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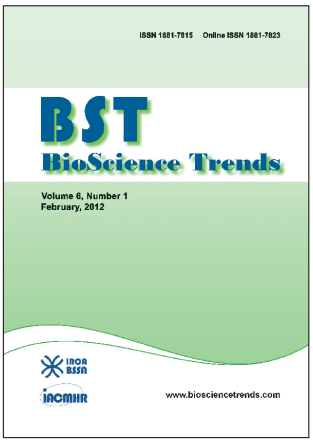

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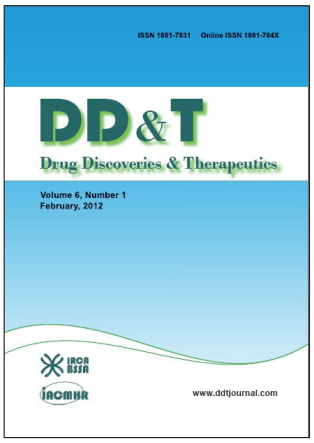

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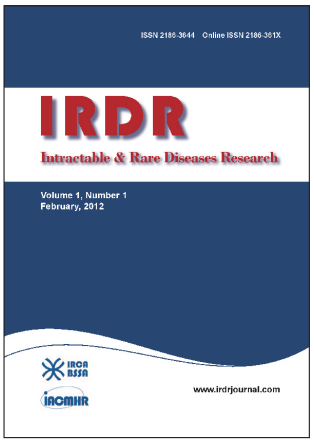

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Editorial Board

CONTENTS

Volume 4, Number 1, 2015

Reviews

1 - 6 **Current research on the treatment of primary sclerosing cholangitis.**
Ahmad H Ali, Elizabeth J Carey, Keith D Lindor

7 - 11 **Current research on pediatric patients with bronchiolitis obliterans in Brazil.**
Silvia Onoda Tomikawa, Joaquim Carlos Rodrigues

12 - 16 **Evaluation of quality of life and risk factors affecting quality of life in adolescent idiopathic scoliosis.**
Jing Han, Qintong Xu, Yi Yang, Zhengjun Yao, Chi Zhang

17 - 23 ***ARID1B*-mediated disorders: Mutations and possible mechanisms.**
Joe C. H. Sim, Susan M White, Paul J. Lockhart

24 - 32 **Budd-Chiari syndrome and liver transplantation.**
Nobuhisa Akamatsu, Yasuhiko Sugawara, Norihiro Kokudo

33 - 38 **Liver transplantation and autoimmune hepatitis.**
Tomohiro Tanaka, Yasuhiko Sugawara, Norihiro Kokudo

39 - 48 **Fragile X syndrome as a rare disease in China – Therapeutic challenges and opportunities.**
Xiaowei Jin, Li Chen

Original Articles

49 - 53 **Heterozygous mutation of c.3521C>T in COL1A1 may cause mild osteogenesis imperfecta/Ehlers-Danlos syndrome in a Chinese family.**
Xianlong Shi, Yanqin Lu, Yanzhou Wang, Yu-ang Zhang, Yuanwei Teng, Wanshui Han, Zhenzhong Han, Tianyou Li, Mei Chen, Junlong Liu, Fengling Fang, Conghui Dou, Xiuzhi Ren, Jinxiang Han

54 - 59 **Different types of androgen receptor mutations in patients with complete androgen insensitivity syndrome.**
Jialiang Shao, Jiangang Hou, Bingkun Li, Dongyang Li, Ning Zhang, Xiang Wang

Case Report

60 - 64 **Cardiac amyloidosis in a heart transplant patient - A case report and retrospective analysis of amyloidosis evolution.**
Svetlana Kintsler, Jörg Jäkel, Vincent Brandenburg, Katrin Kersten, Ruth Knuechel, Christoph Röcken

Guide for Authors

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Mini-Review

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Current research on the treatment of primary sclerosing cholangitis

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Summary **Primary sclerosing cholangitis (PSC) is a progressive disease of the liver characterized by inflammation and destruction of the intra- and/or extra-hepatic bile ducts, leading to fibrosis and ultimately liver failure, cirrhosis and an increased risk of malignancy. The etiology of PSC is unclear. It is often associated with the inflammatory bowel diseases (IBD), particularly Ulcerative Colitis (UC); up to 75% of PSC patients have UC. PSC is more prevalent in men than in women. Ursodeoxycholic acid (UDCA) has been extensively studied in PSC in randomized clinical trials but failed to show a positive impact on the natural course of the disease. Currently, there is no effective medical therapy for PSC, and the majority of patients will eventually require liver transplantation. PSC is one of the leading indications for liver transplantation. In this paper, we review the current research on the potential therapeutic agents for the treatment of PSC.**

Keywords: Primary sclerosing cholangitis, ursodeoxycholic acid, obeticholic acid, vancomycin

1. Introduction

Primary sclerosing cholangitis (PSC) is a progressive liver disease characterized by ongoing destruction of the intra- and extra-hepatic bile ducts leading to cholestasis, advanced fibrosis, liver cirrhosis and eventually liver failure with its consequent complications such as portal hypertension and an increased risk of malignancy (*1-3*). PSC affects nearly 50,000 patients in the United States (*4*). The median life expectancy after diagnosis of PSC is 12 to 18 years without liver transplantation (*3,5*). PSC is often associated with Ulcerative Colitis (UC) (*4*), an inflammatory bowel disease (IBD) characterized by chronic ulceration of the large intestine. PSC can occur in association with autoimmune diseases such as autoimmune hepatitis and autoimmune pancreatitis; commonly referred to as the PSC overlap syndromes (*6*). The diagnosis of PSC is made in patients with a chronic cholestatic biochemical profile when cholangiography shows stricturing of the intra- and/or extra-hepatic bile ducts (*7*). Small-duct PSC is a variant of PSC characterized by chronic cholestasis, normal cholangiography, and histological findings consistent with PSC (*8,9*).

Currently, there is no effective medical therapy for PSC. Ursodexoycholic acid (UDCA) is the single most extensively studied agent in PSC. Several controlled and uncontrolled clinical trials have shown significant improvement in liver biochemistries when PSC patients were treated with UDCA (*10-15*). However, large randomized and controlled prospective clinical trials have failed to demonstrate that UDCA can positively affect the clinical outcomes of patients with PSC (*13,15*). In fact, the safety of long term use of high-dose UDCA in PSC patients has been questioned, as it has been associated with increased rates of serious adverse events (*13,16*) and, more recently, with the development of colon cancer (*16,17*). The American Association for the Study of Liver Disease (AASLD) recommends against the use of UDCA in PSC patients (*7*). Liver transplantation remains the treatment of choice for end-stage PSC, being the fifth leading indication for liver transplantation in the United States (*18*). In some Scandinavian countries, PSC is the leading indication for liver transplantation (*18-20*). Recurrent PSC is an important problem, occurring in the transplanted liver in 20%-40% of PSC patients (*18*).

Several potential therapeutic avenues in PSC have

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been explored over the last 2 decades, some of which have shown promising results. In this paper, we review the current research on the treatment strategies in PSC.

2. Bile acid mimetics and PSC

Twenty-four norUDCA is the C23 homolog of UDCA that is currently being evaluated in a phase II randomized clinical trial in patients with PSC (ClinicalTrials.gov Identifier: NCT01755507). In a rodent model of cholestasis, the administration of norUDCA to *Mdr2* knockout mice improved sclerosing cholangitis, possibly by altering the composition of the bile acid pool through displacing the toxic bile acids and increasing the hydrophilicity of the bile acids (*21*). In a more recent animal model of cholestasis, norUDCA significantly improved indices of liver injury in common bile duct-ligated (CBDL) mice when compared to UDCA (*22*). Taken together, these results suggest that norUDCA could be of potential benefit in patients with PSC.

3. Farnesoid X receptor agonists and PSC

The farnesoid X receptors (FXRs) are a group of nuclear hormone receptors expressed in high amounts in tissues involved in bile acid metabolism such as liver, intestine, and kidney (*23*). Recently, bile acids have been identified as natural ligands of FXRs (*24,25*). FXRs play a key role in bile acid homeostasis by regulation of genes involved in bile acid synthesis, secretion, conjugation, transportation, absorption, and detoxification (*26-30*). An important target of the FXRs is the gene encoding for cholesterol 7α hydroxylase (CYP7A1) the rate-limiting enzyme in bile acid biosynthesis. When bound to bile acids, FXRs repress the gene encoding for CYP7A1 (*24*). Moreover, the expression of an important transport protein (cytosolic intestinal bile acid-binding protein) (*31*) located in the intestines is increased as a result of activation of the FXRs (*24,32*). This protein is believed to play a key role in the regulation of the enterohepatic circulation (*24,32*). In addition to their role in bile acid homeostasis, FXRs have been found to regulate liver regeneration during liver injury (*33-38*).

