide deletion at codon 79 (HBB:c240_240delC) (*3*).

Defining genetic changes will be helpful to explain the clinical findings of the patient and for providing accurate diagnosis and genetic counseling. Therefore, it is important to report rare nucleotide changes in hemoglobin diseases and molecular analysis by Sanger sequencing so that prevalence of various hemoglobin variant can be monitored carefully.

## 4. Discussion

The present study is an expansion of our previous study published in Prenatal diagnosis (*1*) in which we have done screening of thalassemia and other hemoglobin disorders in extended family members of thalassemia
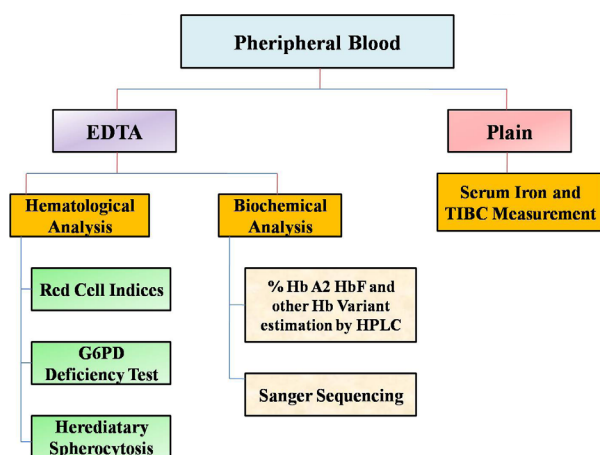
**Table 1. Incidence of different disorders causing anemia in studied cohort**

| Diagnosis | Male | Female | Total |
|---|---|---|---|
| Normal | 2,273 (31%) | 1,643 (16.9%) | 3,916 (22.9%) |
| Megaloblastic anemia | 94 (1.3%) | 117 (1.2%) | 211 (1.2%) |
| Iron deficiency anemia | 3,555 (48.5%) | 6,646 (68.4%) | 10,201 (59.8%) |
| Herediatary Spherocytosis | 37 (0.5%) | 38 (0.4%) | 75 (0.4%) |
| G6PD deficient | 37 (0.5%) | 0 | 37 (0.2%) |
| Thalassemia and Hemoglobin variants | 1,334 (18.2%) | 1,273 (13.1%) | 2,607 (15.2%) |
| Total | 7,330 (43%) | 9,717 (57%) | 17,047 |

() number in parenthesis represents percentages.

patients, couples coming for premarital screening and college going students. In that study we concluded that a cost effective approach for preventing birth of a thalassemia child is to focus on screening of extended family members rather than screening the general population. We have done a year wise comparison and observed that there is no change in frequency of beta thalassemia and other hemoglobinopathies (Figure 2). Even it is almost more or less from our previous studies.

**Table 2. Hematological Parameters in different hemoglobinopathies**

| Category | Hb (g/dL) | MCV (fl) | MCH (pg) | MCHC (g/dL) |
|---|---|---|---|---|
| Normal | 12.2 ± 2.6 | 84.5 ± 12.7 | 29.9 ± 4.6 | 36.8 ± 2.9 |
| β TT | 9.2 ± 2.1 | 69.2 ± 6.7 | 20.6 ± 2.8 | 28.6 ± 2.2 |
| β TM | 8.7 ± 2.2 | 68.7 ± 6.0 | 21.6 ± 2.9 | 25.8 ± 4.7 |
| AS | 9.1 ± 2.4 | 68.6 ± 7.0 | 21.2 ± 3.4 | 30.9 ± 2.6 |
| SS | 8.4 ± 1.6 | 69.2 ± 7.9 | 22.3 ± 3.2 | 30.3 ± 2.4 |
| Sβ | 8.3 ± 2.1 | 68.6 ± 6.2 | 22.5 ± 3.5 | 31.1 ± 2.3 |
| S/E | 7.4 ± 3.2 | 67.1 ± 6.9 | 19.2 ± 4.5 | 28.4 ± 3.9 |
| AE | 8.6 ± 2.4 | 69.6 ± 6.1 | 21.1 ± 3.0 | 29.7 ± 2.7 |
| EE | 6.3 ± 2.0 | 64.1 ± 6.6 | 18.4 ± 2.5 | 27.7 ± 2.5 |
| Eβ | 6.0 ± 1.4 | 66.9 ± 4.1 | 18.9 ± 2.6 | 26.8 ± 3.3 |
| SD | 7.9 ± 1.7 | 91.3 ± 0.2 | 32.8 ± 0.6 | 34.2 ± 4.9 |
| AD | 10.4 ± 2.7 | 69.5 ± 9.7 | 24.0 ± 4.8 | 31.2 ± 3.4 |
| DD | 8.9 ± 3.0 | 76.1 ± 12.7 | 23.7 ± 5.6 | 31.2 ± 1.6 |

Table 5 summarizes the various studies conducted in last ten years which have used the patient's selection criterion similar to our study. We can clearly see from the table that the frequency of thalassemia and other hemoglobinopathies varies significantly from one geographical region to another. The figures of World Health Organization (WHO) estimate that approximately 5% of the world's populations are carriers for genetic hemoglobin disorders. Highest (18.1%) prevalence of beta thalassemia trait was observed in New Delhi (*11*) whereas it was found lowest in Dibrugarh, Assam where the HBE was more prevalent (*7*). Sickle cell trait, which was found in high frequency (9.2%) in Maharashtra was found only in 0.8% of studied subjects in the present study. In our other previous studies HBS and HBE was observed in low frequency (*1*).

Colah *et al.* reported that about 1.5% of the world population are carriers of β thalassemia (*12*). In central India the prevalence of β thalassemia trait has been estimated to be 9.59% (*13*). In the present study the most common Hb abnormality detected was β thalassemia trait present in 1185 (11.0%) patients. These data reveal that for the most part of India β thalassemia trait is a common Hb disorder. In this study hematological parameters were used to distinguish cases of β thalassemia trait

**Table 3. Comparison of percentage of Hemoglobin variants**

| Category | HbA | HbA2 | HbF | HbD | HbS |
|---|---|---|---|---|---|
| NORMAL | 80.5 ± 6.9 | 2.4 ± 0.5 | 0.8 ± 0.3 | - | - |
| β TT | 89.5 ± 2.6 | 5.5 ± 0.7 | 0.6 ± 0.4 | - | - |
| β TM | 24.6 ± 28.8 | 3.6 ± 1.3 | 64.1 ± 28.4 | - | - |
| AS | 55.9 ± 5.9 | 3.4 ± 0.8 | 2.1 ± 2.3 | - | 34.0 ± 11.7 |
| SS | 3.8 ± 1.7 | 3.4 ± 0.7 | 19.0 ± 6.0 | - | 70.1 ± 8.4 |
| Sβ | 3.5 ± 1.7 | 5.5 ± 0.6 | 16.9 ± 8.2 | - | 72.8 ± 8.8 |
| S/E | 2.5 ± 0.2 | 49.8 ± 12.6* | 4.4 ± 0.5 | - | 43.7 ± 13.7 |
| AE | 48.5 ± 7.1 | 33.5 ± 11.3* | 2.6 ± 7.1 | - | - |
| EE | 5.7 ± 2.0 | 65.4 ± 6.6* | 22.8 ± 5.9 | - | - |
| Eβ | 4.4 ± 1.4 | 64.3 ± 14.4* | 24.1 ± 8.8 | - | - |
| SD | 2.6 ± 0.1 | 2.3 ± 0.7 | 16.1 ± 5.7 | 33.2 ± 9.3 | 26.3 ± 12.7 |
| AD | 56.6 ± 7.8 | 2.3 ± 0.6 | 2.5 ± 5.3 | 33.4 ± 5.2 | - |
| DD | 5.8 ± 2.3 | 3.0 ± 1.2 | 2.4 ± 2.5 | 79.8 ± 13.1 | - |

*HBA2+HBE.

**Table 4. Year wise distribution of incidence of anemia causing disorder**

| Year [May-Apr] | No of Samples screened | Normal | Thalassemia and other hemoglobinopathies | IDA | Other* |
|---|---|---|---|---|---|
| 2009-2010 | 1,010 | 203 (20%) | 197 (19.5%) | 590 (58.5%) | 20 (2%) |
| 2010-2011 | 1,274 | 300 (23.5%) | 210 (16.4%) | 729 (57.2%) | 35 (2.7%) |
| 2011-2012 | 1,471 | 470 (31.9%) | 237 (16.1%) | 728 (49.4%) | 36 (2.4%) |
| 2012-2013 | 1,527 | 358 (23.4%) | 229 (14.9%) | 899 (58.8%) | 41 (2.6%) |
| 2013-2014 | 2,058 | 454 (22.0%) | 282 (13.7%) | 1,279 (62.1%) | 43 (2.1%) |
| 2014-2015 | 2,128 | 364 (17.1%) | 291 (13.6%) | 1,444 (67.8%) | 29 (1.4%) |
| 2015-2016 | 2,356 | 415 (17.6%) | 351 (14.8%) | 1,554 (65.9%) | 36 (1.5%) |
| 2016-2017 | 2,542 | 368 (14.5%) | 373 (14.6%) | 1,745 (68.6%) | 56 (2.2%) |
| 2017-2018 | 2,681 | 443 (16.5%) | 515 (19.2%) | 1,674 (62.4%) | 49 (1.8%) |
| TOTAL, 2009-2018 | 17,047 | 3,375 (19.8%) | 2,685 (15.8%) | 10,642 (62.4%) | 345 (2.0%) |
| Chi Square for trend *p* value | | < 0.0001 | 0.0826 | < 0.0001 | 0.038 |

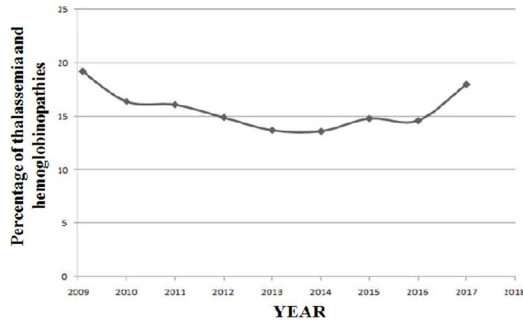() number in parenthesis represents percentage.

**Figure 2. Year wise prevalance of thalassemia and hemoglobinopathies.**

from iron deficiency anemia but these parameters were not useful in patients who have both iron deficiency and thalassemia trait. The patients with iron deficiency anemia showed significantly lower levels of HbA2 and MCV than the patients with a normal iron profile. In this study the rate of occurrence of iron deficiency anemia cases increases by year. There is a significant correlation with serum iron level and HbA2 concentration (*14*) and false diagnosis may occur in patients with iron deficiency anemia and borderline HbA2 (3.5-3.9%). Other causes of borderline HbA2 include β thalassemia trait with silent mutation and α triplication (*15*). So it has been emphasized that interpretation of chromatograms must be done after taking into consideration the clinical history, family history, finding of peripheral blood smear and mutation analysis for accurate diagnosis.

Several countries (Italy, Greece, Cyprus, UK, France, Iran, Thailand, Australia, Singapore, Taiwan, Hong Kong, and Cuba) prevent the birth of a thalassemia child up to 70% by setting up comprehensive national prevention programs, which include public awareness and education, carrier screening, and counseling, as well as information on prenatal diagnosis and preimplantation diagnosis (*16-19*). In our country also prevention programs are currently being done but they are limited to certain urban regions and these programs are done by a handful of tertiary care centers. The biggest problem in prevention of thalassemia in India is our traditions for which our society is restricted to an endogamy pattern of marriage and there is some type of social stigma that prevents them from doing thalassemia screening.

This study provided a detailed prevalence and spectrum of hereditary anemia among the North Indian population and will contribute toward the development of prevention strategies and reduction of excessive health care costs in this area, allowing better management of hemoglobinopathies. Also, there is need of a national level prevention program, which registers the thalassemia child, and sets up a national level campaign for public awareness of thalassemia and other hemoglobinopathies.

## 5. Conclusion

The present study provides a broad overview of the burden

**Table 5. Percent prevalence of hemoglobin variants in Indian subcontinent in past 10 years**

| SR. NO | Year | Name of City/State (Ref.) | Total subject | Normal | Anemic | BTT | BTM | AE | EE | E/β THAL | S/E | E/D | AS | SS | S/β THAL | S/D | AD | DD | Others |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2015 | West Bengal (*4*) | 10,407 | 8,898 (85.5%) | 1,509 (14.5%) | 579 (5.6%) | 11 (0.1%) | 522 (5.0%) | 32 (0.3%) | 92 (0.9%) | - | - | 37 (0.4%) | 7 (0.1%)) | 4 (0.0%) | 1 (0.0%) | 7 (0.1%) | 3 [0.0%] | 214 (2.0%) |
| 2 | 2014 | Kolkata West Bengal (*5*) | 90,210 | 79,897 (88.6%) | 10,313 (11.4%] | 3,870 (4.3%) | 1,135 (1.2%) | 2,418 (2.7%) | 358 (0.4%) | 1,406 (1.6%) | 4 (0.0%) | - | 343 (0.3%) | 415 (0.5%) | 187 (0.2%) | 1 (0.0%) | 141 (0.1%)] | - | 35 (0.0%) |
| 3 | 2014 | Burdwan West Bengal (*6*) | 1,872 | 982 (52.5%) | 890 (47.5%) | 158 (17.8%) | 13 (0.7%) | 306 (16.3%) | 225 (12.0%) | 134 (7.2%) | - | - | 10 (0.5%) | 2 (0.1%) | 30 (1.6%) | 1 (0.0%) | 7 (0.4%) | 0 (0.0%) | 4 (0.2%) |
| 4 | 2014 | Dibrugarh Assam (*7*) | 9,000 | 3,680 (40.9%) | 5,320 (59.1%) | 313 (3.4%) | 32 (0.3%) | 2,294 (25.0%) | 1,892 (21.0%) | 114 (1.2%) | - | 2 (0.0%) | 189 (2.1%) | 203 (2.2%) | 53 (0.0%) | 2 (0.0%) | 6 (0.0%) | - | 220 (4.0%) |
| 5 | 2013 | Gujarat (*8*) | 7,000 | 5,385 (77.0%) | 1,615 (23.0%) | 839 (12.0%) | 292 (4.2%) | 11 (0.2%) | 10 (0.1%) | 4 (0.0%) | - | 3 (0.0%) | 214 (3.0%) | 85 (1.2%) | 52 (0.8%) | 8 (0.1%) | 47 (0.7%) | 11 (0.2%) | 39 (0.5%) |
| 6 | 2012 | Burdwan West Bengal (*9*) | 3,823 | 2,702 (70.7%) | 1,121 (29.3%) | 626 (16.4%) | 1.6 (2.8%) | 176 (4.0%) | 7 (0.2%) | 134 (3.5%) | 3 (0.0%) | - | 24 (0.6%) | 6 (0.2%) | 12 (0.3%) | - | 4 (0.1%) | 1 (0.0%) | 22 (0.6%) |
| 7 | 2012 | Dhule Maharashtra (*10*) | 24,723 | 1,668 (6.7%) | 2,726 (11.1%) | 183 (0.7%) | 3 (0.0%) | 3 (0.0%) | - | 1 (0.0%) | - | - | 2253 (9.2%) | 270 (1.1%) | 10 (0.0%) | - | 3 (0.0%) | - | 20,329 (82.2%) |
| 8 | 2010 | New Delhi (*11*) | 800 | 553 (69.1%) | 247 (30.9%) | 145 (18.1%) | 23 (2.9%) | 9 (1.1%) | 1 (0.1%) | 10 (1.3%) | - | - | 11 (1.4%) | 4 (0.5%) | 6 (0.8%) | 1 (0.1%) | 7 (0.9%) | - | 30 (3.7%) |
| 9 | Present | Lucknow | 17,047 | 4,092 (24%) | 12,955* (76%) | 1,185 (11.0%) | 579 (3.4%) | 204 (1.2%) | 68 (0.4%) | 51 (0.3%) | 17 (0.1%) | - | 187 (1.1%) | 136 (0.8%) | 85 (0.5%) | 34 (0.2%) | 85 (0.5%) | 51 (0.3%) | 511 (3.0%) |

() number in parenthesis represents percentage. *Hospital referral.

and spectrum of hemoglobinopathies in this region. The size of affected and carriers of hemoglobinopathies in the Indian population is very large. As HPLC is a powerful diagnostic tool for early detection and management of hemoglobinopathies, only a few inconclusive and unidentified cases with coexisting nutritional deficiency require further molecular analysis and iron study. Despite doing a national level screening program, extended member screening for thalassemia, antenatal screening and genetic counseling, we have seen that there is no decline in the birth of thalassemia major in India from the past.

As the centers managing hemoglobinopathies in the high prevalence area increase their experience, more and more families with this condition will come forward and cascade screening and counseling of these families will provide additional cases and carriers, and this will also provide a newer area for research. Therefore, funding by government agencies should be liberal and small scale thalassemia screening industries like laboratories should be established at various states and these centers should be linked to tertiary care hospitals so that mutational and prenatal diagnosis could be treated as prevention programs. In conclusion, a national level awareness program should be performed to prevent the birth of a thalassemia child.

## Acknowledgements

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# Original Article

# Filaggrin, major basic protein and leukotriene B4: Biomarkers for adult patients of bronchial asthma, atopic dermatitis and allergic rhinitis

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**Summary**    **Bronchial asthma (BA), atopic dermatitis (AD), and allergic rhinitis (AR) are well known atopic disorders with complex etiologies. This study was undertaken to investigate the role of filaggrin, eosinophil major basic protein (MBP) and leukotriene B4 (LTB4) in patients with BA, AD, and AR. Sera from 1,246 patients with different atopic disorders and 410 normal healthy controls were collected and were evaluated for filaggrin, MBP and LTB4 by specific sandwich ELISAs, whereas immunoglobulin E (IgE) was used as a positive control for atopic patients. Serum analysis showed that filaggrin levels were remarkably high in patients with AD and in patients with multiple (mixed) atopic disorders ($p < 0.001$), whereas its levels in BA and AR patients were low but much higher than in normal human sera ($p < 0.01$). MBP levels were also high in AR, BA and mixed atopic patients, whereas AD patients showed no increase of MBP ($p > 0.05$). In contrast, LTB4 level was found to be significantly low in all tested atopic patients groups as compared to the levels of LTB4 present in normal human sera ($p < 0.001$). In conclusion, these findings support an association between filaggrin, MBP or LTB4 and atopic disorders. Our data strongly suggest that filaggrin, MBP or LTB4 might be useful in elucidating the mechanisms involved in the pathogenesis of these atopic disorders.**

*Keywords:* Bronchial asthma, atopic dermatitis, allergic rhinitis, filaggrin, LTB4, MBP

## 1. Introduction

Bronchial asthma (BA), atopic dermatitis (AD) and allergic rhinitis (AR) are common atopic disorders with complicated etiologies. The atopic march from early AD to BA, AR, or both later in life and the extensive comorbidity among them indicates, that these atopic disorders might share a common mechanism

(*1*). Moreover, heritability of these atopic disorders is high, being 35-95% for BA, 71-84% for AD, 33-91% for AR and 34-68% for allergen-specific serum immunoglobulin E (IgE) levels (*1,2*).

Filaggrin is now considered as a major predisposing gene for many atopic disorders, which result in a major paradigm in dermatology and allergy research (*3*). Many studies pointed out an association of the filaggrin gene with different atopic disorders. More specifically, loss-of-function mutations in the filaggrin gene have been reported to have an association with various atopic/allergic disorders (*3*). Batchelor *et al*., reported that there is a strong and consistent association between filaggrin mutations and development of AD (*4*), but

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an associations between filaggrin mutations with AR and BA are not pronounced (*3,4*). Currently, it is not fully known whether mutation in the filaggrin gene also effects its protein secretion in patients with BA, AD and AR, therefore, the present study was hypothesized to determine the role of production of filaggrin protein in hyperreactivity in these allergic patients. To prove this hypothesis, we determined the serum levels of filaggrin protein in patients with BA, AR, AD and also in those atopic patients, which were affected by multiple atopic disorders (mixed atopic patients). Not only have we measured these, we also determined the levels of IgE as a positive control for the allergic patients as studies have shown a well-defined association between serum IgE levels with allergic disorders (*5,6*). Bronchial hyperactivity has long been recognized as a hallmark of a number of allergic disorders but it's association with the dysfunctioning of mast cells and eosinophils is still not completely defined and remains controversial (*7,8*). We assumed that bronchial hyperactivity might have correlations with eosinophil's major basic protein (MBP) and leukotriene B4 (LTB4) in atopic disorders. Therefore, MBP and LTB4 were also estimated in these atopic patients to determine their roles in these allergic conditions.

## 2. Methods

### 2.1. *Human subjects*

This is a prospective case-control study, which enrolled AD, BA and AR individuals based on having a typical atopic picture according to recent guidelines described by Global Initiatives for Asthma (GINA) for BA (*9*), AR and its Impact on Asthma (ARIA) for AR (*10*) and SCORAD index for AD (*11*). The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in Tokyo 2004) for humans and was approved by National Plan for Science, Technology and Innovation of KSA (NSTIP # 11-BIO1459-09) and was also approved by institutional review board (IRB) of College of Medicine, Qassim University, KSA. Informed consent from all studied subjects was taken before sample collection. All studied atopic individuals were consecutively recruited from Outpatient Clinics affiliated to Qassim University (pulmonology, pediatric and dermatology Clinics). Out

of 1,246 atopic patients, BA (*n* = 445; age 38.1 ± 8.9), AR (*n* = 225; age 41.7 ± 13.9), AD (*n* = 216; age 25.6 ± 10.4), patients having mixed atopic disorders (*n* = 360; age 38.6 ± 11.4) were selected. Normal healthy humans (*n* = 410; age 39.13 ± 11.2) were selected and were used as controls. All selected control humans have no history of allergic disease. Venous blood samples from all studied subjects were taken and sera were stored at -80ºC until analyzed as described previously (*12-14*). Demographic details of all studied subjects are summarized in Table 1.

### 2.2. *Measurement of filaggrin, IgE, eosinophil's major basic protein, LTB4 in atopic patients*

Levels of filaggrin, IgE, MBP and LTB4 were measured in serum samples of all selected atopic patients and their levels were compared with normal healthy controls' sera. Serum filaggrin levels were measured by specific human filaggrin sandwich ELISA according to the manufacturers' instructions (cat. # SEJ103Hu, Cloud-Clone Corp., Hubei, PRC.). Whereas, IgE serum levels were measured by human IgE specific sandwich ELISA (cat. # 20783-72876, GenWay Biotech, CA, USA). Serum MBP and serum LTB4 levels were measured by human MBP and LTB4 specific sandwich ELISAs, respectively (cat. # SEB650Hu; cat. # CEA562Ge) according to their manufacturers' instructions (Cloud-Clone Corp., Hubei, PRC.).

### 2.3. *Statistical analysis*

Results are expressed as the mean+SEM unless stated otherwise. One-way ANOVA of variance followed by Tukey-Kramer multiple comparisons test, or Two-way ANOVA of variance followed by Bonferroni comparisons test. $p < 0.05$ was considered significant. All statistical analysis was carried out by Graph Pad Prism version 5.0 (Graph Pad Software Inc., San Diego, CA, USA).

## 3. Results

### 3.1. *Filaggrin in different atopic disorders*

In this study, we determined the serum levels of filaggrin in patients with different atopic disorders and their levels were compared with healthy human controls. The data

**Table 1. Demographic details of all studied subjects**

| No. | Subjects | Number (*n*) | Age (mean ± SD) | Sex (F/M) |
|-----|----------|--------------|-----------------|-----------|
| 1 | Bronchial Asthma | 445 | 38.1 ± 8.9 | 254 F/191 M |
| 2 | Atopic dermatitis | 216 | 25.6 ± 10.4 | 98 F/118 M |
| 3 | Allergic rhinitis | 225 | 41.7 ± 13.9 | 119 F/106 M |
| 4 | Mixed atopic disorders | 360 | 38.6 ± 11.4 | 178 F/182 M |
| 5 | Normal human controls | 410 | 39.1 ± 11.2 | 209 F/201 M |

SD, standard deviation; F, females; M, males; *n*, number.

showed a significant increase in serum filaggrin levels ($p < 0.001$) in 1,246 different atopic disorders patients compared with 410 healthy controls of the same age group. The average filaggrin levels (± SEM) in all studied atopic subjects and controls humans were 7.13 ± 0.09 and 2.09 ± 0.04 ng/mL, respectively (Figure 1A). More specifically, the average filaggrin levels (± SEM) in the patients sera with AD ($n$ = 216), AR ($n$ = 225), BA ($n$ = 445) and mixed atopic patients ($n$ = 360) were 8.74 ± 0.81, 6.51 ± 0.36, 6.96 ± 0.12, and 8.29 ± 0.33 ng/mL, respectively (Figure 1B). These results showed that filaggrin levels were significantly increased in AD patients as compared with AR or BA patients ($p < 0.05$), whereas patients with multiple atopic disorders had almost similar levels of filaggrin as AD patients ($p > 0.05$). Data of all tested serum proteins including filaggrin in BA, AD, AR and in patients with mixed atopic disorders are summarized in Table 2.
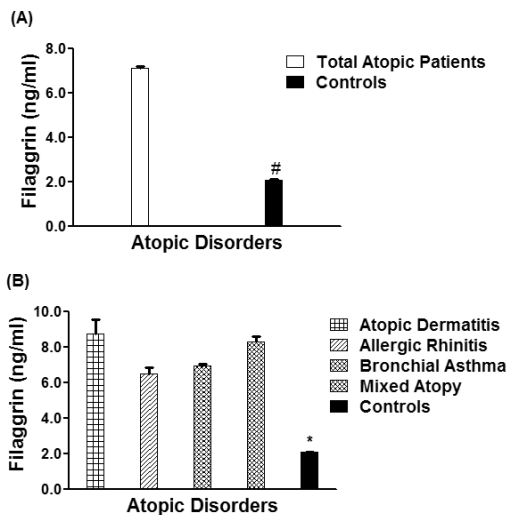
### 3.2. *Total IgE in different atopic disorders*

The serum levels of total IgE in patients with different atopic disorders ($n$ = 1,246) were found to be significantly higher as compared to healthy controls ($n$ = 410) ($p < 0.0001$). Average IgE levels (± SEM) in all studied atopic subjects and human controls were 68.9 ± 2.06 and 45.4 ± 1.98 IU/mL, respectively (Figure 2A). Specifically, the average IgE levels (± SEM) in the patients sera with AD ($n$ = 216), AR ($n$ = 225), BA ($n$ = 445) and mixed atopic patients ($n$ = 360) were 48.65 ± 10.6, 82.17 ± 6.50, 69.53 ± 2.07, and 74.04 ± 6.24 IU/mL, respectively (Figure 2B). Results also pointed out that IgE levels were significantly increased in AR patients as compared to AD or BA patients ($p < 0.05$), whereas patients with multiple atopic disorders had almost similar levels of IgE as AR patients ($p > 0.05$).

### 3.3. *Major basic protein in different atopic disorders*

The serum levels of MBP in patients with different



**Figure 1. Filaggrin in different atopic disorders. (A)** Levels of filaggrin in the sera of all studied atopic patients ($n$ = 1,246) and controls (410). *$p < 0.001$ *vs.* all atopic patients. **(B)** Levels of filaggrin in the patients' sera of atopic dermatitis ($n$ = 216), allergic rhinitis ($n$ = 225), bronchial asthma ($n$ = 445), mixed atopic patients (n = 360) and in controls' sera (n = 410). #$p < 0.0001$ *vs.* atopic dermatitis; #$p < 0.001$ *vs.* allergic rhinitis; #$p < 0.001$ *vs.* bronchial asthma; #$p < 0.0001$ versus mixed atopic patients. Each bar shows the mean ± SEM.
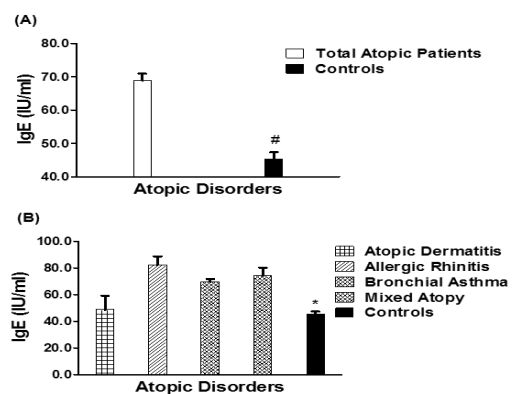


**Figure 2. IgE in different atopic disorders. (A)** Levels of IgE in the sera of all studied atopic patients ($n$ = 1,246) and controls (410). *$p < 0.001$ *vs.* all atopic patients. **(B)** Levels of IgE in the patients' sera of atopic dermatitis ($n$ = 216), allergic rhinitis ($n$ = 225), bronchial asthma ($n$ = 445), mixed atopic patients ($n$ = 360) and in controls' sera ($n$ = 410). #$p < 0.05$ *vs.* atopic dermatitis; #$p < 0.001$ *vs.* allergic rhinitis; #$p < 0.001$ *vs.* bronchial asthma; #$p < 0.0001$ versus mixed atopic patients. Each bar shows the mean ± SEM. IgE, immunoglobulin E.

**Table 2. Serum levels of filaggrin, eosinophil's MBP, LTB4 and IgE in all studied subjects**

| No. | Subjects | Number ($n$) | Filaggrin (ng/mL) | eMBP (ng/mL) | LTB4 (ng/mL) | IgE (IU/mL) |
|---|---|---|---|---|---|---|
| 1 | Bronchial Asthma | 445 | 6.96 ± 0.12[a] | 11.08 ± 0.29[f] | 10.21 ± 0.14[l] | 69.53 ± 2.07[q] |
| 2 | Atopic dermatitis | 216 | 8.74 ± 0.81[b] | 5.47 ± 1.16[g] | 7.12 ± 0.36[m] | 48.65 ± 10.60[r] |
| 3 | Allergic rhinitis | 225 | 6.51 ± 0.36[c] | 14.39 ± 0.92[h] | 9.94 ± 0.69[n] | 82.17 ± 6.50[s] |
| 4 | Mixed atopic disorders | 360 | 8.29 ± 0.33[d] | 9.51 ± 0.78[i] | 8.45 ± 0.29[o] | 74.04 ± 6.24[t] |
| 5 | Normal human controls | 410 | 2.09 ± 0.04[e] | 5.12 ± 0.19[k] | 13.05 ± 0.18[z] | 45.40 ± 1.98[u] |

Statistical significance among studied groups for filaggrin: [b]$p < 0.05$ *vs.* a or b; [d]$p < 0.05$ *vs.* a or b; [e]$p < 0.05$ *vs.* a, b, c or d. Statistical significance among studied groups for eMBP: [h]$p < 0.05$ *vs.* g, h, f or i. [k]$p < 0.05$ *vs.* f, g, h or i. Statistical significance among studied groups for LTB4: [l]$p < 0.05$ versus z; [m]$p < 0.05$ *vs.* z; [d]$p < 0.05$ *vs.* z; [o]$p < 0.05$ versus z. Statistical significance among studied groups for IgE: [s]$p < 0.05$ *vs.* q, r; [t]$p < 0.05$ *vs.* q or r; [u]$p < 0.05$ versus q, r, s or t. Data represented as mean ± SEM. *Abbreviations*: eMBP, eosinophil's major basic protein; LTB4, leukotriene B4; IgE, immunoglobulin E; $n$, number of samples tested.
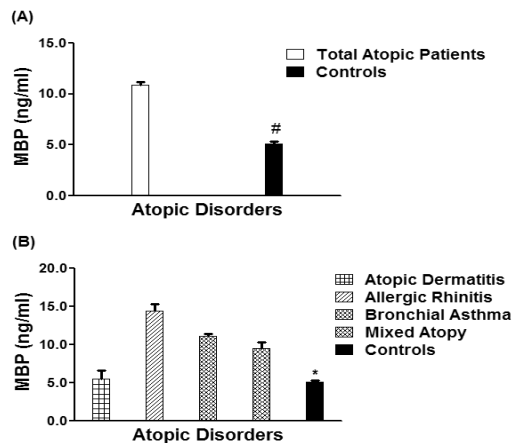
**Figure 3. Eosinophil's major basic protein different atopic disorders. (A)** Levels of major basic protein (MBP) in the sera of all studied atopic patients (*n* = 1,246) and controls (410). **p* < 0.001 *vs.* all atopic patients. **(B)** Levels of MBP in the patients' sera of atopic dermatitis (*n* = 216), allergic rhinitis (*n* = 225), bronchial asthma (*n* = 445), mixed atopic patients (*n* = 360) and in controls' sera (*n* = 410). [#]*p* < 0.05 *vs.* atopic dermatitis; [#]*p* < 0.0001 *vs.* allergic rhinitis; [#]*p* < 0.001 *vs.* bronchial asthma; [#]*p* < 0.001 *vs.* mixed atopic patients. Each bar shows the mean ± SEM.