Obeticholic acid (OCA, INT-747), a 6-ethyl derivative of the natural human bile acid chenodeoxycholic acid (CDCA), is a first-in-class selective FXR agonist with ~ 100-fold greater FXR agonistic activity than CDCA (*39-41*). In a male Wistar rat model of cholestasis, OCA protected hepatocytes against deleterious effects caused by administration of lithocholic acid (LCA) (*41*). In another animal model, the administration of OCA reduced liver fibrosis and indices of hepatic damage in bile duct ligated rats (*42*). Collectively, these results suggest that FXR agonists could be of therapeutic benefit in patients with cholestatic liver diseases.

The safety and efficacy of OCA has been evaluated in 2 randomized clinical trials in patients with primary biliary cirrhosis (PBC) with promising results (*43,44*). The administration of OCA to PBC patients led to a significant reduction of serum alkaline phosphatase (ALP), an important surrogate marker in PBC (*43,44*). One important adverse event was pruritus, occurring in a dose-dependent manner and leading to discontinuation of the drug in 38% of PBC patients (*43,44*). Currently, a phase II clinical trial of OCA in PSC patients is ongoing, using lower doses to help avoid pruritus (ClinicalTrials.gov Identifier: NCT02177136).

4. Apical sodium-dependent bile acid transporter inhibitors and PSC

Abnormal bile acid pool composition is thought to play a key role in the pathogenesis and progression of PSC (*45*). This hypothesis is derived from several animal and human studies. PSC-like lesions occur in mice devoid of the canalicular transporter *Mdr2*, which mediates biliary excretion of phospholipids that normally form mixed micelles with the bile acids, thus protecting the liver against the detergent effects of bile acids (*46*). Bile acid toxicity towards the biliary epithelium could result from decreased biliary HCO_3 secretion (*47*). The bile salt-sensing receptor TGR5 plays a key role in the regulation of HCO_3 secretion, and interestingly, TGR5 has been identified as a likely disease gene in a large genome-wide study of PSC (*48*). In Lindor's high-dose UDCA study in PSC, treatment with high-dose UDCA was associated with an increased rate of serious clinical events when compared with placebo (*13*). Sinakos *et al*. investigated the serum bile acid composition in patients in the high-dose UDCA arm and compared that with serum bile acids in patients in the control group (*49*). They found a significant expansion of the total serum bile acid pool and increased UDCA and LCA enrichment in the UDCA-treated patients versus the placebo group when compared to pretreatment levels (*49*). In addition, they found that the increase in total serum bile acid pool correlated with worse outcomes in patients with PSC (*49*). Together, these observations suggest that changes in the bile acid pool could be deleterious in patients with PSC, and alteration of the bile acid pool may be of therapeutic benefit in PSC.

The apical sodium-dependent bile acid transporter (ASBT), also known as the ileal bile acid transporter, is expressed predominantly in the distal ileal tissue and plays a key role in the reabsorption of bile acids from the lumen of the small intestine, which is critical for the enterohepatic circulation of the bile acids (*50*). Normally, ~ 95% of the secreted bile acids are reabsorbed from the intestine into the portal circulation and back to the liver (*51*). With this biological rationale, interrupting the enterohepatic circulation could result in a decrease bile acid load on the liver, which in turn could be of potential therapeutic benefit in patients with

PSC. Currently a phase II clinical study evaluating the safety and efficacy of LUM001, an ASBT inhibitor, in patients with PSC is ongoing (ClinicalTrials.gov Identifier: NCT02061540).

5. Antimicrobials and PSC

Several animal experiments demonstrated a link between the gut microbiota and development of PSC (*52-59*). Induction of small bowel bacterial overgrowth by ligating the jejunum in rats resulted in development of hepatic lesions compatible with PSC (*53,55,56*). Daily treatment with antibiotics led to significant improvement in these lesions (*55*), suggesting that gut microbiota modification could be of therapeutic benefit in a selected group of PSC patients.

Vancomycin, metronidazole and minocycline have been evaluated in clinical trials in patients with PSC (*60,61*). The use of these antibiotics led to a significant reduction in serum ALP, an important surrogate marker in PSC (*60,61*). Thus antibiotic therapy in PSC patients seems to be a promising tool in the treatment of PSC. However, larger studies are needed to clarify these results.

6. Monoclonal antibodies and PSC

Mucosal adressin cell adhesion molecule 1 (MAdCAM-1), an endothelial cell adhesion molecule, is expressed in high amounts in the gut of patients with IBD and those with IBD and PSC (*62-65*). Vascular adhesion protein 1 (VAP-1) has been found to induce the expression of MAdCAM-1 in the hepatic endothelial cells of human liver tissue (*66*). This, in turn, was associated with increased adhesion of lymphocytes from patients with PSC (*66*). Thus, targeting the VAP-1/MAdCAM-1 could be of beneficial effect in patients with PSC. Vedolizumab is a monoclonal antibody against α4/β7, which is a cell surface glycoprotein expressed on B and T cells and interacts with MAdCAM-1, has shown a beneficial effect in UC (*67*). This agent could also be of therapeutic benefit in patients with PSC. The VAP-1-blocking agent, BTT1023, is currently being investigated in a phase II clinical trial in patients with PSC (ClinicalTrials.gov Identifier: NCT02239211).

It has been previously shown that the liver-infiltrating lymphocytes in PSC include mucosal T cells recruited to the liver by aberrant expression of the gut-specific chemokine CCL25 that activates α4/β7 binding to MAdCAM-1 on the hepatic endothelium (*63,68*). Therefore, targeting the CCL25-MAdCAM-1 axis could be of therapeutic benefit in PSC. CCX282-B, a CCR9 antagonist that inhibits CCR9- and CCL25-dependent chemotaxis, has shown efficacy in Crohn's disease (*69*). This agent deserves to be investigated in patients with PSC.

Lysyl oxidase-like protein 2 (LOXL2) belongs to the lysyl oxidase family and has been shown to contribute to progressive liver damage in experimental models (*70*). Simtuzumab, a monoclonal antibody against LOXL2, is currently being investigated in a phase II clinical trial in patients with PSC (ClinicalTrials.gov Identifier: NCT01672853).

7. Special cases

Primary sclerosing cholangitis-Autoimmune hepatitis (PSC-AIH) overlap syndrome is a disorder characterized by clinical, biochemical and histological features of AIH in the presence of cholangiographic findings consistent with PSC (*71,72*). Data on PSC-AIH overlap patients are scarce and long-term outcome is not well-defined. Because of these reasons, there is no consensus on the treatment of patients with PSC-AIH. Recently, Zenouzi *et al*. (*73*) reported the long-term follow up on three cases originally described in 1996. All three patients were alive (22, 27, and 25 years after initial presentation) and have shown disease progression, two of whom are on the liver transplantation list (both developed esophageal varices and one developed weight loss). The third patient underwent liver transplantation 22 years after initial presentation. All three patients were on UDCA and immunosuppression therapy (azathioprine) (*73*). These data suggest that PSC-AIH may have a better prognosis than the classic PSC, and that the combination of UDCA and immunosuppression in PSC-AIH may be of therapeutic benefit and warrants investigation. Given the rarity of PSC-AIH, randomized clinical trials are unlikely to occur.

Autoimmune pancreatitis (AIP) is a chronic pancreatic condition characterized by narrowing of the pancreatic duct, raised immunoglobulin G4 (IgG4) levels, lymphocytic infiltration on biopsy, and response to steroids (*74*). AIP in association with intra-hepatic and/or extra-hepatic bile duct stricturing similar to those present in PSC is termed autoimmune pancreatitis-sclerosing cholangitis (AIP-SC) (*7*). It is unclear whether PSC and AIH represent different ends of the same disease or are separate clinical conditions. Patients with AIP-SC seem to respond to corticosteroids (*75*). However, studies are needed to clarify the long-term effects of corticosteroid therapy in patients with AIP-SC which are unlikely to occur given the rarity of the disease.

8. Conclusion

PSC is progressive disease of the liver that ultimately leads to cirrhosis and liver failure. There is no effective medical therapy for PSC. Recent advances in understanding the pathological mechanisms that contribute to the hepatobiliary damage in PSC have

led to the development of clinical programs to evaluate potential candidates as therapeutic tools in PSC. Several early-phase clinical trials evaluating these agents are underway.

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Mini-Review

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Current research on pediatric patients with bronchiolitis obliterans in Brazil

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Summary **Bronchiolitis obliterans (BO) is a rare but severe disease, characterized by inflammation and fibrosis of the terminal bronchioles. BO in children usually occurs after a severe lung viral infection. Diagnosis is based on clinical history of acute bronchiolitis followed by persistent obstruction of the airways and characteristic findings in HRCT. There is no consensus on treatment beyond supportive measures, but bronchodilators and corticosteroids are often used. This review describes the clinical and radiological characteristics and outcomes of BO in pediatric patients, with an emphasis on current research in Brazil.**

Keywords: Bronchiolitis obliterans, children, obstructive pulmonary disease

1. Introduction

Bronchiolitis obliterans (BO) is a rare chronic obstructive lung disease that occurs following a severe injury to the lower respiratory tract and results in partial or complete obliteration of the small airways (*1,2*). BO has several etiologies, but in children the disease usually occurs post-infection (*1,2*). Several viruses have been associated with BO (*2,3*), such as respiratory syncytial virus, parainfluenza, influenza, and adenovirus; the latter in particular is associated with the most severe form of the disease.