**Figure 4. LTB4 in different atopic disorders. (A)** Levels of LTB4 in the sera of all studied atopic patients (*n* = 1,246) and controls (410). **p* < 0.001 *vs.* all atopic patients. **(B)** Levels of filaggrin in the patients' sera of atopic dermatitis (*n* = 216), allergic rhinitis (*n* = 225), bronchial asthma (*n* = 445), mixed atopic patients (*n* = 360) and in controls' sera (*n* = 410). [#]*p* < 0.0001 *vs.* atopic dermatitis; [#]*p* < 0.001 *vs.* allergic rhinitis; [#]*p* < 0.001 *vs.* bronchial asthma; [#]*p* < 0.0001 *vs.* mixed atopic patients. Each bar shows the mean ± SEM. LTB4, leukotriene B4.

atopic disorders (*n* = 1,246) were found to be significantly higher as compared with healthy controls (*n* = 410) (*p* < 0.01). The average MBP levels (± SEM) in all studied atopic subjects and human controls were 10.90 ± 0.27 and 5.12 ± 0.19 ng/mL, respectively (Figure 3A). Importantly, the average MBP levels (± SEM) in the patients sera with AD (*n* = 216), AR (*n* = 225), BA (*n* = 445) and mixed atopic patients (*n* = 360) were 5.47 ± 1.16, 14.39 ± 0.92, 11.08 ± 0.29, and 9.51 ± 0.78 ng/mL, respectively (Figure 3B). These results showed that MBP levels were significantly increased in AR patients as compared with AD, BA, or mixed atopic patients (*p* < 0.05). Moreover, results also indicated that MBP levels were not increased in AD patients as compared with the levels found in controls' sera (*p* > 0.05).

### 3.4. *LTB4 in different atopic disorders*

Serum levels of LTB4 in patients with different atopic disorders (*n* = 1,246) were found to be significantly low as compared with normal healthy controls (*n* = 410) (*p* < 0.001). The average LTB4 levels (± SEM) in all studied atopic subjects and human controls were 9.92 ± 0.13 and 13.05 ± 0.18 ng/mL, respectively (Figure 4A). Specifically, the average LTB4 levels (± SEM) in the patients sera with AD (*n* = 216), AR (*n* = 225), BA (*n* = 445) and mixed atopic patients (*n* = 360) were 7.12 ± 0.36, 9.94 ± 0.69, 10.21 ± 0.14, and 8.45 ± 0.29 ng/mL, respectively (Figure 4B). These results showed that LTB4 levels were almost similar in all tested atopic patients groups including AD, AR, BA and also mixed atopic patients, but were significantly low as compared with their respective controls (*p* < 0.05).
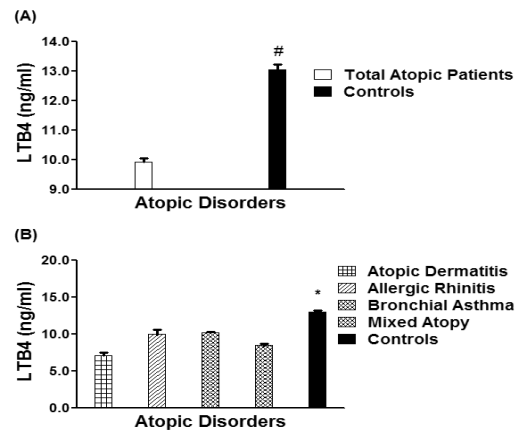
## 4. Discussion

This study demonstrated the role of filaggrin, IgE, eosinophil major basic protein, and LTB4 in patients with BA, AD, AR and in those patients, which had multiple atopic disorders. Filaggrin is a key structural protein required for the normal biogenesis and physiology of the stratum corneum (*15*). The findings of genetic variants in the gene encoding filaggrin in up to 50% of AD patients enhanced our understanding of the role of filaggrin in skin barrier defect, AD pathogenesis, and the subsequent progression along the atopic march (*16,17*). The atopic march concept describes the progression of atopic disorders from AD in infancy to AR and BA in childhood (*17*). It is now well documented that the mutations in the filaggrin gene are major risk factors for AD (*18,19*). Not only in patients with AD, mutations in the filaggrin gene also had significant association with BA and AR (*20,21*). In this study, we determined the protein levels of filaggrin in patients BA, AD, AR and patients with mixed atopic disorders and their levels were compared with normal healthy controls. The data showed a significant increase in serum filaggrin levels in 1,246 different atopic disorders patients compared with 410 healthy controls of the same age group. More specifically, filaggrin levels were significantly increased in AD patients as compared with AR or BA patients, whereas patients with multiple atopic disorders had almost similar levels of filaggrin as AD patients. These results indicated that filaggrin protein is clearly associated with almost all atopic disorders particularly with AD patients and with those patients, which have multiple disorders.

Atopy is defined as a personal or familial propensity to produce IgE antibodies and sensitization in response

to many factors particularly environmental triggers (*22*). The IgE sensitization and severity of many atopic disorders were well studied and connected with each other particularly for AD progression and BA persistence (*23,24*). Previously we also concluded that IgE is useful in evaluating the progression of AD and in elucidating the mechanisms of disease pathogenesis (*6*). In this study, we found that the serum levels of total IgE in patients with different atopic disorders were significantly higher as compared with healthy controls. Not only have we measured these, our data also pointed out that IgE levels were significantly increased in AR patients as compared with AD or BA patients, whereas patients with multiple atopic disorders had almost similar levels of IgE as AR patients, indicating that diagnostic values of IgE in serum are more important for AR patients and patients with mixed atopy as compared to AD or BA patients.

Eosinophils have a vital role in allergic inflammatory processes and MBP is present in the secretory granules of the eosinophil (*25*). Evidence implicates that the eosinophil and its granule proteins are assumed to mediate hypersensitivity disorders, as MBP-1 levels are elevated in sputum and bronchoalveolar lavage of BA patients (*25*). Studies have also shown that MBP-1 has a role in tissue damage as tissue damage is directly associated with eosinophil infiltration in BA (*25*). Morital *et al*. demonstrated that serum major basic protein is elevated in patients with AD (*26*). In addition, activated eosinophils and depositions of eosinophil granule proteins have also found in AD skin biopsies (*26*). Serum eosinophil cationic protein and eosinophil peroxidase is a sensitive indicator of the disease activity in AR (*27*). Serum eosinophil cationic protein and eosinophil peroxidase in patients with seasonal rhinitis demonstrated a high predictive ability for later development of BA (*28*). In view of these, it is important to know the protein level of MBP in patients with various atopic disorders, therefore in this study we determined the levels of MBP in the serum samples of AD, AR, BA and in those patients which have multiple atopic disorders. The serum levels of MBP in patients with different atopic disorders were found to be significantly increased and high as compared with healthy controls. Moreover, our results also pointed out that MBP levels were remarkably high in AR patients as compared with AD, BA, or mixed atopic patients. However, MBP levels were not increased in AD patients. These data indicate that MBP serum level has more value in the diagnosis of AR rather than AD.

LTB4 is a well-known mediator of leukocyte pathways involved in chemotactic properties of neutrophils, macrophages, monocytes, eosinophils, and dendritic cells (*29*). Studies have shown that LTB4 plays important roles in inflammatory and immune responses by activating phagocytic cells, differentiated T-cells, and dendritic cells (*29,30*). Dysfunction of LTB4 has been reported in various allergic disorders (*29,30*), therefore in this study we determined serum levels of LTB4 in patients with different atopic disorders and they were found to be significantly lower as compared with normal healthy controls. Specifically, our results also showed that LTB4 levels were almost similar in all tested atopic patients groups including AD, AR, BA and also mixed atopic patients, but were significantly lower as compared with their respective controls ($p < 0.05$). These data indicated that LTB4 might play a role in the pathogenesis of BA, AD and AR. As a whole, the present findings clearly suggest the roles of multiple proteins in the pathogenesis of BA, AR and AD. Our results are fully supported by numerous studies performed in different disorders including allergic disorders (*31-34*). In our previous studies we have also reported pathogenic effects of multiple proteins in patients with systemic lupus erythematosus (*35-37*). Moreover, studies have also shown dysfunction of multiple proteins in diabetes patients (*38-40*). Not only have these, inhibition of a wide array of enzyme activities have also been reported in the same group of patients (*41-43*). All these reports further strengthen our findings that the pathogenic effects can be generated by the abnormal behavior of multiple proteins rather than the involvement of a single protein. With the support of these studies, the findings from the present study in various atopic disorders strongly support an association between protein levels of filaggrin, MBP or LTB4 and AD, AR or BA. Our results suggest that filaggrin, IgE, MBP and LTB4 may be useful in elucidating the mechanisms of pathogenesis of these atopic disorders. In conclusion, our data clearly show that the levels of filaggrin, MBP and LTB4 were abnormal in patients with BA, AD, AR, and in those patients, which had multiple atopic disorders. These data clearly conclude that serum levels of filaggrin, MBP and LTB4 might be useful in the diagnosis of BA, AD and AR.

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## Brief Report

# Prune belly syndrome: Approaches to its diagnosis and management

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**Summary**  **Prune Belly syndrome (PBS) or Eagle-Barrett syndrome is an anatomo-radiological syndrome consisting of a complex and rare malformation characterized by the following triad of symptoms: deficiency of the abdominal muscles, malformations of the urinary tract, and bilateral cryptorchidism. The exact etiology is unknown, though PBS predominantly occurs in males. The clinical manifestations can vary widely, from stillbirth to renal and major respiratory dysplasia to almost normal children. The current study included a total of 3 patients. The findings included clinical characteristics, diagnostics, therapy, and clinical outcomes. All patients were diagnosed with congenital aplasia of the abdominal wall and a variety of urogenital malformations. Cryptorchidism and a mega-bladder were observed in 2 patients and distinctive renal malformations, such as renal dysplasia, were observed in 1 patient. Treatment varies but usually includes surgical management of symptoms. One patient required urgent urinary surgery; a vesicotomy was urgently performed due to anuria. These aspects explain the great diversity of opinions on the approach to this syndrome, but the severity of renal dysplasia is the main prognostic factor. Two newborns died a few days later due to severe renal failure. Despite these concerns, many patients with PBS report being in physical and mental health and having a good quality of life.**

*Keywords:* Prune belly, complex malformation, diagnosis, prognosis

## 1. Introduction

Prune belly Syndrome (PBS), also known as Eagle-Berrett syndrome, is an extremely rare congenital disorder (1 in 40,000 live births) that almost exclusively affects boys (*1,2*). This syndrome is defined by a characteristic clinical triad consisting of abdominal muscle deficiency, severe urinary tract abnormalities, and bilateral cryptorchidism in males. The term "prune-belly" reflects the characteristic wrinkled appearance of the abdominal wall in the newborn due to the complete or partial absence of abdominal wall muscles (*2*).

PBS is a complex malformation disorder with wide variability in severity and clinical manifestations. Its clinical manifestations vary from stillbirth due largely to major renal and respiratory dysplasia to an almost normal child (*2,3*). Its etiology has yet to be determined and its prognosis depends on renal involvement (*3,4*). Diagnosis of the syndrome should be considered during antenatal care with a thorough examination and regular prenatal follow-up.

The purpose of the current study was to describe the authors' clinical experience with children identified as having PBS and to describe the syndrome's clinical features, its diagnosis, and its treatment.

## 2. Patients and Methods

Three patients seen in the Neonatology Department of the Maternity and Neonatology Center between September 2015 and March 2016 were retrospectively examined. The following parameters were examined for each patient: age, sex, consanguinity between parents, reasons for admission, results of a physical examination,

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**Figure 1. AXR showing a flaccid and distended abdomen in PBS.**



**Figure 2. Hypoplasia of abdominal musculature with abdominal bloating.**



**Figure 3. Significant bilateral pyelocalyceal dilatation on renal ultrasound.**

paraclinical results (biological and radiological), and treatment (medical or surgical treatment).

2.1. *Case 1*

Baby E is a male born from a non-consanguineous marriage. The second twin of a bi-chorionic bi-amniotic twin pregnancy, Baby E. was delivered by caesarean section at 36 weeks due to breech presentation. There was no family history of genetic or congenital diseases. At birth, the newborn was admitted to this Department for severe bilateral uretero-pyelo-calyceal dilatation, diagnosed antenatally by ultrasound. An MRI was done at 32 weeks' gestation. The scan showed a megabladder, a small quantity of ascites, hypotonic intestinal loops, and a short femur. PBS was therefore considered. The twin was not affected.

At admission, the abdomen was bloated and hypoplasia of the abdominal musculature was evident. No abdominal masses were palpated and no obvious malformations, and especially those of the genitalia or spine, were noted. An abdominal x-ray (AXR) was performed upon admission and it showed a distended abdomen (Figure 1). An abdominal ultrasound was performed on day 2 of life, and it showed significant bladder distension associated with bilateral hydronephrosis and renal parenchymal dysplasia. Cranial sonography was normal.

The syndrome was progressively fatal. Anuria occurred on day 3, necessitating an emergency vesicotomy. Death occurred 7 days later on day 10 of life due to severe renal failure.

2.2. *Case 2*

Baby A is a male born from a non-consanguineous marriage. The pregnancy was uncomplicated. An antenatal ultrasound was done in the fifth month of pregnancy, and it showed dilatation of the entire urinary tract with thinning of the renal parenchyma. The newborn was delivered vaginally. The newborn experienced

respiratory distress, so it was admitted at hour 1 of life, and oxygen supplementation was required.

Upon admission, the newborn had an overdistended abdomen with hypoplasia of the abdominal musculature (Figure 2). Biological renal tests revealed abnormalities that caused severe renal failure, so an emergency abdominal ultrasound was performed. It showed significant bilateral urinary dilatation with renal parenchymal thinning and multicystic renal dysplasia (Figure 3).

Given the complexity of the urinary malformations and the poor renal prognosis, the newborn was entrusted to his parents and his chronic renal failure was managed conservatively. He died a few days later.

2.3. *Case 3*

Baby M is a male born from a first-degree consanguineous marriage. The newborn was delivered by emergency cesarean section at 32 weeks' gestation due to acute fetal distress and breech presentation. An antenatal ultrasound performed at 30 weeks of gestation revealed polyhydramnios, dilatation of the digestive tract suggesting a small intestinal atresia, and talipes equinovarus (clubfoot). Amniocentesis was normal. At birth, the newborn had respiratory distress. The abdomen was bloated, and the newborn had bilateral

**Figure 4. Abdominal bloating with bilateral cryptorchidism.**

cryptorchidism and clubfoot (Figure 4).

The newborn was admitted to manage respiratory distress related to hyaline membrane disease. Continuous positive airway pressure (CPAP) was administered, and the newborn's respiration subsequently improved. An abdominal ultrasound was performed on day 2 showing a moderate dilatation of the pyelocalyceal cavities. However, renal function was normal. A point worth noting is that the fetus was diagnosed with atresia of the small intestine antenatally, but that condition was ruled out due to the passage of flatus. Thus, feeding per os was gradually introduced.

When the baby was discharged, it was referred to Pediatric Surgery. Afterwards, contact with the family was lost.

### 3. Results and Discussion

All three newborns studied were males. Consanguinity was only reported in the case of Baby M (Case 3). Moreover, Baby E had an unaffected twin, so consanguinity seemed to not be the direct cause of PBS.

In the 3 patients studied, ultrasonographic abnormalities were identified prenatally, but PBS was considered prenatally in only one case (Case 1). The syndrome was diagnosed too late into the pregnancy, precluding termination. The other two cases were diagnosed postnatally based on clinical and ultrasonographic findings.

Clinical manifestations can vary. Indeed, ectopic testes may be missed, as was true in Case 1. A skeletal abnormality (clubfoot) was noted in Case 3. Progression of the syndrome is often unpredictable. Rapid deterioration of renal function was observed in Baby M (Case 3); a vesicotomy was urgently performed due to anuria. This was not true in Case 2, where abdominal ultrasound showed significant bilateral urinary dilatation with thinning of the renal parenchyma and multisystem renal dysplasia. Given the complexity of the urinary malformations and the poor renal prognosis, the syndrome was treated conservatively. Both infants

died a few days later due to severe renal failure.

PBS, also known as Eagle-Berrett Syndrome, is an extremely rare complex malformation with an estimated incidence of 1 in 30,000 to 50,000 births (*1-4*).

More than 95% of affected individuals are males (*1,3*). In fact, fewer than 30 cases of PBS in girls have been reported in the literature (*5,6*).

This pathology seems to be rarer nowadays as early antenatal ultrasound diagnosis is routinely performed, thus allowing termination of an undesired pregnancy (*3*). The etiology of PBS remains unclear. It does not seem to have a genetic basis. However, the syndrome's marked predominance in males suggests a possible genetic basis with autosomal recessive inheritance linked to sex (*4*). Consanguinity is not uncommon in patients with PBS, so familial factors may be involved (*2*).

PBS was first identified in 1950 by Eagle and Barrett (*5*) as a triad of congenital anomalies: deficient musculature in the anterior abdominal wall, urinary tract abnormalities, and bilateral cryptorchidism. The origin of these anomalies is not yet fully understood. Several theories have been formulated. According to one, those abnormalities are due to a prenatal obstruction of the urinary tract that leads to dilatation of the urinary tract and abdominal distension in the fetus, resulting in hypoplasia of the muscles of the abdominal wall and undescended testes (*1,5*). According to another theory, the underlying defect in PBS is abnormal mesoderm development secondary to defective migration or differentiation of the lateral mesoderm into the abdominal musculature or muscles of the urinary tract occurring between the sixth and tenth week of gestation (*3,5*). Aplasia of the abdominal musculature induces protrusion of the anterior abdominal wall, which gives the skin a crumpled appearance reminiscent of the skin of a "prune" and hence the name PBS.

Various urinary malformations have been observed and three clinical forms may occur (*3*): *i*) A non-viable oligoanuric form with severe renal dysplasia; *ii*) A severe form with significant renal dysplasia associated with megaureters, a megabladder, and progressive renal failure; and *iii*) A form with moderate renal dysplasia and dilatation of the urinary tract to an extent but a good prognosis.

Bilateral cryptorchidism completes the PBS triad. The causes of undescended testes are idiopathic (*3*). However, those clinical manifestations vary in terms of severity and when they develop. Indeed, ectopic testes may be missed in some cases along with abnormalities in the abdominal wall, which are typical of PBS (*3,5*).

Although PBS is defined as deficient musculature in the anterior abdominal wall, urinary tract abnormalities, and bilateral cryptorchidism, it is in fact a multisystem disease, with patients displaying cardiopulmonary, gastrointestinal, and musculoskeletal anomalies to a varying degree. Indeed, several cases have been reported in the literature. According to a study by

Routh *et al*. involving 133 newborns with PBS (*6*), pulmonary abnormalities were found in 58% of cases, and particularly pulmonary hypoplasia secondary to oligohydramnios or thoracic deformities. Cardiac abnormalities were also found in 25% of cases, as well as gastrointestinal (24%) and skeletal abnormalities (23%).

PBS should be diagnosed based on an antenatal ultrasound. This is of crucial importance since it would allow prompt management of newborns with the syndrome at birth, thus resulting in improved survival (*2,6*). Indeed, PBS can be diagnosed as early as 12 weeks of gestation (*6*). An antenatal diagnosis should be considered whenever the following ultrasound anomalies are evident: oligohydramnios, urinary abnormalities (dilatation of the urinary tract, a megabladder, mega-ureters, and hydronephrosis), and the absence of abdominal musculature.

However, the rarity of this condition means that it is identified only in certain settings, and it is clinically diagnosed when its characteristic features are identified postnatally (*1,6*). Patients with PBS have had a generally poor prognosis. Most patients die during the first days of life. The severity of renal dysplasia and urinary tract abnormalities and the presence of pulmonary hypoplasia are the two principal characteristics that determine the clinical manifestations of PBS and eventual outcomes for patients. Renal function can be assessed soon after birth by measuring plasma levels of urea and creatinine (*1,7*).

The management of PBS has yet to be specifically defined because of the paucity of data. There is still debate over the appropriate treatment for PBS in early childhood. Surgical treatment includes abdominoplasty, bilateral orchiopexy, and the treatment of urinary abnormalities. Surgery is usually performed around the age of 1 year (*1*). In addition to its esthetic benefits, abdominal wall reconstruction helps to improve respiratory function and facilitates defecation. Moreover, it has a greater impact on the improvement of bladder emptying than that of cystoplasty (*2,8*). Surgery is considered the only effective treatment for cryptorchidism (*2*). With current advances in reproductive medicine, undescended testes must be routinely repositioned to prevent infertility in patients.

The management of urinary malformations is still a subject of debate (*2*). Management must be tailored to each individual by striking a balance between early intervention and the undesired effects of treatments in order to improve survival and limit sequelae. The main goal of treatment should be to preserve the kidneys (*3*). In the event of rapid deterioration of renal function, emergency urinary diversion must be performed to allow consistent renal function, provide adequate urinary drainage, and avoid recurrent infections. Regardless of the treatment, the long-term renal prognosis is unclear. In fact, about 1/3 of survivors are said to develop renal failure secondary to their renal dysplasia, obstructive nephropathy, or recurrent pyelonephritis (*1*). When urinary malformations are evident, the key to successful treatment is a well-trained surgeon.

In conclusion, PBS is a complex and rare malformation that is predominant in males. Renal failure is the leading cause of death (*2*). This syndrome is essentially a problem of treatment. The complexity of urinary malformations makes conservative treatment an important part of the therapeutic arsenal. Surgical treatment of urinary malformations must be tailored to the particular case, along with when and how that surgery is performed. These malformations should be managed by an experienced team. Orchiopexy should be performed for undescended testicles in the neonatal period to increase the chances of paternity; the same is true for abdominoplasty because of its aesthetic and functional benefits. Finally, prenatal ultrasound diagnosis is vital since it allows better management of PBS at birth.

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## Case Report

# Ataxia with ocular apraxia type 2 not responding to 4-aminopyridine: A rare mutation in the *SETX* gene in a Saudi patient

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**Summary**        **Ataxia with ocular apraxia type 2 is an autosomal recessive disorder caused by a mutation in the senataxin (*SETX*) gene. The disease is characterized by early onset cerebellar ataxia, cerebellar atrophy, axonal sensorimotor neuropathy, oculomotor apraxia, and increased levels of α-fetoprotein. Reported here is a rare homozygous frameshift deletion c.5308_5311del, p.(Glu1770Ilefs\*15) in the *SETX* gene in a Saudi family. Ataxia with ocular apraxia type 2 was diagnosed based on the patient's history, an examination, and genetic testing. Genetic testing remains the only definitive method with which to identify the gene responsible. This is the third case report of this rare mutation in the literature. Ataxia with ocular apraxia type 2 continues to be a challenging disease to manage with no therapeutic options available to date. In the current case, the medication 4-aminopyridine was inefficacious in improving walking or balance. Further research is needed to identify potential treatments for this challenging condition.**

*Keywords: SETX, senataxin, ataxia with ocular apraxia type 2, rare mutation, Saudi Arabia*

## 1. Introduction

The inherited ataxias are a clinically and genetically heterogeneous group of diseases that occur due to dysfunction of the cerebellum and its connections (*1*). In most of these disorders, the disease develops between 30-50 years of age. Several mutations in a variety of genes have been identified as causing these ataxias (*2*). Ataxia with ocular apraxia type 2 is an autosomal recessive disorder caused by a mutation in the senataxin (*SETX*) gene. The disease typically develops between 10-25 years of age. The disease is characterized by early onset cerebellar ataxia, cerebellar atrophy, axonal sensorimotor neuropathy, and oculomotor apraxia. Other clinical features include abnormal movements such as chorea and dystonic posturing of the hands and head or postural tremors. Serum α-fetoprotein is elevated in more than 90% of affected individuals. Other possible biochemical changes include high levels of serum cholesterol and creatine kinase (*3*). A defective response to DNA damage has been noted in several autosomal recessive ataxias. Senataxin is a nuclear 2,667-amino acid protein involved in the response to DNA damage and RNA metabolism (*4*). The patient in the current case presented clinically with ataxia with ocular apraxia and elevated serum α-fetoprotein, and a molecular analysis was performed. Reported here is a rare homozygous frameshift deletion c.5308_5311del, p.(Glu1770Ilefs*15) in the *SETX* gene causing ataxia with ocular apraxia type 2 in a Saudi patient with no response to 4-aminopyridine therapy. To the extent known, this is the only reported case of this mutation in an Arab county.

## 2. Case Report

A 33-year-old Saudi woman presented with a history

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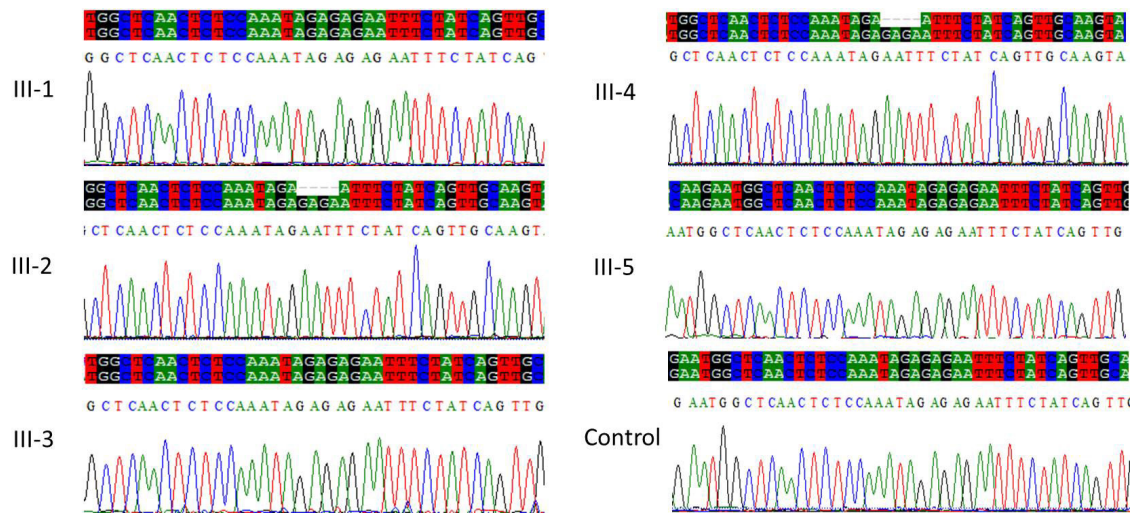E-mail: halgahtani@hotmail.com

**Figure 1. Sanger sequence analysis for validation of exome sequencing.** Probands III-2 and III-4 are affected family members who clearly exhibit a deletion of 4 bases from the *SETX* gene compared to the normal and wild-type control groups.

of gradually progressive ataxia, a lack of balance, frequent falls, and tremors in the trunk for many years. There was no history of weakness or sensory changes, a previous infection, a fever, a skin rash, mouth or genital ulcers, or photosensitivity. The patient denied having memory loss or psychiatric symptoms. Her past medical history was unremarkable and did not include endocrine disorders, infections, sleep disorders, or trauma. Her family history indicated a similar condition in her older sister. Her parents are first-degree relatives. She was not on any medications. On examination, the patient was conscious and oriented with normal cognitive functions. The patient had limited language skills and scanning speech. She was able to comprehend language, *i.e.* naming and repetition were intact, but she was unable to read or write. Her short-term memory was affected, but her long-term memory was normal. There was no telangiectasia in her mucous membranes or sclera, and a cranial nerve examination was normal. She had nystagmus in all directions, and a motor examination revealed decreased tone all over with 5/5 power. Hyporeflexia was noted. A cerebellar examination revealed dysmetria and dysdiadokinesia. She had difficulty walking without assistance. There were no associated skeletal deformities, and a fundus examination was normal. A gait examination revealed a wide-based gait with a tendency to fall to either side.

Extensive blood work, including both routine and specific tests to rule out the cause of ataxia in the patient's age group, was negative. These included vitamin E levels, vitamin B-12 levels, a blood smear for acanthocytes, viral serology, a thyroid function test, serology for celiac disease, and lipoprotein electrophoresis. Alpha-fetoprotein was elevated at 64.8 ng/mL (normal < 10 ng/mL). Genetic testing was performed and sequencing identified a homozygous frameshift deletion c.5308_5311del, p.(Glu1770Ilefs*15) in the *SETX* gene. Probands III-2

and III-4 are affected family members clearly exhibiting a deletion of 4 bases (Figure 1). This mutation was verified in 100 unrelated healthy persons, but none had this sequence variation. This variant has been identified in three heterozygotes in the Exome Aggregation Consortium (ExAC) dataset, which includes a total of over 60,000 unrelated individuals. The variant has four nucleotides deleted from exon 11/26 of the *SETX* gene. This causes a change from Glutamic acid to Isoleucine at amino acid residue 1,770/2,677 of the full-length protein and a shift in the reading frame that introduces a premature termination at codon 15 of the new reading frame. This is predicted to cause a lack of or abnormal protein function either through protein truncation or nonsense-mediated mRNA decay.

The patient was a heterozygous carrier of three missense novel mutations in *GBA2* gene c.2201G>A, p.(Arg734His), *SETX* c.5360A>T, p.(Tyr1787Phe), and *WDR81* c.2051A>C, p.(Gln684Pro). In addition, she was a heterozygous carrier of a novel mutation in *VAMP1* gene c.2+12C>T. The diseases caused by mutations in *GBA2* and *WDR81* are inherited in an autosomal recessive manner, while both autosomal recessive and dominant patterns have been identified with *SETX*, and the disease caused by mutations in *VAMP1* is inherited in an autosomal dominant manner.