Post-infectious BO is prevalent especially in some regions in Asia and the southern cone of South America (the south of Brazil, Uruguay, Argentina, and Chile) (*4,5*). The reasons for this high prevalence of post-infectious BO in these regions are not clear and may be due to more aggressive infectious agents, load or infection due to crowding, some constitutional/genetic predisposition, or even environmental factors (*5*). Therefore, there are several centers in Brazil like the current author's that study BO in children.

Although the prevalence of BO in Brazil is not known, it is presumably quite low. In the city of Curitiba, 48 pediatric patients were studied over 11 years of outpatient treatment (*6*). In the city of Porto Alegre, Zhang *et al*. (*7*) studied 31 pediatric patients over 18 years of monitoring and Bosa *et al*. (*8*) was studied the nutritional status of 57 pediatric patients followed up at two referral hospitals. In the city of Fortaleza (*9*), 35 pediatric patients were studied over five years. Finally, a study by the current authors at the Clinical Hospital affiliated with the Faculty of Medicine of the University of São Paulo (*10*) included 40 patients over 11 years of outpatient treatment.

The reason why some children develop BO is not fully understood. Factors such as viral genotype, host immune response, genetic predisposition, and environmental influences may be associated with disease severity in acute and long-term sequelae (*11*). Previous studies (*2,12-14*) found that risk factors for developing BO in children are adenoviral infection and the severity of acute illness (duration of hospitalization, admission to an intensive care unit, mechanical ventilation, oxygen use, corticosteroid treatment, and β2 agonist administration).

2. Histopathology

BO is characterized by inflammation and fibrosis of the terminal bronchioles with narrowing or complete obliteration of the lumens of the small airways, as

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shown in Figue 1 (*15*). Mauad *et al*. of the Faculty of Medicine of the University of Sao Paulo examined 34 biopsy specimens from open lung lobectomy, lobe excision, and autopsies, and they described histological aspects of BO (*16*). Their study found that 97% of childhood BO was constrictive, with variable degrees of inflammation and airway obliteration.

3. Clinical and radiological aspects

The initial symptoms and signs of BO are similar to acute viral bronchiolitis: fever, cough, tachypnea, and wheezing (*1,3*). But the disease does not progress as expected and symptoms and signs persist for weeks or months. Patients with BO have tachypnea, dyspnea, hypoxemia, crackles, wheezing, an increased antero-posterior diameter of the chest, digital clubbing, and cyanosis (*3,7,12,17*). In a previous study by the current authors (*10*), the most common symptoms and signs of BO in 40 patients were wheezing, dyspnea, and coughing (Table 1).

Imaging techniques play an important role in the diagnosis for post-infectious BO (*15*). The three most common methods of imaging are conventional chest radiographs (CRX), lung ventilation/perfusion scans (V/Q scan), and high-resolution chest tomography (HRCT) (*15*). HRCT is more sensitive than CRX and V/Q scan at detecting airway and parenchymal abnormalities (*15*). Brazilian studies (*6,9,10,18*) found that characteristic findings in HRCT were: a mosaic pattern of perfusion, bronchiectasis, bronchial wall thickening, air trapping, and atelectasis. These findings are shown in Figure 1. A previous study by the current authors (*10*) revealed the most frequent findings for HRTC, as shown in Table 2.

Table 1. Symptoms and signs at time of diagnosis of 40 children with BO

Characteristics (n = 40)	n (%)
Symptoms	
Persistent cough	23 (57.5)
Dyspnea	26 (65)
Persistent wheezing	40 (100)
Cyanosis (reported episodes)	9 (22.5)
Physical examination	
Increased antero-posterior diameter of the chest	23 (57.5)
Aspects similar to Cushing syndrome	8 (20)
Clubbing of the fingers	7 (17.5)
Watch-glass nails	2 (5)
Pulmonary auscultation	
Diffuse crackles	24 (60)
Localized crackles	7 (17.5)
Wheezin	33 (82.5)

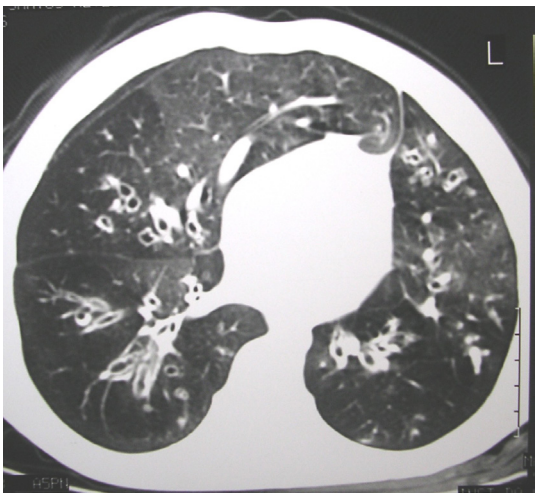

Figure 1. HRTC of a pediatric patient with post-infectious BO.

Table 2. HRCT findings in children with BO

HRCT findings (n = 40)	n (%)
Mosaic pattern of perfusion	29 (72.5)
Bronchial wall thickening	18 (45)
Atelectasis	16 (40)
Alveolar filling	12 (30)
Bronchiectasis	11 (27.5)
Hyperinflation	5 (12.5)
Air trapping	5 (12.5)
Swyer-James-MacLeod syndrome	1 (2.5)

4. Pulmonary function testing

Pulmonary function is severely compromised in children with post-infectious BO (*19*). Patients typically exhibit severe fixed air-flow obstructions with little or no response to bronchodilator, increased airway resistance, decreased compliance, a reduced expiratory flow, air trapping, and increased residual volume (*1,7,11,17,19*).

Mattiello *et al*. studied the lung function of children with BO (*20*). Seventeen patients had a reduced FVC, FEV_1, and $FEF_{25-75\%}$, an increased total lung capacity (TLC) and residual volume (RV), and a reduced functional exercise capacity undergoing a cardiopulmonary exercise test (CPET) and a 6-minute walk test (6MWT) (Table 3).

5. Diagnosis

Studies have proposed the following criteria for the diagnosis of post-infectious BO (*5,11*) : *i*) A history

Table 3. Spirometric and plethysmographic parameters of 20 patients with BO (adapted from Mattiello)

Parameter	Mean value + S.D.	% predicted + S.D.
FVC (L)	1.7 + 0.6	66.8 + 17.3
FEV_1 (L)	0.9 + 0.4	57.7 + 17.9
FEV_1/FVC (%)	57.9 + 12.5	
$FEF_{25-75\%}$, (L)	0.5 + 0.2	20.4 + 12.6
TLC (L)	4.1 + 1.1	121.2 + 23.2
RV (L)	2.4 + 0.7	294.3 + 83.3
RV/TLC (%)	59.1 + 8.4	

* PFT = pulmonary function test, TLC = total lung capacity, RV = residual volume

of acute bronchiolitis in a previously healthy infant; *ii*) Airway obstruction detected either by physical examination and/or by lung function tests that persists for over 6 weeks after the initial event despite the use of bronchodilators and steroids; *iii*) HRTC exhibiting bronchiectasis and/or a mosaic pattern; *iv*) Exclusion of other chronic obstructive pulmonary diseases, such as cystic fibrosis, severe asthma, bronchopulmonary dysplasia, foreign body aspiration, aspiration pneumonia associated with gastroesophageal reflux, tracheomalacia, congenital malformation, tuberculosis, AIDS, and other immunodeficiency diseases.

Thus, an open lung biopsy should be considered only when histological confirmation is needed. Furthermore, a biopsy cannot always confirm a diagnosis due to the heterogeneous distribution of pulmonary lesions; the specimens obtained may exhibit only mild histological changes that may go unnoticed (*1,5,11*).

Table 4. Differences in all time point assessment vs. baseline in the tiotropium vs. placebo group in the main PFT measurements (reprinted from Teixeira)

PFT parameter	Friedman Test	*P* value
FVC	18.171	0.33
FEV1	48.184	< 0.0001
RV	45.037	< 0.0001
Resistance	101.10	< 0.0001
Conductance	136.83	< 0.0001

6. Treatment

There is no consensus on bronchiolitis obliterans treatment. Supportive measures are important and include not smoking, vaccination against influenza, respiratory physiotherapy, supplemental oxygen at home for hypoxemic patients, and nutritional assistance (*1,3,5,11,21*). Bosa *et al*. (*8*) assessed the nutritional status of 57 children with BO and found a high rate of malnutrition (21.7%) and risk of malnutrition (17.5%), indicating the need for nutritional intervention in those patients.

Bronchodilators are used to treat symptomatic wheezing, although BO has been considered a fixed obstructive disease of the small airways that does not respond or that responds poorly to bronchodilators (*19*). In a Brazilian study by Teixeira *et al*., administration of a single dose of tiotropium to 30 patients with post-infectious BO resulted in a continued decrease in bronchial obstruction and air trapping for up to 24 hours (*19*) (Table 4).

The use of corticosteroids in the treatment of BO is based on the study by Moran and Hellstrom (*22*), who used a rabbit model of BO to demonstrate the natural course of the disease and that corticosteroid therapy in the early phase of illness modified fibroblastic response. However, similar studies were not performed in humans, so use of corticosteroids to treat BO remains controversial (*1,7,11*).

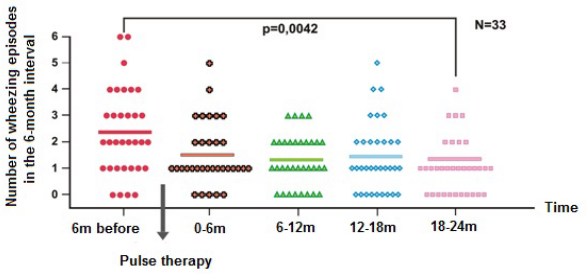

Figure 2. Exacerbation of wheezing before and after pulse therapy (*n* = 33).

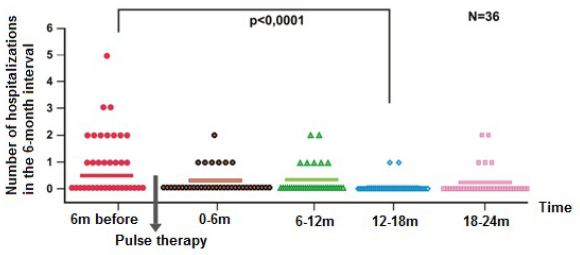

Figure 3. Hospitalization before and after pulse therapy (*n* = 36).

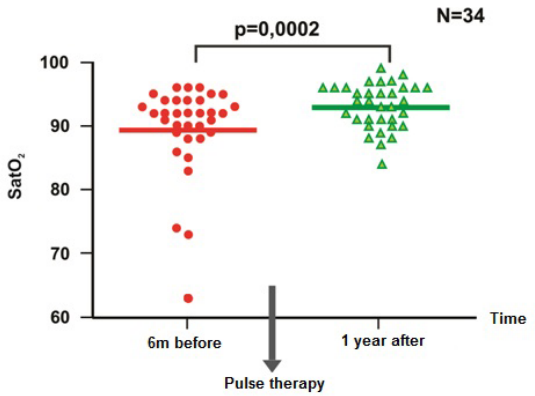

Figure 4. Oxygen saturation (SatO2) before and after 1 year of pulse therapy (*n* = 34).

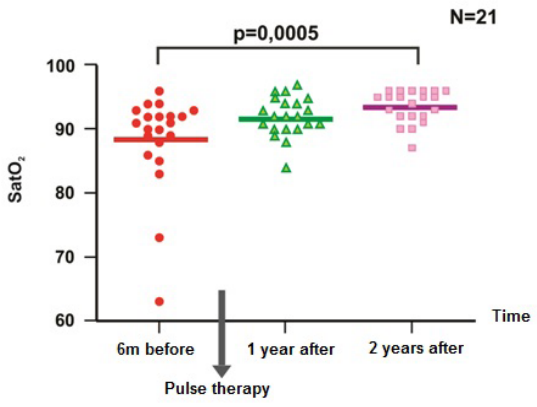

Figure 5. Oxygen saturation (SatO2) before and after 1 and 2 years of pulse therapy (*n* = 21).

Zhang *et al*. (*7*) advocated systemic use of corticosteroids, arguing that severe respiratory obstruction would prevent an aerosol spray from reaching the peripheral airways. Other clinicians prefer to use inhaled corticosteroids to minimize systemic adverse effects and to reduce bronchial hyperreactivity (*21*).

Methylprednisolone intravenous pulse therapy has been proposed to reduce adverse reactions to prolonged systemic administration of oral corticosteroids and is an alternative for patients with more severe disease (*1,11,17*). In a previous study by the current authors (*10*), 40 children with BO were treated with high-dose methylprednisolone pulse therapy, and these children exhibited clinical improvement as indicated by decreased exacerbation of wheezing and improved oxygen saturation. As a result, these patients had fewer instances of hospitalization (Figures 2-5).

7. Outcome and prognosis

During an acute adenoviral infection, mortality can be as high as 18.4% (*13,14*), but once an infection has been established BO has a low mortality rate (*15*). Patients with post-infectious BO, in contrast to those with post-transplant BO, usually exhibit clinical improvement after 2-3 years of supportive treatment, although clinical and radiological changes and changes in pulmonary function may persistent (*7,9,12,23*).

As the lungs develop, the diameter of the airways increases and the airways become less susceptible to obstruction. Thus, the clinical improvement observed may occur as a result of normal lung development and not represent a regression of lesions (*7*). In a study by Zhang *et al*. (*7*), 22.6% of patients had clinical remission, 67.7% of patients continued to have symptoms, and 9.7% of patients died.

However, a study by Cazzato *et al*. (*24*) yielded worrying findings indicating increased lung dysfunction (a decline in FEV_1 of 1.01% and a decline in $FEF_{25-75\%}$ of 1.04% per year) over time in patients with post-infectious BO, suggesting that BO may be a progressive lung disorder. Additional studies need to be performed to understand the process of inflammation caused by the disease and the better approaches to its treatment.

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Mini-Review

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Evaluation of quality of life and risk factors affecting quality of life in adolescent idiopathic scoliosis

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Summary **Adolescent idiopathic scoliosis (AIS) is a common disease leading to spinal deformity in children ages 10 and over. With advances in the study of health-related quality of life (HRQoL), greater attention has been given to the quality of life (QoL) of patients with AIS and their perception of deformity instead of just focusing on improving the rate of surgical correction. This article provides an overview of the methods of evaluating HRQoL and it analyzes several main factors affecting QoL, such as severity of disease, method of treatment, gender, and social environment, based on previous studies of patients with AIS. The authors believe that radiological studies should no longer be taken as the only indicator of postoperative therapeutic evaluation and hope to build a new evaluation system with assessment of QoL for patients with AIS.**

Keywords: Adolescent idiopathic scoliosis, quality of life, evaluation, questionnaire, risk factors

1. Introduction

Adolescent idiopathic scoliosis (AIS) is defined by the Scoliosis Research Society (SRS) as an unknown spinal deformity with a coronal Cobb angle > 10 degrees occurring in a child over the age of 10 whose skeleton is still developing. AIS occurs frequently in teenagers.

The rate at which AIS is corrected has greatly improved with the formulation of the theory of three-dimensional scoliosis correction and rapid advances in internal fixation, such as multi-level pedicle screw fixation. Changes in healthcare models and constant advances in research into health-related quality of life (HRQoL) have led to the realization that greater attention should be paid to the quality of life (QoL) of patients with AIS and their perception of deformity instead of just focusing on improving the rate of surgical correction.

Although scoliosis is far from life-threatening, social, family, and surgery-related factors might lead patients to develop mental disorders (*1*) or even attempt suicide (*2*). In order to improve patient QoL and satisfaction with treatment, attention must be paid to research on QoL in patients with AIS.

2. Advances in methods of evaluating HRQoL in patients with AIS

HRQoL refers to health status and is an individual's level happiness or satisfaction with personal life events in the face of disease, accident or injury, or medical treatment. HRQoL reflects a patient's subjective assessment of his or her QoL. Dimensions like health status, function, pain, and satisfaction can be evaluated using comprehensive scales and questionnaires that assess general health or the state of a specific disease. Common HRQoL scales are divided into two categories, general instruments to evaluate HRQoL and specific instruments to evaluate HRQoL.

2.1. *General instruments to evaluate HRQoL*

The questionnaire most commonly used to evaluate general health is the Short Form-36 Health Survey (SF-36). Other instruments include the Pediatric Outcomes Data Collection Instrument (PODCI) and the Child Health Questionnaire (CHQ).

The SF-36 covers the 8 aspects of physiological functioning (PF), bodily pain (BP), physiological

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functioning (RP), general health (GH), social functioning (SF), vitality (VT), mental health (MH), and emotional functioning (RE), and the SF-36 can be used to evaluate the QoL of patients with various diseases (*3*). Although the SF-36 is widely used in clinical practice, it is a general instrument to evaluate HRQoL and it not specific to scoliosis. Other drawbacks are problems like repeated questions and the long time needed to administer the questionnaire. Moreover, evaluation of self-image, which is of particular significance in patients with AIS, is not included in SF-36.

2.2. *Specific instruments to evaluate HRQoL*

Specific instruments to evaluate HRQoL are specifically designed for a specific disease like spinal deformity. Instruments to assess QoL in patients with scoliosis include the Scoliosis Research Society Outcomes Instruments (SRS-22 and SRS-24), the Quality of Life Profile for Spinal Deformities (QLPSD), the Spinal Appearance Questionnaire (SAQ), the Scoliosis Quality of Life Index (SQLD), the Walter Reed Visual Assessment Scale (WRVAS), and the Bad Sobernheim Stress Questionnaire (BSSQ) (*4-7*).