This variant is classified as pathogenic in light of the current evidence (established association between the gene and the patient's phenotype, rarity in control populations, identification of the variant in a homozygous state in four related individuals with the same phenotype, proof of segregation, and mutation type (frameshift)). Ataxia caused by a mutation in *SETX* is inherited in an autosomal recessive manner. The patient is homozygous for the variant, which is in line with autosomal recessive inheritance.

A nerve conduction study and electromyography revealed a mixed axonal and demyelinating motor and
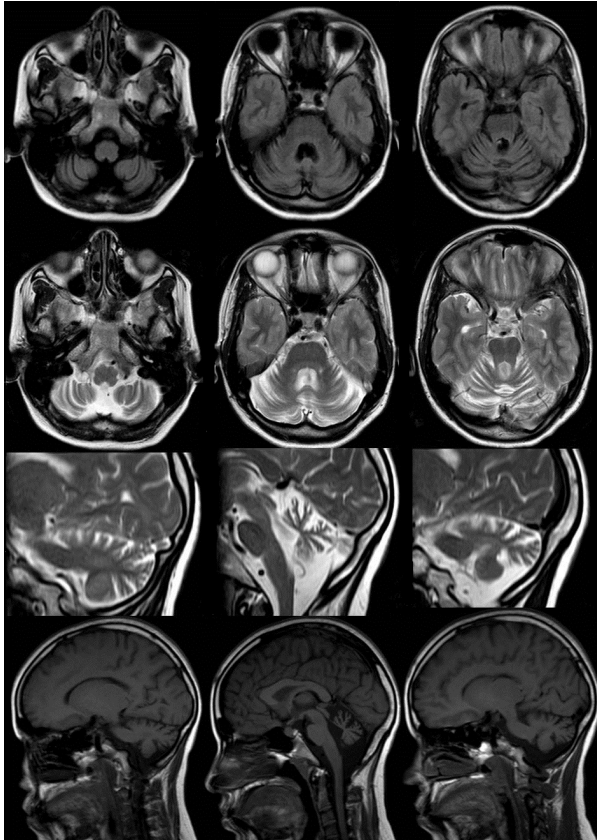
**Figure 2. MRI of the brain showing cerebellar atrophic changes with prominent cerebrospinal fluid spaces.**
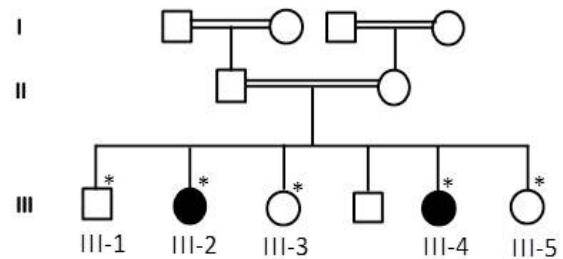


**Figure 3. A family pedigree was drawn after receiving detailed information from family members.** Individuals marked with asterisks were available for genetic testing.

sensory neuropathy. Magnetic resonance imaging (MRI) of the brain revealed cerebellar atrophic changes with prominent cerebrospinal fluid spaces (Figure 2).

A detailed family pedigree was drawn after obtaining detailed information from the parents, as shown in Figure 3. Family and genetic counseling were offered through a genetic consultant. The patient was tentatively given 4-aminopyridine for four months with no improvement. Pre-, periodic, and post-treatment assessment of gait consistently indicated a lack of response to this medication. The patient was last seen two months prior to publication of this report and is being followed up by the outpatient department at regular intervals every 4 months. The course of this disease is slow and progressive, and the patient is currently wheelchair-bound.

**3. Discussion**

SETX encodes senataxin, which is a 2,667-amino acid protein that contains a DNA/RNA helicase domain at its *C*-terminal end. The function of senataxin is poorly understood. It is believed to be involved in DNA repair, replication, recombination, and transcription, RNA processing, transcript stability, and translation initiation (*4*). In addition, *SETX* has been found to play a role in the defense against oxidative DNA damage (*5*).

Mutations in this gene have been associated with autosomal recessive ataxia with ocular apraxia type 2 and an autosomal dominant form of juvenile amyotrophic lateral sclerosis. Ataxia with ocular apraxia type 2 is considered to be the second most common autosomal recessive cerebellar ataxia after Friedreich ataxia (*6*). Of > 120 unique *SETX* mutations reported to date, different types including missense, nonsense, splice site, frameshift, and deletion/insertion mutations have been noted in patients with ataxia with ocular apraxia type 2. These mutations are presumed to silence the functioning of *SETX*, resulting in a recessively inherited disorder (*7*).

A review of the literature revealed two reports of an *SETX* c.5308_5311del, p.(Glu1770Ilefs*15) variant (*8,9*). One report described a Cypriot family with four affected members with autosomal recessive cerebellar ataxia. The c.5308_5311del variant was found to co-segregate with the disease in the family, and it was homozygous in all four affected members. The affected family members had an age of onset of 8-14 years, and they presented with clinical manifestations such as slow progressive ataxia of the limbs and trunk, eye movements with multidirectional nystagmus, peripheral neuropathy, spasticity, the Babinski sign, and areflexia (*8*). This variant was heterozygous in a singular patient with ataxia in combination with the *SETX* variant c.6547-1G>C variant (*9*). The previously reported cases are similar to the current case in terms of the age of onset, sex, clinical manifestations, disease severity, family history, and the method of genetic diagnosis. Minor differences in the current case were the race of the patient (Arab descent) and tentative treatment with 4-aminopyridine.

Ataxia with ocular apraxia type 2 is clinically characterized by a group of symptoms involving the cerebellum, oculomotor apparatus, and peripheral nervous system and other findings involving other parts of the neuraxis (*10*). Cerebellar symptoms include ataxia, gait difficulties, head or postural tremors, dysmetria, dysdiadochokinesia, and nystagmus. Oculomotor symptoms (present in approximately 50% of patients) include oculomotor apraxia, saccadic pursuit, gaze-evoked nystagmus, poor horizontal optokinetic nystagmus, and square-wave jerks.

Oculomotor apraxia is characterized by a dissociation between the movement of the eyes and head when the head is free, *i.e.* the head reaches the lateral target before the eyes. Peripheral nervous system involvement includes areflexia and subsequent peripheral axonal sensorimotor neuropathy. The disease is progresses slowly with no cardiac involvement, cancer predisposition, or immunodeficiency. Other clinical manifestations include pyramidal signs, a dystonic posture of the hands, choreic movements, and mild cognitive impairment (*11*).

Ataxia with ocular apraxia type 2 is diagnosed based on the patient's history, an examination, and genetic testing. A history and a physical examination are of paramount importance to diagnosing cerebellar ataxia, and this is especially true if other family members are affected. MRI of the brain is crucial to evaluating structural abnormalities such as cerebellar atrophy. Genetic testing remains the only method with which to definitively identify the gene responsible (*12*). The only biochemical marker that is typically found is elevated serum α-fetoprotein, but that finding is not specific to ataxia with ocular apraxia type 2. Electromyography typically reveals signs of axonal neuropathy. A nerve biopsy typically reveals chronic axonal neuropathy with preferential loss of large (and to a lesser degree small) myelinated fibers, but it is rarely performed (*13*).

Management of ataxia with ocular apraxia type 2 mainly includes occupational and physical therapy for gait dysfunction and speech therapy. There is currently no treatment that can cure the disease or alter its progression (*14*). 4-aminopyridine (prolonged-release fampridine) is a lipid-soluble selective potassium channel blocker that readily crosses the blood-brain barrier and that can improve walking in adult patients with multiple sclerosis. It acts on the surface of nerve fibers to reduce the leakage of ionic current from potassium channels in demyelinated axons, thereby inhibiting repolarization and prolonging the duration of action potentials, presumably allowing for more action potential propagation along the cell membrane. This medication is used off-label to improve walking in patients with hereditary ataxia, and it has had success in some patients. However, the current case suggests that this medication is not effective in the management of ataxia with ocular apraxia type 2. Patients with that condition have a varied prognosis, but improvement in their condition is unlikely (*15*).

Saudi Arabia has a high rate of consanguinity, which according to some studies ranges from 25% to 65% (*16*). This favors the occurrence of autosomal recessive diseases. Proper education emphasizing this fact should be offered to the Saudi community. Premarital or preimplantation genetic screening allows a genetic diagnosis early on and can help couples who carry the same disease-causing variants to make an informed decision regarding their marriage and the consequences

of their decision. The current authors have previously reported a wide variety of novel and rare genetic mutations causing a broad spectrum of diseases and clinical manifestations in the Saudi community (*17-24*). Molecular testing of potential carriers of those mutations may be urgently needed.

In conclusion, reported here is a rare homozygous frameshift deletion c.5308_5311del, p.(Glu1770Ilefs*15) in the *SETX* gene in a Saudi family. This is the third case report of this rare mutation in the literature. Ataxia with ocular apraxia type 2 continues to be a challenging disease to manage, with no therapeutic options available to date. In the current case, the medication 4-aminopyridine was inefficacious in improving walking or balance. Further research is needed to identify potential treatments for this challenging condition.

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# A case of leg cellulitis caused by multidrug-resistant *Streptococcus pseudoporcinus*

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**Summary**   **A 94-year-old woman was admitted to our hospital with a 5-day history of painful redness in the left lower leg. She was diagnosed with cellulitis and initiated antibiotic therapy with cefazolin. After two days, she presented with an extremely high fever (39.9°C), high C-reactive protein level (256 mg/L; normal, < 3), and signs of disseminated intravascular coagulation. In bacteriological examination, *Streptococcus pseudoporcinus* was detected from her lower leg wound purulence. An antibiogram revealed multidrug resistance except for cefepime, carbapenems, and vancomycin. We changed the antibiotics to cefepime and vancomycin according to the antibiogram and administered immunoglobulin concurrently. As the result of these therapies, her conditions gradually resolved over two weeks. *S. pseudoporcinus*, one of the β-hemolytic Streptococcus species recently described, has been isolated from the genitourinary tract of women. To our knowledge, this is the first case of cellulitis caused by *S. pseudoporcinus*. Typically, most antibiotics indicate adequate drug susceptibilities of *S. pseudoporcinus*, but in our case, multidrug resistance contributed to the prolonged duration of treatment. Because the colonization of *S. pseudoporcinus* in healthy individuals is not rare, it could become an important pathogen in elderly people and in those who have underlying medical conditions, as with other β-hemolytic Streptococci.**

*Keywords:* Soft tissue infections, streptococcus agalactiae, aged, antibacterial drug resistance

## 1. Introduction

*Streptococci* are gram-positive cocci in chain, which traditionally classified by hemolytic pattern on blood agar (α, partial hemolysis, resulting in greenish zone around colonies; β, complete lysis of erythrocytes; and γ, lack of visible hemolysis) and the use of Lancefield group antigens (*e.g.*, *Streptococcus pyogenes*, group A; *Streptococcus agalactiae*, group B) (*1*). Major human streptococcal pathogens belong to pyogenic group of β hemolytic *streptococci* and are classified as Lancefield groups A, B, C or G (*2*). In particular, *S. pyogenes*, commonly known as group A *Streptococcus* (GAS), can cause severe skin or invasive infections including necrotizing fasciitis and streptococcal toxic

shock syndrome (*3*). On the other hand, *S. agalactiae* called group B *Streptococcus* (GBS) is the common cause of neonatal sepsis and meningitis because of colonization in the pregnant women (*4*). *Streptococcus pseudoporcinus*, one of the β-hemolytic *Streptococcus* species recently described, has been also isolated from the genitourinary tract of women (*5*). The pathogenicity of *S. pseudoporcinus* remains unknown, except for causing obstetric disorders such as chorioamnionitis and preterm delivery (*6,7*). Previous report described skin infections related with by *S. pseudoporcinus* is only a few, so far (*8*). To our knowledge, this is the first case of cellulitis caused by *S. pseudoporcinus*.

## 2. Case Report

A 94-year-old woman visited to our hospital with a 5-day history of painful redness in the left lower leg. Physical examination revealed diffuse edema and redness of her lower leg with a high fever (38.5°C) (Figure 1A). She had a long history of foot tinea and

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**Figure 1. (A) Swelling and redness of the left lower leg on admission. (B) After total 4 weeks treatments, the symptoms were cured with scarring.**

**Table 1. Drug susceptibility results**

| Antimicrobial agent | MIC[a] (μg/mL) | Susceptibility[b] |
|---|---|---|
| Penicillin | 0.5 | R |
| Ampicillin | 1 | R |
| Ceftriaxone | 2 | R |
| Cefepime | 0.5 | S |
| Meropenem | 0.12 | S |
| Vancomycin | ≤ 0.25 | S |
| Azithromycin | 2 | R |
| Clarithromycin | > 4 | R |
| Levofloxacin | > 4 | R |
| Clindamycin | > 0.5 | R |

[a]MIC, minimum inhibitory concentration. [b]The Clinical & Laboratory Standards Institute (CLSI) interpretive standards for *Streptococcus* spp. β-Hemolytic Group were used.

stasis dermatitis, but no underlying medical problems, except for hypertension. Laboratory examinations revealed moderate elevation of C-reactive protein (CRP; 24 mg/L; normal, < 3). Computed tomography showed no abscesses or abnormal air patterns in the subcutaneous tissue. She was diagnosed with cellulitis based on the above findings and hospitalized our hospital to initiate antibiotic therapy (cefazolin), immediately. After two days, she presented with an extremely high fever (39.9°C), high CRP level (256 mg/L), and signs of disseminated intravascular coagulation (white blood cell count, $10.5 \times 10^9$/L; platelet count, $95 \times 10^9$/L; prothrombin time/international normalized ratio, 1.18; fibrin degradation products, 20.7 mg/L). In bacteriological examination using VITEK® 2 compact (bioMérieux), *S. pseudoporcinus* was detected by a swab culture from her lower leg wound purulence. An antibiogram revealed multidrug-resistance except for cefepime, meropenem, and vancomycin (Table 1). We changed cefazolin to meropenem according to

the antibiogram and administered immunoglobulin concurrently. Because her symptoms persisted despite receiving treatment, after one week, we changed the antibiotics to cefepime and vancomycin. As the result of these therapies, her conditions gradually resolved over two weeks (Figure 1B). Because of disuse syndrome, she was transferred to another rehabilitation hospital. At follow-up after three months, she remains free of symptoms.

## 3. Discussion

*S. pseudoporcinus* is a β-hemolytic gram-positive coccus that was identified as pyogenic *Streptococcus* in 2006 (*5*). Because *S. pseudoporcinus* often exhibits cross-reactivity with standard GBS antigen agglutination kits and is normally isolated from the female genitourinary tract, it could be confused with GBS (*7*). Stoner *et al.* (*9*) reported that 5.4% of women had genital cultures that were positive for *S. pseudoporcinus*, which suggests that the colonization of *S. pseudoporcinus* in healthy individuals is not rare. Typically, except for tetracycline, most antibiotics including β-lactam antibiotics, vancomycin, clindamycin, macrolides and fluoroquinolones indicate adequate drug susceptibilities of *S. pseudoporcinus* (*8,10*). In a previous study, a patient with *S. pseudoporcinus* isolated from a skin wound was cured promptly with cephalexin alone (*8*). However, in our case, multidrug resistance in addition to tetracycline contributed to the prolonged duration of treatment. Some GBS with multidrug resistance have been described, especially in Japan (*11*); furthermore, it has been recognized as an important pathogen in elderly people and in those who have underlying medical conditions (*12*). β-lactam resistance in GBS is reportedly due to multiple amino acid substitutions found in some penicillin-binding proteins (13). It is unknown what caused multidrug resistance in our case, however, since *S. pseudoporcinus* has some microbiological similarities to GBS, it may be likely to acquire multidrug resistance similarly.