2.2.1. *SRS-24*

Haher *et al*. (*8*) created the simple and practical SRS-24 HRQoL questionnaire for patients with scoliosis in 1999 and they contended that a child's HRQoL and subjective satisfaction should also be assessed as part of the evaluation after surgery for scoliosis. The SRS-24 is divided into two parts. The first part includes assessment of pain, functioning, self-image, and activity, and this portion can be used to evaluate any patient with scoliosis. The second part includes postoperative self-image, functioning, and satisfaction with treatment. This portion can only be used to evaluate patients after surgery for scoliosis.

2.2.2. *SRS-22*

The SRS-24 has several advantages like being distinct and concise and having a high response rate. However, the second part of the scale is limited to patient evaluation after surgery. In 2000 and 2003, Asher introduced a modified SRS and SRS-22 questionnaire including five aspects of functional status, self-image, pain, psychological status, and satisfaction with treatment. This modified scale is also more accurate in some dimensions than the SF-36.

In addition, the SRS-22 can be used to evaluate QoL in patients after surgery for scoliosis as well as QoL in patients receiving conservative treatment of scoliosis. The SRS-22 is the world's most widely used scale to evaluate the QoL of patients with scoliosis. The SRS-22 has been translated into numerous languages such as Spanish, Japanese, and Turkish. Based on the English version of the SRS-22, Li *et al*. (*9,10*) created a Chinese (simplified) version of the SRS-22 that is culturally adapted. They concluded that it has good reliability and validity and can be used to clinically assess patients with AIS after surgery in China.

2.2.3. *SAQ*

Based on the WRVAS, Sanders *et al*. (*11*) created a new HRQoL questionnaire called the SAQ in 2007. The SAQ combines standardized images with a questionnaire to assess how patients and their families subjectively feel about a spinal deformity. Sanders also found that the SAQ was more sensitive and reliable in distinguishing an improvement in QoL after surgery than the SRS-22. Wei *et al*. (*12*) created a Chinese (simplified) version of the SAQ that was culturally adapted in accordance with international guidelines. They demonstrated its good reliability and validity in gauging how patients with AIS in China rated their appearance.

3. Analysis of the factors affecting QoL in patients with AIS

With the development of the Bio-Psycho-Social model of human behavior (*13,14*) and continuous revisions to relevant questionnaires, greater importance has been attached to factors that affect a patient's QoL. A study by Payne *et al*. (*3*) indicated that the presence of a spinal deformity was a risk factor for psychological depression regardless of the treatment the patient received. Adolescence is a sensitive period of personal and psychological maturity, so many factors like a deformity and physical discomfort can affect the QoL of patients with AIS.

3.1. *Disease factors: Severity of scoliosis*

Patients with AIS are most often seen for an abnormality such as incorrect body posture or left-right asymmetry of the shoulders. Since adolescence is a critical period of psychological development, the deformity caused by scoliosis may place a certain degree of social and psychological pressure on patients, and a more severe deformity will cause greater psychological stress.

AIS is a complex three-dimensional deformity. Spinal deformity in any plane can affect a patient's results on an HRQoL questionnaire. For postoperative patients with AIS, the Cobb angle of the instrumented thoracic curve is the main factor influencing QoL. A study by Helenius *et al*. (*15*) examined 98 consecutive patients who underwent surgery with a Harrington distraction rod and posterior spondylodesis. They found that the magnitude of thoracic curvature as assessed during follow-up an average of 21 years later

was significantly inversely correlated with scores for cosmetic aspects on the SRS-24. A study by Watanabe *et al*. (*16*) found that general self-image was inversely correlated with the Cobb angle and the rotation angle of the thoracic curve and that self-image after surgery was correlated with the extent of correction of the thoracic Cobb angle. These results indicate that the Cobb angle of the thoracic curve and radiographic parameters for evaluation of scoliosis in the axial plane greatly affect patient outcomes, and particularly how patients with AIS gauge perceive their appearance.

A study by Shang *et al*. (*17*) scored 46 patients with AIS using the Symptom Checklist 90 (SCL-90), Self-rating Depressive Scale (SDS), and Self-rating Anxiety Scale (SAS) as recommended by the Psychology Department of the 4th Military Medical University. They compared those scores to those of 50 healthy adolescent volunteers of the same age. They found that patients with severe AIS were more likely to have psychological problems, thus affecting their QoL, than patients with medium or mild AIS.

3.2. *Treatment factors*

3.2.1. *Conservative treatment*

Brace therapy is an important form of conservative treatment for patients with AIS, and this therapy can significantly reduce the severity and slow the progress of AIS (*18,19*). However, bracing is likely to cause adverse psychological stress that affects QoL. A study by Climent *et al*. (*20*) used QLPSD to assess the effect of various types of braces on QoL, and they found that patients treated with a Milwaukee brace scored significantly higher than patients treated with a Boston brace, especially in terms of psycho-social functioning. This which means that the Milwaukee brace has a greater impact on QoL. A study by Maruyama *et al*. (*21*) used the SRS-22 to evaluate the QoL of patients treated with a Milwaukee brace and they reached the same conclusion. Matsunaga *et al*. (*22*) used the Maudsley Personality Inventory to assess 145 adolescent females with idiopathic scoliosis to compare changes in personality after brace therapy. Of the 134 patients rated as normal before the start of therapy, 108 were rated as abnormal when tested 1 month after the start of therapy. After psychological intervention, 47 patients were finally rated as abnormal, which suggests that psychological testing combined with psychological treatment may reduce the negative psychological effects of brace therapy and facilitate modified bracing.

Some researchers believe that patients with AIS who undergo brace therapy may feel shy and have internal pressure as a result of lifestyle or studying. Therefore, the mental health of patients with AIS should be evaluated and monitored to reduce the negative psychological effects of brace therapy. Possible approaches include psychological testing to assess patient personality types before bracing and formulating personalized treatment plans for individual patients to provide a better QoL.

3.2.2. *Surgical treatment*

The effect of surgery on a patient's social and psychological functioning has received less attention in the literature than the effects of brace therapy on that functioning. Surgery is a major challenge for patients with AIS due to problems like pain and emotional distress during hospitalization, worries about surgical complications, and the disruption to one's social life during post-surgical recovery (*23*).

A complex disease, AIS is not readily treated with surgery and patients with AIS also have a high risk of suffering psychological illness, particularly as a result of characteristics like preoperative trait anxiety and a low level of cognitive development. Therefore, close attention must be paid to a patient's psychological state and psychological intervention must be provided when necessary in addition to correcting scoliosis.

3.3. *Individual factor: Gender*

Gender is a factor that affects the psychology of patients with AIS. Payne *et al*. (*2*) used the Adolescent Health Survey (AHS) to study 685 patients with AIS, 269 males and 416 females ranging in age from 12-18 years. The AHS is a comprehensive assessment of health status that attempts to ascertain all medical, social, and family circumstances that might have an impact on the health status of adolescents. The study's results indicated that scoliosis was an independent risk factor for more frequent suicidal thoughts, more concern about abnormal body development, and a greater worry and concern about peer relations. Male adolescents with scoliosis were 60% more likely to think they were underweight while female adolescents with scoliosis were 52% more likely to have suicidal thoughts than their peers. This implies that the impact of scoliosis and gender differences in patients may be greater than previously thought.

3.4. *Social factors*

3.4.1. *Differences between urban and rural areas*

China is a developing country with unevenly developing regional economies. There are considerable disparities in living conditions, income, and medical systems in urban and rural areas. Compared to rural areas, urban areas allow a more open lifestyle with a higher income and a better medical insurance system. These social factors are sure to affect the evaluation of a patient's QoL and these differences will be reflected in SRS-22

scores. A study by Wang *et al*. (*24*) used the SRS-22 to study the regional factors that affected patient QoL, and they found that urban patients had significantly higher scores in satisfaction with management of their disease and lower scores in self-image than did rural patients. This indicates that differences between urban and rural areas affect the evaluation of QoL.

3.4.2. *Family environment*

For patients with AIS, the family environment is also a factor that affects QoL. Kahanovitz and Weiser (*25*) studied 72 female adolescents with scoliosis ages 12-16 years, and they found that patients from single-parent families had a lower QoL and that the mother's attitude towards her child's illness had a highly positive effect on a child's attitudes toward treatment, thus improving his or her QoL. Unlike adults, most children fail to comply with the treatment and recovery process because of their specific physiological characteristics. The psychological state of parents directly affects their children, who are likely to adopt the behaviors and opinions of their parents. Therefore, the parent-child relationship should be emphasized when treating adolescent patients.

4. Discussion

In conclusion, the QoL of patients with AIS can be directly or indirectly affected by factors like disease, treatment, individual traits, and social circumstances. This fact is being realized by spinal surgeons. Comprehensive and effective questionnaires or scales for the specific disease (scoliosis, in this case) must be used to follow-up on a patient's QoL and early psychological intervention must be provided when needed. Radiological studies should not serve as the only method of postoperative evaluation in patients with AIS and evaluation should include assessment of QoL.