In conclusion, we described the first case of cellulitis caused by *S. pseudoporcinus*. Owing to multidrug resistance and her advanced age, we took more time to treatment in this case. Therefore, we should keep in mind that *S. pseudoporcinus* could emerge as a serious medical problem in the near future as with other β-hemolytic *streptococci*.

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## Case Report

# Intensive care unit-acquired complicated necrotizing pneumonia caused by *Enterobacter cloacae*: A case report

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**Summary**    A 58-year-old man with a history of diabetes mellitus and end-stage renal disease acquired pneumonia with acute respiratory failure during his stay in an intensive care unit (ICU). Empirical antimicrobial therapy with ceftazidime and vancomycin was initiated, and imipenem replaced ceftazidime 2 days later due to the patient's pulmonary condition failed to improve. However, within 5 days, pulmonary consolidation rapidly progressed to necrotizing pneumonia complicated by lung abscess, empyema, pyopneumothorax, and tension pneumothorax, leading to the patient's death. After the patient had died, all bacterial isolates from cultures of pleural effusion, blood, and tracheal aspirate were identified as *Enterobacter cloacae* (*E. cloacae*), which was susceptible to imipenem but resistant to ceftazidime. *E. cloacae* should be considered in the differential diagnosis of complicated necrotizing pneumonia with lung abscess, empyema, pyopneumothorax, and tension pneumothorax. Carbapenem therapy should be immediately initiated until the pathogen in such rapidly progressive ICU-acquired pneumonia is confirmed. Increased awareness among physicians regarding *E. cloacae*-induced complicated necrotizing pneumonia acquired in ICUs could enable earlier detection and appropriate antimicrobial therapy for this invasive disease.

*Keywords:* Enterobacter cloacae, necrotizing pneumonia, lung abscess, pyopneumothorax, intensive care unit

## 1. Introduction

*Enterobacter cloacae* (*E. cloacae*) is one of the most frequently isolated and virulent human pathogens in its genus, and is increasingly significant as an intensive care unit (ICU)-acquired pathogen (*1,2*). This microorganism can cause a wide variety of infections, including bacteremia, soft tissue and ophthalmic infections (*1*), pneumonia (*2*), endocarditis (*3*), urinary tract infections (*4*), central nervous system infections (*5*), and osteomyelitis (*6*). Two cases of necrotizing pneumonia caused by *E. cloacae* were also reported

(*7,8*). *E. cloacae* has emerged as a nosocomial pathogen from intensive care patients, especially who are on mechanical ventilation (*9*). However, no cases of *E. cloacae*-induced complicated necrotizing pneumonia with lung abscess, empyema, pyopneumothorax, and tension pneumothorax occurring simultaneously in an ICU has been reported. This paper reports an intractable case of ICU-acquired *E. cloacae* as the cause of fatal complicated necrotizing pneumonia. Ceftazidime or piperacillin–tazobactam is usually used empirically to treat ICU-acquired pneumonia. However, the increasing prevalence of extended-spectrum-beta-lactamase-producing *E. cloacae* is becoming a concern, not only for infection therapy and empirical use of antibiotics but also for infection control programs (*10*). This case also highlights the importance of choosing appropriate empirical antimicrobial therapy for *E. cloacae*-induced complicated necrotizing pneumonia in ICUs.

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## 2. Case Report

A 58-year-old man with a history of diabetes mellitus and end-stage renal disease was admitted to an ICU of Tainan Municipal Hospital in March 2017 for hyperkalemia (serum potassium level: 7.06 mmol/L) resulting in bradycardia, shock, and acute respiratory failure. After cardiopulmonary cerebral resuscitation, endotracheal intubation with ventilator support, vasopressor therapy, and emergent hemodialysis, the patient's general condition gradually improved and he regained consciousness on day 3. He was successfully weaned from the ventilator and the endotracheal tube was removed on day 5.

However, the patient subsequently developed shortness of breath that progressed to respiratory distress on day 7. Arterial blood gas (ABG) analysis revealed a pH of 7.287, $PaCO_2$ level of 58.5 mmHg, $PaO_2$ level of 113.8 mmHg, and bicarbonate concentration of 27 mmol/L while the patient was breathing through a non-rebreathing mask. He underwent secondary endotracheal intubation with ventilator support for acute hypercapnia respiratory failure. Laboratory examinations yielded a white blood cell count of 14,950/µL with 84% neutrophils; a platelet count of 92,000/µL; and a C-reactive protein level of 6.28 mg/dL (reference range: < 0.3 mg/dL). A chest radiograph (Figure 1A) revealed bilateral consolidation, suggesting pneumonia. Empirical broad-spectrum antimicrobial therapy consisting of ceftazidime (2 g every 8 hours) and vancomycin (1 g every 12 hours) was immediately initiated. The patient's pulmonary condition failed to improve within the first 2 days (days 8 and 9) of antimicrobial therapy. Imipenem (500 mg every 6 hours) replaced ceftazidime on day 10. However, tachypnea, respiratory distress, decreased consciousness, and shock occurred on day 11. A physical examination revealed absent breath sounds on the right side, subcutaneous crepitus over the right chest wall, and a distended abdomen. Under the impression of tension pneumothorax, a needle was promptly inserted into the right upper intercostal space for chest decompression. A large amount of air immediately flowed from the needle, thereby increasing the patient's blood pressure. ABG analysis revealed a pH of 7.081, $PaCO_2$ level of 83.4 mmHg, $PaO_2$ level of 130.4 mmHg, and bicarbonate concentration of 24.2 mmol/L while the patient was breathing 100% $FiO_2$. Laboratory examinations yielded a white blood cell count of 19,320/µL with 88% neutrophils; a platelet count of 79,000/µL; and a serum potassium level of 3.89 mmol/L. A chest radiograph (Figure 1B) revealed cavitation in the consolidation of the right lower and left upper lobes. Moreover, subcutaneous emphysema was observed. A right chest tube thoracostomy was promptly performed, and air and 500 mL of a foul-smelling reddish pleural effusion were drained. The patient's right pleural effusion was cloudy and contained 7 mg/dL glucose (serum glucose:

180 mg/dL), 4.6 mg/dL protein (serum protein: 5.4 mg/dL), 18,720 U/L lactate dehydrogenase (serum lactate dehydrogenase: 510 U/L), 100,000/µL red blood cells, and 80,000/µL white blood cells with 87 % neutrophils. Pleural effusion, blood, and tracheal aspirate cultures were performed. Computed tomography (CT; Figure 2) revealed necrotizing pneumonia, lung abscess, pyopneumothorax, pneumoperitoneum, and subcutaneous emphysema. The patient's condition rapidly deteriorated and he died on day 12.

Bacterial isolates were identified using the BD Phoenix automated microbiology system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Antimicrobial susceptibility was tested using the disc diffusion method. Interpretation was performed according to the criteria of the Clinical and Laboratory Standards Institute (*11*). After the patient had died, all bacterial isolates were identified as *E. cloacae*, and the results of antibiotic susceptibility testing indicated susceptibility to imipenem, levofloxacin, aminoglycosides, and tigecycline but resistance to ceftriaxone, ceftazidime, cefmetazole, trimethoprim-sulfamethoxazole (TMP-SMX), and ampicillin-sulbactam.

## 3. Discussion

We searched PubMed Advanced Search Builder for papers published in English until 30 September 2018 using the following terms: ("*Enterobacter cloacae*" [All Fields] AND ("necrotizing pneumonia" [All Fields]). Of the 5 results, 3 were not relevant. Of the rest 2 results, necrotizing pneumonia caused by *E. cloacae* was reported in an adult and a neonate, respectively (*7,8*). According to our review of relevant studies, this case report is the first to describe *E. cloacae* as the cause of life-threatening pulmonary complications including necrotizing pneumonia, lung abscess, empyema, pyopneumothorax, and tension pneumothorax occurring simultaneously in an ICU. In our case, the patient likely acquired an *E. cloacae* infection through the respiratory tract during his ICU stay because of his underlying immunocompromised status. Common organisms associated with necrotizing pneumonia include *Streptococcus pneumoniae*, *Staphylococcus aureus* (*S. aureus*), methicillin-resistant *S. aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (*12*). *E. cloacae* should be considered in the differential diagnosis of complicated necrotizing pneumonia with lung abscess, empyema, pyopneumothorax, and tension pneumothorax. *E. cloacae* is predictably resistant to ampicillin and cefoxitin; however, aminoglycosides and ciprofloxacin are active against most strains of *E. cloacae*, whereas TMP-SMX has variable activity (*1*). Although ceftazidime or piperacillin-tazobactam is usually used empirically to treat ICU-acquired pneumonia, these are not appropriate antimicrobial therapies for ICU-acquired complicated necrotizing
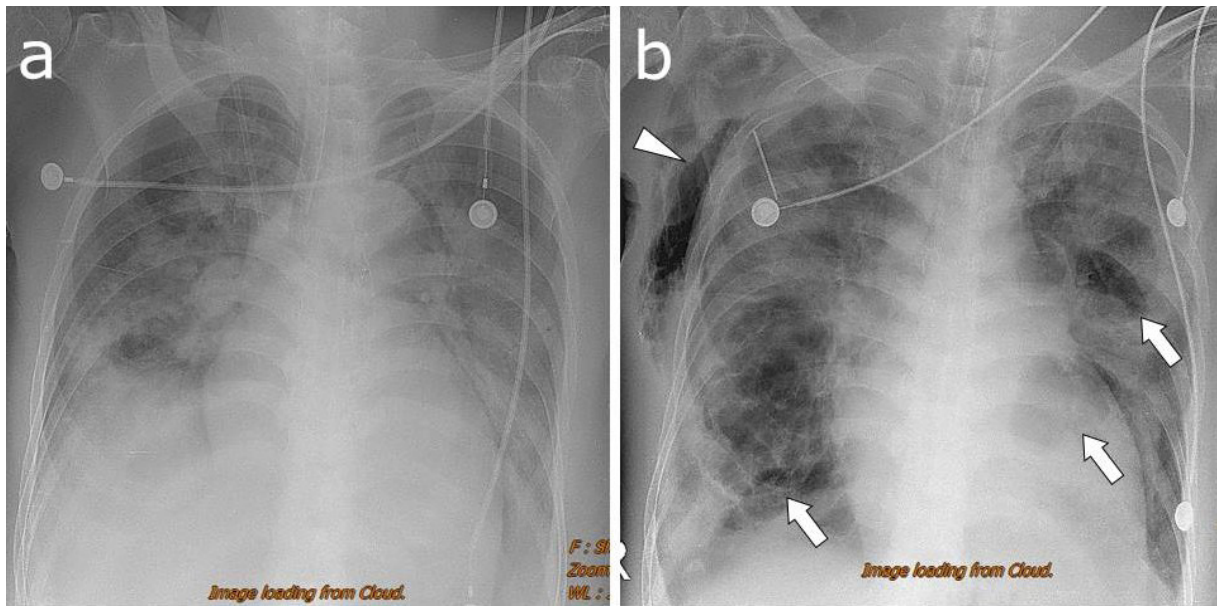
**Figure 1. (A) Chest radiography on hospital day 7 reveals a diffuse consolidation of the left lung and patchy areas of the consolidation in the right lung. (B) Radiography on hospital day 12 shows multiple cavities of variable sizes within the consolidation of the bilateral lungs (arrows). Subcutaneous emphysema of the right chest can be observed (arrowhead). A needle is inserted in the right upper intercostal space.**
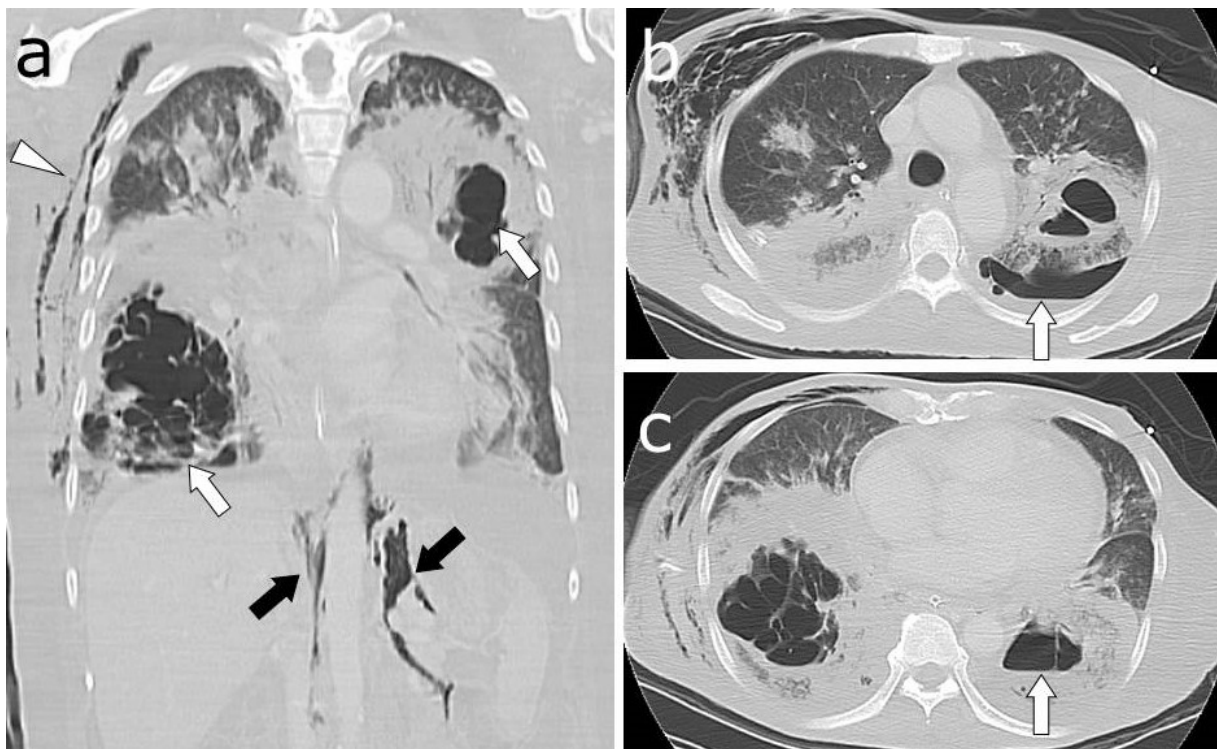


**Figure 2 (A) Lung window of a CT shows necrotizing pneumonia in the right lower and left upper lobes (white arrows). Subcutaneous emphysema (arrowhead) and pneumoperitoneum (black arrows) can be observed. (B) An air-fluid level is present in the left pleural space (arrow), representing pyopneumothorax. (C) A cavity contains an air-fluid level within the left lower lobe consolidation (arrow), suggesting lung abscess. CT, computed tomography.**

pneumonia caused by *E. cloacae*. *E. cloacae* strains that possess chromosomally encoded ampC β-lactamases can develop resistance to broad-spectrum cephalosporins (*13*). In addition, *E. cloacae* strains with extended-spectrum β-lactamases are increasingly described worldwide (*14*), and the emergence of greater resistance to carbapenems has become a global concern (*15*). However, carbapenems remain among the most active β-lactam antibiotics used to fight *E. cloacae* (*1*). In our case, the patient's underlying

immunocompromised status was the major cause of his death. Early initiation of carbapenems therapy in patients with underlying immunocompromised status could exert an appropriate therapeutic effect, thereby yielding an improved prognosis. Therefore, carbapenems therapy should be immediately initiated until the pathogen in such rapidly progressive ICU-acquired pneumonia is confirmed.