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Review

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ARID1B-mediated disorders: Mutations and possible mechanisms

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Summary **Mutations in the gene encoding AT-rich interactive domain-containing protein 1B (ARID1B) were recently associated with multiple syndromes characterized by developmental delay and intellectual disability, in addition to nonsyndromic intellectual disability. While the majority of *ARID1B* mutations identified to date are predicted to result in haploinsufficiency, the underlying pathogenic mechanisms have yet to be fully understood. ARID1B is a DNA-binding subunit of the Brahma-associated factor chromatin remodelling complexes, which play a key role in the regulation of gene activity. The function of remodelling complexes can be regulated by their subunit composition, and there is some evidence that ARID1B is a component of the neuron-specific chromatin remodelling complex. This complex is involved in the regulation of stem/progenitor cells exiting the cell cycle and differentiating into postmitotic neurons. Recent research has indicated that alterations in the cell cycle contribute to the underlying pathogenesis of syndromes associated with *ARID1B* haploinsufficiency in fibroblasts derived from affected individuals. This review describes studies linking *ARID1B* to neurodevelopmental disorders and it summarizes the function of *ARID1B* to provide insights into the pathogenic mechanisms underlying ARID1B-mediated disorders. In conclusion, ARID1B is likely to play a key role in neurodevelopment and reduced levels of wild-type protein compromise normal brain development. Additional studies are required to determine the mechanisms by which impaired neural development contributes to the intellectual disability and speech impairment that are consistently observed in individuals with *ARID1B* haploinsufficiency.**

Keywords: Intellectual disability, chromatin remodelling, Coffin-Siris syndrome, ARID1B mutation, cell cycle, haploinsufficiency

1. Introduction

Intellectual disability (ID) is an incapacitating condition that imposes a significant burden on affected individuals and their families. ID affects approximately 0.5% of all newborns and the overall incidence of ID is estimated to be 2-3% (*1*). Studies of X-linked, autosomal-recessive, syndromic, and sporadic cases of ID have resulted in the identification of several hundred genes associated with ID. In general, the relative incidence of mutations in each gene appears to be quite low. The latest findings have indicated that mutations in chromatin remodelling genes can cause ID in nonsyndromic and syndromic individuals.

The control of gene expression is an intricately regulated process that requires many multi-protein complexes. Chromatin remodelling regulates gene expression by modulating the access of transcription machinery proteins to the condensed genomic DNA *via* dynamic modification of the chromatin architecture. This modification is mediated by either covalent histone modifications *via* specific enzymes such as histone acetyltransferases or ATP-dependent alteration of DNA-nucleosome topology (*2*). The latter mode of modification is mediated by a class of protein complexes called ATP-dependent chromatin-remodelling complexes, which are known to regulate gene expression in specific cellular contexts or at

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defined time points (*3*). Mutations in the genes encoding subunits of these complexes have recently been linked to both developmental disorders and cancer (*4*).

A DNA-binding subunit of the Brahma-associated factor (BAF, also referred to as switching defective and sucrose non-fermenting SWI/SNF-α) chromatin remodelling complex named AT-rich interactive domain-containing protein 1B (ARID1B) was recently found to cause ID in both nonsyndromic and syndromic individuals. The first report of an individual with a phenotype likely attributable to a mutation in *ARID1B* was in 1998 (*5*). A large heterozygous deletion of 26 genes (including *ARID1B*) was identified in an individual with ID and agenesis of the corpus callosum. In 2009, Nagamani *et al*. reported heterozygous interstitial deletions affecting 6q.25.2-q25.3 (spanning *ARID1B*) in three individuals with developmental delay, microcephaly, facial characteristics, and hearing and speech impairments (*6*). Backx *et al*. subsequently documented a balanced translocation t(6;14)(q25.3;q13.2) that led to reciprocal fusion transcripts of *ARID1B* and *MRPP3* in an individual with ID and agenesis of the corpus callosum (*7*) and Nord *et al*. described an individual with autism who had a deletion of three exons in *ARID1B* (*8*). In the following year, haploinsufficiency of *ARID1B* was identified in four individuals with ID, autism, and corpus callosum abnormalities (*9*). Similarly, Michelson *et al*. reported an interstitial 1.19 Mb deletion of 6q25.2 including *ARID1B* and *ZDHHC14* in an individual with global developmental delay, facial characteristics, dysgenesis of the corpus callosum, limb anomalies, and genital hypoplasia (*10*). The phenotypic spectrum of *ARID1B*-mediated disorders was further broadened when Hoyer *et al*. noted haploinsufficiency for *ARID1B* in eight nonsyndromic/unselected individuals (approximately 1% of cases analyzed) with unexplained ID (*11*). In addition, mutations in *ARID1B* leading to haploinsufficiency were later identified in Coffin-Siris syndrome (CSS), which is characterized by ID, severe speech impairment, coarse facial features, microcephaly, developmental delay, and hypoplastic nails of the fifth digits (MIM 135900) (*12-14*). Mutations in other genes within the BAF complex have also been found to cause CSS, but *ARID1B* mutations account for approximately 70% of cases (*15*). In 2014, the phenotypic spectrum of CSS was further broadened when an individual with CSS with a *de novo* frameshift mutation in ARID1B presented with extreme obesity, macrocephaly, hepatomegaly, hyperinsulinism, and polycystic ovarian syndrome (*16*). Sim *et al*. reported a heterozygous 1.2 Mb deletion of 6q25.3, which contains *ARID1B*, *ZDHHC14*, and *TMEM242*, in an individual with a phenotype overlapping CSS but with distinctive features including plantar fat pads and facial dysmorphism (*17*). Additional analysis identified heterozygous *de novo ARID1B* frameshift and nonsense mutations in four additional affected individuals with a strikingly similar phenotype (*17*). Most recently, an individual with an apparently balanced, *de novo* translocation [t(5;6)(q11;q?24)], that resulted in the heterozygous loss of *ARID1B* and *ADAMTS6* was described. The phenotype included developmental delay, speech impairment and mild ID, hypotonia, hypermetropia, and microstrabismus (*18*). Dysmorphic features included thin upper lip vermilion, single transverse palmar creases, a funnel chest, brachydactyly, clinodactyly, fragile and grooved nails, and skewed flat feet.

Collectively, the heterozygous deletions and mutations of *ARID1B* are predicted to cause haploinsufficiency of ARID1B, leading to the aforementioned disorders. What is striking is the considerable clinical variability associated with reduced levels of ARID1B. A recent review identified the major features associated with ARID1B haploinsufficiency to be ID, speech delay, coarse facies, and hypertrichosis. Minor features, present in a smaller but significant proportion of individuals, included finger/toe abnormalities, feeding difficulties, agenesis of the corpus callosum, seizures, myopia, and growth delay (*15*). However, the data were primarily from individuals with a prior clinical diagnosis of CSS and therefore there is likely to be significant ascertainment bias. Although a range of clinical features have been mentioned here, the clinical features of nonsyndromic individuals with mutations in *ARID1B* may broaden the phenotypic spectrum considerably.

2. What is ARID1B?

ARID1B is a large, ubiquitous nuclear-localized protein of approximately 250 kDa. To date, the Consensus Coding Sequence (CCDS) database has reported that *ARID1B* encodes a large isoform of 2,249 amino acids (CCDS55072.1) and a smaller isoform of 2,236 amino acids (CCDS5251.2). The first functional studies of Arid1b analyzed the *Drosophila* protein (initially termed eyelid and subsequently renamed Osa), which was found to be important in embryonic segmentation, development of the notum and wing margin, and photoreceptor differentiation in flies (*19*). Subsequent studies using genetic and biochemical approaches indicated that the protein binds to DNA without sequence specificity and that the protein is a subunit of BAF complexes containing a core ATP-dependent helicase called Brahma (BRM) (*20,21*).

In humans, there are two paralogues of both Osa [AT-rich interactive domain-containing protein 1A (ARID1A) and ARID1B] and Brahma [BRM and Brahma Related Gene 1 (BRG1)]. ARID1B was found to bind to DNA without sequence specificity and is a component of BAF complexes containing either BRM or BRG1 (*22,23*). However, ARID1B and ARID1A are

mutually exclusive in BAF complexes (*22,24*).

Like Osa in *Drosophila* development, both ARID1A and ARID1B are important in mammalian embryogenesis. Haploinsufficiency for ARID1A was found to cause late embryonic lethality, whereas complete loss of ARID1A arrested development at E6.5 without formation of a primitive streak and mesoderm in mice (*25*). Deficiency of ARID1A was also reported to disrupt the pluripotency of mouse embryonic stem (ES) cells by inhibiting their self-renewal capacity and by promoting their differentiation into primitive endoderm-like cells. Similarly, ARID1B deficiency also reduced the self-renewal capacity of ES cells (*26*). In addition, ARID1B-deficient ES cells displayed features of differentiated cells, such as reduced expression of several pluripotency-related genes and increased expression of some differentiation-related genes.