The true pathogenesis of *E. cloacae*-induced complicated necrotizing pneumonia remains unclear. *E. cloacae* is known to produce enterotoxins, α-hemolysin and thiol-activated pore-forming cytotoxins similar to the Panton-Valentine leukocidin produced by *S. aureus*, which causes leukocyte destruction and tissue necrosis (*16,17*). Thus, the potential pathogenesis of complicated necrotizing pneumonia is associated with pore-forming cytotoxins produced by *E. cloacae*.

In this case, although necrotizing pneumonia could be diagnosed using a plain radiograph, the degree of parenchymal destruction was underestimated. Moreover, detection of other pulmonary complications including empyema, lung abscess, and pyopneumothorax on a plain radiograph was difficult. Therefore, CT should be employed immediately after plain radiography if a patient's condition progressively deteriorates to confirm the diagnosis and assess parenchymal complications that are not evident on the plain radiograph.

In conclusion, *E. cloacae* should be considered in the differential diagnosis of complicated necrotizing pneumonia with lung abscess, empyema, pyopneumothorax, and tension pneumothorax acquired in an ICU. Empirical carbapenems therapy should be immediately initiated until the pathogen in rapidly progressive pneumonia is confirmed. Increased awareness among physicians regarding *E. cloacae*-induced complicated necrotizing pneumonia acquired in ICUs could enable earlier detection and appropriate antimicrobial therapy for this invasive disease.

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# Successful percutaneous treatment of coronary steal syndrome with the amplatzer vascular plug 4 and coil embolization

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**Summary**     The left internal mammary artery (LIMA) is widely used in coronary artery bypass grafting surgery due to its long term perfect patency rates. However, coronary steal syndrome can occur because of unligated LIMA side branches and it causes blood flow from coronary artery to LIMA. Even though the optimal therapy of coronary steal syndrome is still controversial, some percutaneous and surgical treatment modalities can be used in the treatment of steal phenomenon for relieving angina and resolving ischemia. It was demonstrated that percutaneous treatments such as the use of gelatin sponge particles or drug-eluting stents with covered stent, and coil and vascular plug embolization were used to treat this phenomenon successfully. Several studies revealed that these percutaneous treatments can reduce the ischemic area and results in prevention of blood flow from coronary artery to LIMA side branches. Supporting these findings, we herein present a 48-year-old male patient with objective ischemia with coronary steal syndrome treated successfully with the Amplatzer vascular plug (AVP) 4 and coil embolization in the same procedure. To the best of our knowledge, the combined therapy has not been described in the literature yet. Supporting the literature findings, successful treatment of LIMA side branches in our case with two different percutaneous modalities results in improvement of coronary flow and a reduced ischemic area and angina.

*Keywords:* Coil embolization, coronary steal syndrome, left internal mammary artery, vascular plug embolization

## 1. Introduction

The left internal mammary artery (LIMA) is widely used in coronary artery bypass grafting (CABG) surgery due to its long term perfect patency rates and having better clinical outcomes than saphenous grafts (*1*). Despite being an excellent conduit, unligated side branches of the LIMA can cause coronary ischemia due to coronary steal syndrome. Although the benefit of treatment of unligated side branches is shown, optimal

therapy for coronary steal syndrome is controversial. Several methods have been described to treat coronary steal syndrome with perfect clinical benefits such as coil (*2*) and vascular plug embolization (*3*), the use of gelatin sponge particles or drug-eluting stents with covered stent (*4*) and surgical ligation of side branches (*5*). It was demonstrated that occlusion of unligated side branches is related to ischemia and angina regression (*6*). However, in a study coronary steal syndrome was evaluated with coronary flow measurements at rest or following adenosine hyperemia and left arm exercise, and there was not any clear evidence of true coronary steal phenomenon (*7*). Even though mentioned methods have been used in the treatment of coronary steal syndrome successfully, to the best of our knowledge using two different percutaneous treatments together in the same procedure has not been described in the literature yet. We herein present a case of successful

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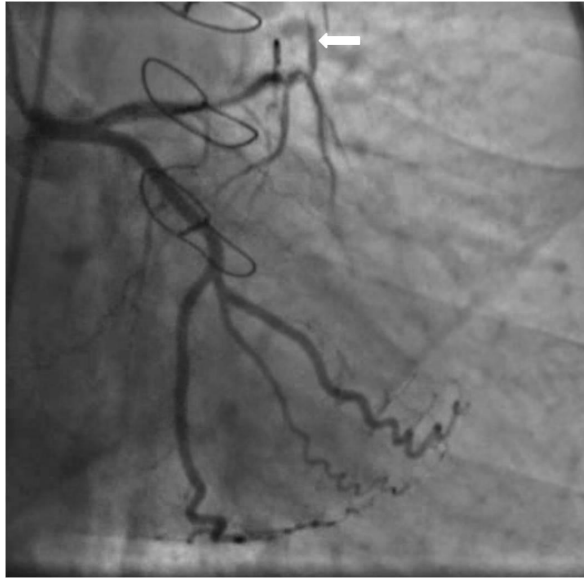E-mail: serkankahraman_86@outlook.com

**Figure 1. Coronary steal syndrome.** It was demonstrated by coronary angiography with arterial blood flow from LAD to LIMA (arrowhead).



**Figure 2. Percutaneous treatment of coronary steal syndrome. (A)**, Coronary angiography revealed that LIMA had two separate unligated side branches (arrowheads); **(B)**, The 4x10mm Amplatzer vascular plug 4 was released into the proximal side branch; **(C)**, The 2 × 3 mm coil was released into the small caliber side branch; **(D)**, The procedure was terminated showing complete flow cessations into the mentioned arteries (arrowheads).

percutaneous treatment of coronary steal syndrome with the Amplatzer vascular plug (AVP) 4 and coil embolization in the same procedure.

## 2. Case Report

A 48-year-old male patient was admitted to department of cardiology one year ago with worsening angina that had been ongoing for 2 months. He had undergone a coronary artery bypass surgery 10 years ago and percutaneous coronary intervention to the left anterior descending (LAD) artery 3 years ago. He had no property on physical examination. Electrocardiography indicated normal sinus rhythm with anterior T wave negativity. Echocardiography imaging revealed apical hypokinesia with a normal ejection fraction. Myocardial perfusion scintigraphy showed extensive ischemia in the anterior wall and then coronary angiography was performed. It was shown that there was an instent restenosis into the proximal LAD artery before surgical anastomosis site and the LIMA-LAD grafting was patent. However, coronary steal syndrome was detected due to unligated LIMA side branches from LAD to the LIMA artery (Figure 1). Also there was no significant lesion in the left circumflex artery or the right coronary artery. The patient was evaluated by our cardiology and cardiovascular council and percutaneous treatment of unligated LIMA side branches was planned. Digital subtraction angiography was performed *via* the right common femoral artery using 7-French (F) femoral sheath. LIMA was selectively cannulated with an internal mammary artery (IMA) 7F guiding catheter (Medtronic, New York, NY). It was demonstrated that LIMA had two separate side branches (Figure 2A). The large branch arose from the proximal LIMA and the
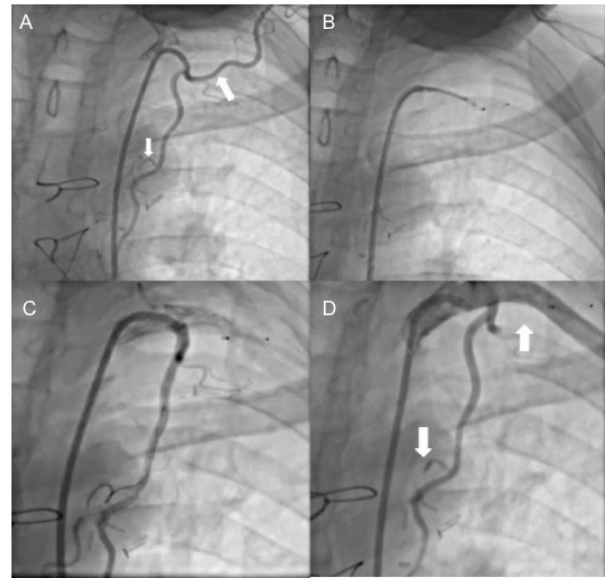
small one arose from the mid LIMA. The proximal side branch was selectively cannulated with a Judkins Right (JR) 5F guiding catheter (Medtronic, New York, NY) through the IMA guiding catheter. The 4 × 10 mm AVP 4 was released into the proximal side branch *via* JR 5F guiding catheter and flow was ceased (Figure 2B). Then, a 0.014" wire (Fielder XT, Asahi Intecc) was passed into the mid side branch. The microcatheter (Codman Prowler, Johnson&Johnson) was then advanced over the wire into the side branch. The 2 × 3 mm coil was released into the side branch *via* microcatheter (Figure 2C). The procedure was terminated showing complete flow cessations into the mentioned arteries (Figure 2D). The patient was discharged from the hospital 2 days later after intervention with no symptoms. The patient follow-up visits were done every 3 months. He is still being followed-up without any symptoms and also myocardial perfusion scintigraphy showed regression of ischemia.

## 3. Discussion

LIMA is still preferred as a graft for surgical myocardial revascularization with an excellent long term patency (*1*). However, some factors can cause acute or long term graft malfunction. Intraoperative technical problems, spasm or stretch of the graft are related to acute graft malfunction. Contrary to the mentioned reasons, progression of atherosclerosis, kinking of graft, competitive flow and steal due to unligated side branches are some of the factors to determine long

term graft patency. Although sternal, intercostal and perforating branches of the LIMA are small caliber arteries and generally do not create ischemia, the lateral internal thoracic artery is the large side branch of the LIMA with a prevalence of 10% in the main population and is found to be associated with clinically evident ischemia (*8*). Thus, unligated side branches of the LIMA are potential facilitative factors for coronary ischemia. Obese patients and patients treated with minimal invasive CABG surgery are having low success rates for ligation of LIMA side branches (*9,10*). In the literature analysis, ischemia is observed more commonly in the myocardial perfusion scintigraphy in these patients having unligated LIMA side branches (*11*). Conversely, Guzon *et al*. demonstrated that there was no clear evidence of ischemia in patients with coronary steal syndrome (*7*).

The optimal treatment of coronary steal syndrome due to unligated side branches of the LIMA is still controversial. Percutaneous or surgical treatment can be performed in patients with progressive worsening angina that is clearly related to competitive run off into the side branches and having objective evidence of ischemia. It was demonstrated that generally anginal symptoms improve and ischemia resolves after occlusion of LIMA side branches (*6*). Percutaneous treatment options such as stent grafts, coil and vascular plug embolization have some advantages and disadvantages. The use of drug-eluting stents with covered stent seems to have high success rates. However, high long term restenosis rates with an incidence of 31.6% is the main disadvantage of the procedure (*12*). Coil embolization is a safe and effective modality having a rapid occlusion time. While coil embolization is a preferable method, it cannot be a suitable option to treat large caliber vessels. The distance between the coil and exit of the vessel, the number of coils used and the coil diameters and features are the responsible factors for recanalization after occlusion of LIMA side branches. The AVP 4 is a self-expanding Nitinol mesh occlusion device having the ability to safely remove the problem. The AVP device has some advantages such as ease of delivery, wide range of device sizes, shorter operation time, lower radiation rates and lower risk of recanalization. On the other hand, small caliber vessels are not suitable to occlude with the AVP. Mentioned treatment modalities were successfully done in different cases in the literature. However, in these cases, generally a large side branch of LIMA was treated and relatively small caliber vessels were ignored. Although we treated our patient with percutaneous intervention like other cases in the literature, the major difference was the usage of two different percutaneous treatments for both the large and the small caliber side branches together in the same procedure. While the AVP was used to treat the large caliber side branch, coil embolization was used to treat the small one. This approach provided complete resolution of coronary steal phenomena. We also demonstrated that blood flow cessation of all unligated side branches are important to reduce ischemia and angina. To the best of our knowledge, our strategy was the first study of percutaneous treatment of all side branches unexceptionally. However, large-scale studies are needed to evaluate optimal therapy and selection of suitable cases.

In conclusion, coronary steal syndrome is one of the most important reasons for ischemia and anginal symptoms in patients undergoing CABG surgery. Although the optimal therapy is controversial, percutaneous treatment modalities such as coil and AVP embolization seem to have a broad use to treat these patients with higher procedural success and lower complication rates.

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# System building and improvement for the diagnosis and treatment of rare diseases in Shanghai, China

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| Summary | **Shanghai has always attached importance to the prevention and treatment of rare diseases and has been at the forefront in China. The Shanghai Rare Diseases Diagnosis and Treatment Center, Shanghai Children's Rare Diseases Diagnosis and Treatment Center, and Shanghai Rare Diseases Specialist Clinic were established in February 2018. Moreover, with the development of clinical pathways for rare diseases and the provision of related services such as diagnosis, treatment, screening, information and training, the service system for diagnosis and treatment of rare diseases in Shanghai has formed, which greatly improves the accessibility of medical services for patients with rare diseases in Shanghai and surrounding areas, and is of great significance in reducing the burden on patients with rare diseases. Meanwhile, it also gives an important reference for other regions of China for providing rare disease diagnosis and treatment services.** |
|---|---|

*Keywords:* Rare disease, diagnosis and treatment, service system

The establishment of national or regional rare disease clinical medical centers and their network construction is an effective means to promote the availability of appropriate health services for patients with rare diseases (*1*). As an advanced area for the prevention and treatment of rare diseases in China, Shanghai has initially established a clinical diagnosis and treatment service system for rare diseases. This paper intends to introduce the system from the aspects of clinical diagnosis and treatment centers, clinical pathways, diagnosis, screening, information and training.