Functional studies by Nagl *et al*. suggested that ARID1A and ARID1B are important to mammalian development by regulating the cell cycle during differentiation. Their studies indicated that ARID1A deficiency delayed the cell cycle arrest of mouse MC3T3-E1 pre-osteoblasts during osteogenic differentiation induced by ascorbic acid, while ARID1B deficiency had no impact on the kinetics of cell cycle arrest (*27*). Subsequent analyses of the kinetics of the cell cycle using serum deprivation and replenishment indicated that ARID1A and ARID1B have important and opposing roles in regulating cell cycle. ARID1A-deficient MC3T3 cells displayed delayed cell cycle arrest induced by serum starvation, whereas ARID1B deficiency had no impact on serum-starved cells (*28*). However, ARID1B deficiency delayed cell cycle entry of serum-starved cells during serum replenishment. Conversely, ARID1A-deficient cells shared similar kinetics of cell cycle entry with parental cells. The current authors also observed delayed cell cycle entry of serum-starved human fibroblasts derived from an individual with *ARID1B* haploinsufficiency and fibroblasts with ARID1B deficiency mediated by shRNAmir (*17*), a finding that coincides with the results of previous studies.

Molecular analysis using chromatin immunoprecipitation (ChiP) indicated that ARID1B regulates cell cycle entry by mediating the expression of *c-Myc*. ARID1B deficiency prevented the association of BAF complexes containing the subunits BAF155 or BAF170 with the *c-Myc* promoter. Therefore, the expression of *c-Myc* was not upregulated when serum-starved MC3T3 cells were replenished with serum, leading to delayed cell cycle entry (*28*). Conditional deletion of *c-Myc* is embryonically lethal in mice and has been found to decrease the size of the brain by disrupting the development of forebrain and hindbrain (*29,30*). Consistent with these findings, microcephaly has been noted in some individuals with *ARID1B* haploinsufficiency (*6,12,13*).

Both BAF155 and BAF170 are also important for correct neural development. BAF155 was reported to drive chromatin restructuring in mouse ES cells during neural differentiation induced by retinoic acid (*31*). Down-regulation of BAF155 resulted in reduced chromatin compaction and prolonged expression of self-renewal genes such as *Nanog* and *Oct4*, resulting in delayed neural differentiation of ES cells. In addition, heterozygous deletion of *Baf155* is embryonic lethal in mice and results in defective neural tube closure leading to exencephaly (*32,33*). BAF170 has been found to be an intrinsic factor that controls cortical size. Conditional deletion of *Baf170* was found to promote indirect neurogenesis by increasing the pool of intermediate progenitors and in turn result in an enlarged cortex (*34*). Similarly, overexpression of Baf170 promoted direct neurogenesis and resulted in the development of a smaller cortex. Collectively, these findings suggest that *ARID1B* haploinsufficiency partially impairs the function(s) of BAF complexes containing BAF155 and/or BAF170. This leads to dysregulation of the expression of *C-MYC*, delaying cell cycle entry of developmentally arrested cells, such as neural progenitors. These deficits may explain why

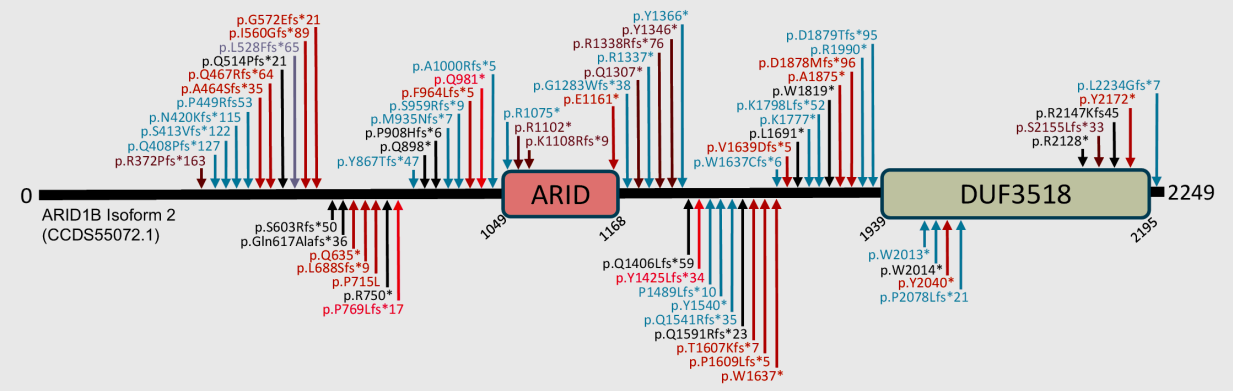

Figure 1. The protein domain organization of ARID1B and the distribution of ARID1B mutations associated with intellectual disability. Mutations in brown were identified by Hoyer *et al*. (*11*), those in blue were identified by Santen *et al*. (*12*), those in orange were identified by Tsurusaki *et al*. (*13*), those in black were identified by Wieczorek *et al*. (*14*), those in red were identified by Sim *et al*. (*17*), and those in purple were identified by Vals *et al*. (*16*).

Table 1. Summary of individuals with deletions affecting *ARID1B* and neighboring genes

Reference	Case	Approximate Deleted Region (Hg19)	Genes Affected
Pirola *et al.*, 1998	1	chr6:151,225,045-158,663,897	*MTHFD1L, AKAP12, ZBTB2, RMND1, C6orf211, ESR1, SYNE1, MYCT1, VIP, FBXO5, MTRF1L, RGS17, OPRM1, IPCEF1, CNKSR3, SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5*
Nagamani *et al*., 2009	1	chr6:155,085,617-158,876,467	*SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4*
	2	chr6:154,841,486-161,623,426	*SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4, TMEM181, DYNLT1, SYTL3, EZR, OSTCP1, RSPH3, C6orf99, TAGAP, FNDC1, SOD2, WTAP, ACAT2, TCP1, SNORA20, SNORA29, MRPL18, PNLDC1, MAS1, IGF2R, AIRN, SLC22A1, SLC22A2, SLC22A3, LPAL2, LPA, PLG, MAP3K4, AGPAT4-IT1*
	3	chr6:149,951,406-160,276,072	*KATNA1, LATS1, NUP43, PCUMT1, LRP11, RAET1E, RAET1G, ULBP2, ULBP1, RAET1K, RAET1L, ULBP3, PPR1R14C, IYD, PLEKHG1, MTHFD1L, AKAP12, ZBTB2, RMND1, C6orf211, ESR1, SYNE1, MYCT1, VIP, FBXO5, MTRF1L, RGS17, OPRM1, IPCEF1, CNKSR3, SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4, TMEM181, DYNLT1, SYTL3, EZR, OSTCP1, RSPH3, C6orf99, TAGAP, FNDC1, SOD2, WTAP, ACAT2, TCP1, SNORA20, SNORA29, MRPL18, PNLDC1*
	4	chr6:155,336,861-169,178,124	*TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4, TMEM181, DYNLT1, SYTL3, EZR, OSTCP1, RSPH3, C6orf99, TAGAP, FNDC1, SOD2, WTAP, ACAT2, TCP1, SNORA20, SNORA29, MRPL18, PNLDC1, MAS1, IGF2R, AIRN, SLC22A1, SLC22A2, SLC22A3, LPAL2, LPA, PLG, MAP3K4, AGPAT4-IT1, PARK2, PACRG, CAHM, QKI, C6orf118, PDE10A, LINC00473, LINC00602, T, PRR18, SFT2D1, MPC1, RPS6KA2, RNASET2, FGFR1OP, CCR6, GPR31, TCP10L2, UNC93A, TTLL2, TCP10, MLLT4, HGC6.3, KIF25, FRMD1, DACT2, SMOC2*
Nord *et al*., 2011	1	chr6:157,250,871-157,462,426	*ARID1B*
Halgren *et al.*, 2012	2	chr6:157,210,495-157,467,930	*ARID1B*
	3	chr6:157,079,676-157,806,675	*ARID1B, TMEM242, ZDHHC14*
	4	chr6:156,190,443-158,076,922	*ARID1B, TMEM242, ZDHHC14*
	5	chr6:155,797,565-158,517,307	*ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2*
	6	chr6:152,497,968-157,996,910	*SYNE1, MYCT1, VIP, FBXO5, MTRF1L, RGS17, OPRM1, IPCEF1, CNKSR3, SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14*
	7	chr6:151,019,422-159,187,660	*PLEKHG1, MTHFD1L, AKAP12, ZBTB2, RMND1, C6orf211, ESR1, SYNE1, MYCT1, VIP, FBXO5, MTRF1L, RGS17, OPRM1, IPCEF1, CNKSR3, SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4, TMEM181, DYNLT1, SYTL3, EZR*
	8	chr6:153,073,486-167,754,128	*VIP, FBXO5, MTRF1L, RGS17, OPRM1, IPCEF1, CNKSR3, SCAF8, TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4, TMEM181, DYNLT1, SYTL3, EZR, OSTCP1, RSPH3, C6orf99, TAGAP, FNDC1, SOD2, WTAP, ACAT2, TCP1, SNORA20, SNORA29, MRPL18, PNLDC1, MAS1, IGF2R, AIRN, SLC22A1, SLC22A2, SLC22A3, LPAL2, LPA, PLG, MAP3K4, AGPAT4-IT1, PARK2, PACRG, CAHM, QKI, C6orf118, PDE10A, LINC00473, LINC00602, T, PRR18, SFT2D1, MPC1, RPS6KA2, RNASET2, FGFR1OP, CCR6, GPR31, TCP10L2, UNC93A, TTLL2*
Santen *et al.*, 2012	5	chr6:157,079,676-157,806,675	*ARID1B, TMEM242, ZDHHC14*
	6	chr6:157,144,644-158,028,969	*ARID1B, TMEM242, ZDHHC14*
Hoyer *et al.*, 2012	1	chr6:155,364,154-157,681,073	*TIAM2, TFB1M, CLDN20, NOX3, ARID1B*
	2	chr6:157,299,982-157,474,352	*ARID1B*
Wieczorek *et al.*, 2013	K2428	chr6:157,402,040-157,460,542	*ARID1B*
	K2438	chr6:156,960,439-158,889,653	*ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4*
Santen *et al*., 2014	24	not available	*Deleted ARID1B exons 1-20*
	47	not available	*Deleted ARID1B exons 6-9*
Sim *et al*., 2014	1	chr6:156,897,183-158,222,240	*ARID1B, TMEM242, ZDHHC14*
Vengochea *et al*., 2014	1	chr6:155,538,131-158,756,793	*TIAM2, TFB1M, CLDN20, NOX3, ARID1B, TMEM242, ZDHHC14, SNX9, SYNJ2, SERAC1, GTF2H5, TULP4*