## 1. Clinical diagnosis and treatment centers of rare diseasess

The clinical diagnosis and treatment center is a guarantee for patients with rare diseases to receive good quality medical services. As early as in 2011,

in order to guarantee the diagnosis and treatment of children with Pompe disease, Gaucher's disease, Mucopolysaccharidosis, and Fabry disease, the Shanghai Children's Hospitalization Mutual Fund Management Office appointed three hospitals for this responsibility (*2*). With increasing attention to prevention and treatment of rare diseases in recent years, the former Shanghai Municipal Health and Family Planning Commission (SMHFPC) officially established Shanghai Rare Diseases Diagnosis and Treatment Center, Shanghai Children's Rare Diseases Diagnosis and Treatment Center, and the first batch of 5 Shanghai Rare Diseases Specialist Clinics (Figure 1) in February 2018 (*3,4*). Identification of the above centers and outpatient clinics mainly follow the principles of early research, more case accumulation, rich clinical experience, greater social impact, and higher academic status. It also requires them to benchmark the highest and best standards internationally to build a domestically leading and internationally first-class rare disease diagnosis and treatment institution. Additionally, in order to speed up the construction of a first-class medical center city and implement the national strategy of the Yangtze River Delta integration, Shanghai has proposed to build a laboratory diagnostic center for rare

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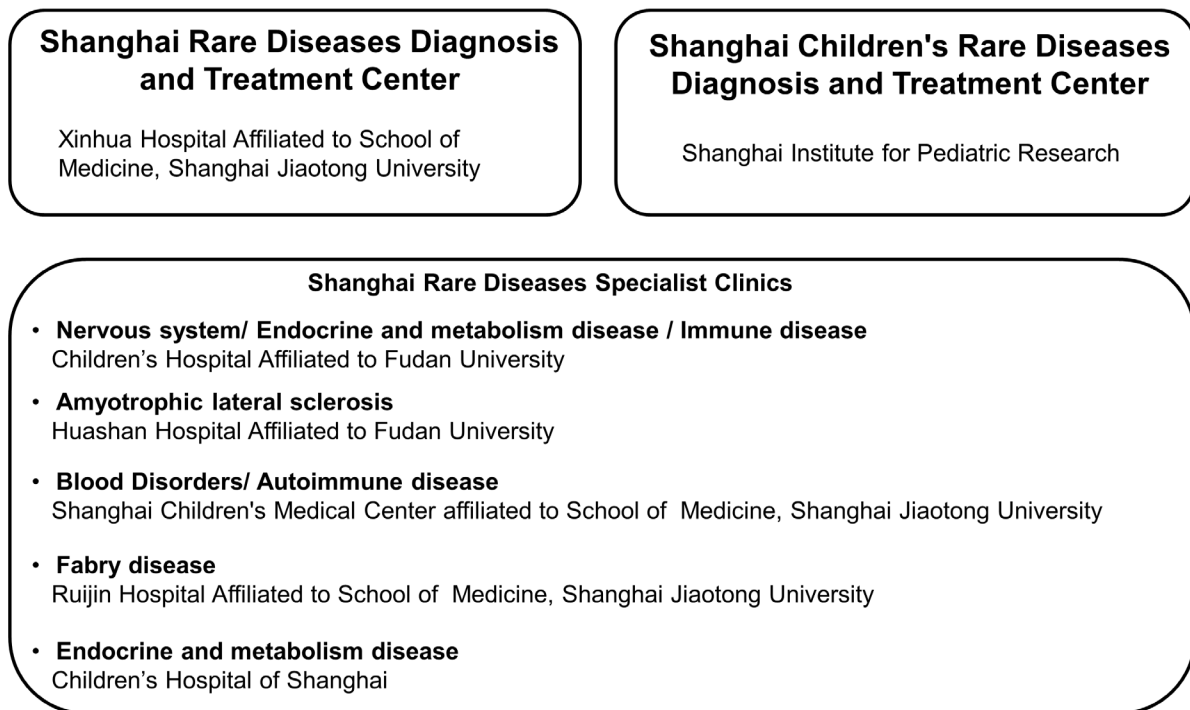E-mail: hejiangjiang@shdrc.org

## Shanghai Rare Diseases Diagnosis and Treatment Center

Xinhua Hospital Affiliated to School of Medicine, Shanghai Jiaotong University

## Shanghai Children's Rare Diseases Diagnosis and Treatment Center

Shanghai Institute for Pediatric Research

## Shanghai Rare Diseases Specialist Clinics

- **Nervous system/ Endocrine and metabolism disease / Immune disease**
  Children's Hospital Affiliated to Fudan University

- **Amyotrophic lateral sclerosis**
  Huashan Hospital Affiliated to Fudan University

- **Blood Disorders/ Autoimmune disease**
  Shanghai Children's Medical Center affiliated to School of Medicine, Shanghai Jiaotong University

- **Fabry disease**
  Ruijin Hospital Affiliated to School of Medicine, Shanghai Jiaotong University

- **Endocrine and metabolism disease**
  Children's Hospital of Shanghai

**Figure1. The clinical diagnosis and treatment system of rare diseases in Shanghai.**

diseases in the Yangtze River Delta (*5*).

## 2. Clinical pathways of diagnosis and treatment of rare disease

A clinical pathway is an important guide for the diagnosis and treatment of rare diseases. In 2011, Shanghai Municipal Science and Technology Commission listed research for prevention and treatment for 10 rare diseases such as maple syrup urine disease, tyrosinemia, methylmalonicacidemia, multiple carboxylase deficiency, hereditary tyrosinemia, multiple carboxylase deficiency, Gaucher's disease, Fabry disease, Pompe disease and mucopolysaccharidoses in a major scientific and technological project called *Science and Technology Innovation Action Plan* (*6*). Based on this project, relevant diagnosis and treatment standards for the above rare diseases were formulated. In addition, Shanghai Foundation for Rare Disease (SFRD) organized the compilation of *Treatable Rare Diseases*, which was published in 2017 (*7*). The book provided recommendations for diagnosis and treatment of 117 rare diseases, including 56 rare diseases listed in the List of Major RareDiseases in Shanghai (2016 Edition) (*8*) and another 61 rare diseases with clear diagnosis and viable treatment.

## 3. Diagnosis and treatment procedure for rare diseases

Timely and reasonable diagnosis and treatment is one of the main demands of patients with rare diseases. At present, the rare disease diagnosis and treatment center or outpatient clinic in Shanghai has established a group of rare disease experts, which is participated in by many clinical specialties and medical technology departments and is organized by experts from the hospital, the provincial level and even the national level. The multiple disciplinary team (MDT) is adopted to diagnose and treat rare diseases (Figure 2). Meanwhile, organizations have also vigorously strengthened the construction of various laboratory diagnostic platforms, including molecular genetics, cytogenetics, and genome-wide platforms.

## 4. Newborn screening for early detection of rare diseases

At present, newborn screening plays a major role in screening of rare diseases. In 1981, screening of phenylketonuria among all newborns was first achieved nationwide in Shanghai (*9*). In 2007, screening for congenital adrenal cortical hyperplasia and glucose-6-phosphate dehydrogenase deficiencywas available for all newborns. In addition, tandem mass spectrometry can be independently selected to detect a variety of rare diseases.

## 5. Referral information service for patients with rare diseases

Some rare disease diagnosis and treatment institutions have begun to implement patient registry, and established a patient's clinical information database and biobank. SFRD is also organizing construction of a registration
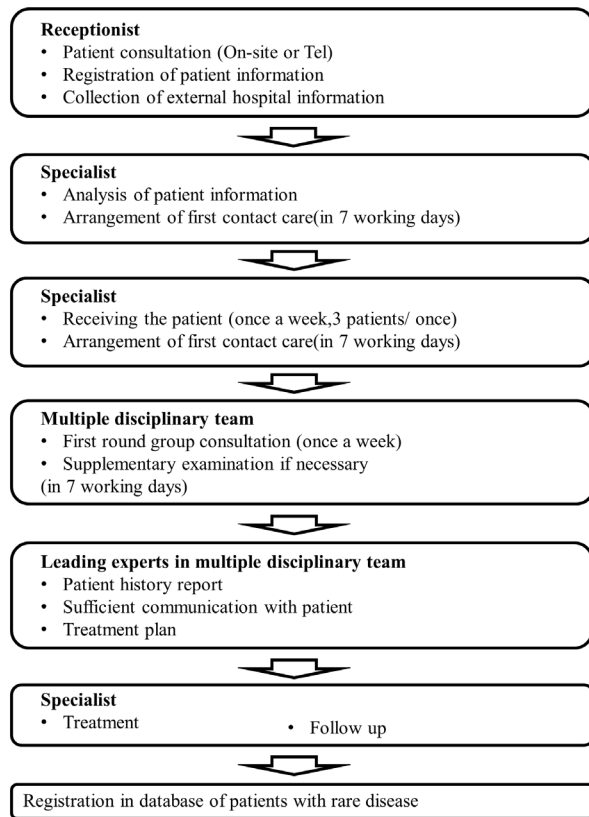
**Receptionist**
- Patient consultation (On-site or Tel)
- Registration of patient information
- Collection of external hospital information

**Specialist**
- Analysis of patient information
- Arrangement of first contact care(in 7 working days)

**Specialist**
- Receiving the patient (once a week,3 patients/ once)
- Arrangement of first contact care(in 7 working days)

**Multiple disciplinary team**
- First round group consultation (once a week)
- Supplementary examination if necessary
(in 7 working days)

**Leading experts in multiple disciplinary team**
- Patient history report
- Sufficient communication with patient
- Treatment plan

**Specialist**
- Treatment                        • Follow up

Registration in database of patients with rare disease

**Figure 2. The diagnosis and treatment process of rare diseases in Shanghai Rare Diseases Diagnosis and Treatment Center.**

platform for rare diseases patients in Shanghai. The first batch of registered units includes two centers and five specialist units (*10*). In addition, Shanghai has also actively organized Rare Disease Day activities (three sessions so far) and academic annual conferences (nine sessions so far) to convey important information such as rare disease research, diagnosis, treatment, services, policies, *etc*., and to raise social concerns and awareness.

**6. Knowledge training for increasing the awareness of rare diseases**

Increasing the awareness of medical staff about rare diseases is of great significance in reducing the rate of misdiagnosis and missed diagnosis of rare diseases. In March 2017, directed by the former SMHFPC, SFRD and the Shanghai Medical Association Rare Diseases Specialist Branch launched the Shanghai Rare Disease Prevention and Control Training Project for Medical Staff (*11*). The project aimed to gradually improve awareness and capacity of diagnosis and treatment for rare diseases among medical staff. The project would last for three years and would be available to medical staff in upper secondary and above medical institutions. Up to now, 10 sessions of training courses have been carried out.

**7. Perspectives**

It can be said that the Shanghai rare disease diagnosis and treatment service system has taken initial shape. This is not only a gospel for rare disease patients in Shanghai, but also for patients in the surrounding areas and even the whole country. This is of great significance for improving accessibility of diagnosis and treatment services for patients and reducing the burden on them. As a highlight of domestic medical education and technology, Shanghai should continue to strengthen the research, teaching and training of rare diseases, and make the current medical staff training programs long-term and institutionalized, and further build regional and national rare disease diagnosis and treatment centers. Moreover, it is necessary to speed up establishment and improvement of the rare disease registration system and research collaboration network based on the clinical system, and comprehensively collect important data such as physiological, psychological and economic burdens of patients with rare diseases, and provide an important basis for the formulation of relevant research, diagnosis, treatment, policies and laws (*12*). In addition, with the introduction of China's First National List of Rare Diseases (*13,14*) and the prioritized evaluation and approval system for orphan drugs policy, Shanghai should actively seek relevant drugs to be used in the above-mentioned medical institutions as soon as possible, and further expand the scope of rare diseases to be diagnosed and treated.

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*Letter*

# What is the Ocular phenotype associated with a dystrophin deletion of exons 12-29?

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**Summary**       Duchenne muscular dystrophy (DMD) is a result of a X-linked recessive inherited mutation of the *DMD* gene which contains 79 exons. This rare disease is passed on by the mother who is called a carrier. Primarily it affects boys, but in rare cases it can affect girls. Dystrophin protein is mostly located in skeletal and cardiac muscles, which explains muscular and cardiac manifestations in symptomatic female DMD-carriers. Dystrophin is also present in extramuscular tissues. Some dystrophin isoforms are exclusively or predominantly expressed in the brain or the retina. It has been reported that DMD patients and DMD-carriers present normal visual acuity, but abnormal electroretinographic findings. As symptomatic female DMD are very rare, ophthalmic screening of the female patient with deletions of exons 12-29 is valuable. Studying the functional relationship between ocular symptoms and related different deletions of exons dystrophin gene may further elucidate the pathophysiology in DMD.

*Keywords:* Duchenne muscular dystrophy, female carrier, ophthalmology, retina, electroretinogram

We read with interest the case reporting for the first time a female Duchenne muscular dystrophy (DMD)-carrier harboring deletions of exons 12-29, and presenting a muscular/myocardiac phenotype (*1*). It would be instructive to study the ocular phenotype in this patient, for assessing whether these deletions affect the retina.

Indeed, according to existing literature (Table 1), dystrophin isoforms (Dp427, Dp260, Dp140, Dp71) are expressed highly in different retinal layers (*2*). Any disturbance of these protein products is responsible for electroretinogram (ERG) abnormalities. Position of the mutation is the key factor of the ERG phenotype of DMD patients.

According to more recent studies in DMD patients and heterozygous DMD carriers, dystrophin is required for normal function of retinal mechanisms underlying ON-OFF, contrast sensitivity, luminance and red-green cone opponent responses (*3,4*). Retinal phenotype by electroretinography (ERG) was already studied in the 90's, for DMD patients and DMD carriers. Most of them had abnormal ERG with reduced amplitude of the b-wave under scotopic conditions (*5-7*). Abnormal dark-adapted ERGs were reported to be more frequent in DMD patients with more distal mutations (*8*). Moreover, deletion downstream of exon 30 was more frequently associated with red-green color defect among DMD patients (*9*).

Despite the occurrence of a dystrophin deletion upstream of exon 30 in this DMD-carrier (*1*), we suggest our colleagues to propose a screening of visual function including color vision, contrast sensitivity, and ERG, along with a retinal OCT (Optical Coherence Tomography) to the patient. This may enhance our understanding of the pathophysiology in DMD and optimize the comprehensive treatment.

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**Table 1. Major research findings from cited references** (*2-9*)

| | |
|---|---|
| Muntoni *et al.* 2003 (*2*) | • Patients with Duchenne mauscular dystrophy (DMD) may have normal visual acuity and abnormal electroretinography (ERG): reduction of the b-wave amplitude in the scotopic ERG.<br>▪ A relation seems to exist between ERG abnormalities and position of deletions of the dystrophin gene (*DMD*). |
| Barboni *et al.* 2013 (*3*) | ERGs were recorded using mesopic and photopic stimuli in DMD patients (*n* = 19), heterozygous DMD carriers (*n* = 7), and healthy controls (*n* = 19).<br>▪ DMD patients had normal visual acuity, but a reduced b-wave in ERG and abnormal contrast sensitivities.<br>▪ Carriers had normal ERG and contrast sensitivities. |
| Barboni *et al.* 2016 (*4*) | ERGs were recorded in DMD patients (*n* = 10), and healthy controls (*n* = 16). ON and OFF cone-driven retinal responses were analyzed.<br>In DMD patients:<br>▪ ERGs were abnormal<br>▪ Function of luminance and red-green cone opponent mechanisms were abnormal. |
| Sigesmund *et al.* 1994 (*5*) | Ophthalmologic examination including ERGs and DNA analysis were performed in DMD patients (*n* = 21):<br>▪ Scotopic ERGs were abnormal<br>▪ Patients with deletions in the central region of the dystrophin gene had the most severe ERG changes. |
| Girlanda *et al.* 1997 (*6*) | ERG was performed in DMD patients (n=18), and DMD carriers (*n* = 12):<br>▪ Reduction of the b-wave amplitude in the scotopic ERG, mainly in DMD patients<br>▪ Oscillatory potentials were altered, even in carriers, suggesting that dystrophin may be also involved in retinal circulation. |
| Pascual Pascual *et al.* 1998 (*7*) | The ratio of B-wave amplitude to A-wave amplitude (B/A amplitude ratio of ERG) was evaluated. It was :<br>▪ Normal ( > 2) in all controls (*n* = 12),<br>▪ Abnormal (< 2) in 100% of DMD patients (*n* = 16),<br>▪ Abnormal (< 2) in 50% of DMD carriers (*n* = 4). |
| Pillers *et al.* 1999 (*8*) | ▪ ERGs recorded in DMD patients with known deletions (*n* = 37) were abnormal in 90% (*n* = 33) and normal in 10% (*n* = 4) of patients.<br>▪ Review of literature: 64 DMD patients with known mutations.<br>The most important determinant in the reduction of the b-wave amplitude in dark-adapted ERG is the mutation position: 94% of DMD patients with more distal mutations had abnormal ERG (versus 46% with mutations of the Dp260 transcript start site). |
| Costa *et al.* 2007 (*9*) | ▪ Color vision was evaluated in 44 DMD patients (12 with deletion upstream of exon 30 and 32 with deletion downstream of exon 30), and 70 healthy controls with no ophthalmological complaints.<br>▪ Red-green color vision impairment in 66% of DMD patients with deletion downstream of exon 30.<br>▪ DMD patients with deletion upstream of exon 30 had normal color vision<br>▪ A positive correlation between abnormal scotopic ERGs and neurodevelopmental disturbances, and the most severe findings were in patients with Dp71 disruption. |

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# IRDR

**Intractable & Rare Diseases Research**

# Guide for Authors

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Intractable & Rare Diseases Research 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.

**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.

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