Case 1 reported by Pirola *et al*. (*5*) has a heterozygous deletion between FISH loci D6S1496 and D6S437. Case 1 described by Sim *et al*. (*17*) has a heterozygous deletion between (Hg18) chr6:156938875-158142228. For both individuals, the deleted region was converted to Hg19 coordinates using UCSC genome browser.

ID is consistently found in individuals with *ARID1B*-mediated disorders.

3. Impact of ARID1B mutations

Despite being a protein of over 2,000 amino acid residues, ARID1B has only two defined protein domains, an AT-rich Interactive Domain (ARID) and Domain of Unknown Function 3518 (DUF3518) (Figue 1). ARID consists of approximately 100 amino acid residues and has been found to bind to DNA without sequence specificity (*22,23*). Missense mutations in this domain are likely to disrupt the DNA-binding ability of ARID1B and compromise the function of the BAF complex. DUF3518 is approximately 260 amino acids long and biochemical studies have indicated that the domain interacts with the helicase subunits BRG1 and BRM in BAF complexes (*24,35*). Therefore, missense mutations in DUF3518 are likely to disrupt the interaction between ARID1B, BRG1, and BRM. Collectively, missense mutations in either domain would presumably have a negative impact by rendering BAF complexes dysfunctional (if the resulting mutant ARID1B protein was stable). However, a striking feature of studies investigating *ARID1B*-mediated disorders is that there is only a single reported missense mutation (p.Pro715Leu) in comparison to more than 60 nonsense or frameshift mutations (Figure 1). However, this may reflect ascertainment bias in the clinical cohorts studied to date, as was mentioned earlier. Most nonsense and frameshift mutations activate nonsense-mediated mRNA decay (NMD) because the mutation causes premature termination of translation that results in incomplete displacement of exon junction protein complexes by the ribosomes (*36*). Thus, these mutations are likely to cause NMD of the *ARID1B* transcript rather than the expression of mutant ARID1B protein. Truncating mutations that avoid NMD usually cause a distinct and more severe phenotype than that observed in NMD due to the dominant negative effects of the mutant protein (*37*).

The other major class of *ARID1B* mutations observed to date involves copy number variations (CNV), and particularly heterozygous deletions (Table 1). In most affected individuals, the additional genes lost could potentially contribute to phenotypic variability. However, no obvious correlation between variable clinical phenotypes and specific types of *ARID1B* mutations has been observed thus far. Moreover, there are several individuals with deletion of *ARID1B* and multiple additional genes that present with a phenotype indistinguishable from individuals with truncating and frameshift *ARID1B* mutations (*11,12,17,38*). In a recent study by the current authors, affected individuals with frameshift and truncating *ARID1B* mutations had a phenotypic presentation very similar to that of an affected individual with a heterozygous deletion of *ARID1B*, *ZDHHC14*, and *TMEM242* (*17*). Therefore, the clinical presentation appears likely to manifest predominantly from *ARID1B* haploinsufficiency rather than the deletion of other genes. Collectively, these findings indicate that the primary pathogenic mechanism in most individuals with an *ARID1B*-mediated disorder who have been described thus far is the result of *ARID1B* haploinsufficiency. Additional studies are required to delineate the mechanisms underlying phenotypic variability associated with *ARID1B* haploinsufficiency and a consortium has recently been established to address this issue (*15*).

4. Conclusions

The predominant mechanism underlying *ARID1B*-mediated disorders appears to be *ARID1B* haploinsufficiency. Why the phenotypic presentation is so variable is a question that has yet to be answered, although there is evidence from *in vitro* studies and animal models that reduced levels of ARID1B can disrupt regulation of the cell cycle. Given that the BAF complex consists of over 25 core and interchangeable protein subunits that give rise to functionally distinct and cell-type specific complexes, variation in these components is likely to contribute to the observed phenotypic variability of *ARID1B*-mediated disorders (*39*). Nonetheless, ID and speech impairment are consistently observed. Although a specific role for ARID1B in early brain development has yet to be identified, the gene is predominantly expressed in neural tissues in the developing mouse embryo (*40*). Thus, *ARID1B* is likely to be important to development of the brain when multipotent neuroepithelial cells are actively proliferating. Future studies will need to investigate if impaired neural development contributes to the ID and speech impairment that characterize individuals with *ARID1B* haploinsufficiency.

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Review

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Budd-Chiari syndrome and liver transplantation

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Summary **Budd-Chiari syndrome involves obstruction of hepatic venous outflow tracts at various levels from small hepatic veins to the inferior vena cava and is the result of thrombosis or its fibrous sequelae. There is a conspicuous difference in its etiology in the West and the East. Myeloproliferative disease predominates in the West and obstruction of the vena cava predominates in the East. The clinical presentation and clinical manifestations are so varied that it should be suspected in any patient with acute or chronic liver dysfunction. It should be treated with step-wise management. First-line therapy should be anticoagulation with medical treatment of the underlying illness, and interventional revascularization and TIPS are indicated in the event of a lack of response to medical therapy. Liver transplantation may be indicated as a rescue treatment or for fulminant cases with promising results. This step-by-step strategy has achieved a 5-year transplant-free survival rate of 70% and a 5-year overall survival rate of 90%. Living donor liver transplantation can also be used for patients with Budd-Chiari syndrome if deceased donor livers are scarce, but it requires a difficult procedure particularly with regard to venous outflow reconstruction.**

Keywords: Budd-Chiari syndrome, liver transplantation, deceased donor, living donor

1. Introduction

Budd-Chiari syndrome (BCS) is a rare disease with a multifactorial etiology and is characterized by obstruction of the hepatic venous outflow anywhere from the intrahepatic venules to the suprahepatic portion of the inferior vena cava (IVC). Regardless of its etiology, the subsequent increase in hepatic sinusoidal pressure will lead to portal hypertension and related clinical sequelae (*1*).

BCS varies greatly in terms of its etiology, clinical presentation, and management. The clinical presentation may be asymptomatic, chronic, or fulminant. The treatment strategy varies from medical anticoagulant and antithrombotic treatment to surgical therapy including liver transplantation (*2-4*).

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The aim of this review was to encompass the updated practical management of this disease entity with special reference to liver transplantation.

2. Etiology and epidemiology

BCS is not a primary disease of the liver parenchyma but subsequent liver dysfunction following obstruction of hepatic veins or the suprahepatic IVC. Hepatic venous outflow obstruction results in an elevated sinusoidal pressure and leads to hepatic congestion. Usually, congestion is followed by subsequent centrilobular fibrosis and nodular regenerative hyperplasia that lead to chronic liver dysfunction and cirrhosis; in some instances, however, it results in fulminant hepatic failure requiring emergency liver transplantation (*5*).

There is an interesting but not as yet understood difference in the etiology and epidemiology of this condition in the West and East (*6*). Recent studies from Western countries have revealed that primary BCS can be regarded as a multifactorial disease in which several prothrombotic conditions additively predispose patients to develop thrombosis in hepatic veins (*3,7,8*). Common prothrombotic conditions associated with BCS include inherited and acquired hypercoagulable states.

Inherited conditions such as factor V Linden mutation (*9,10*), protein C/S deficiency (*3,11,12*), antithrombin III deficiency (*12*), and the prothrombin G20210A mutation (*13*) are common causes of hepatic venous thrombosis resulting in BCS. Acquired hypercoagulable condition such as myeloproliferative disorders (MPD) including polycythemia vera, paroxysmal nocturnal hemoglobinuria, essential thrombocytosis, agnogenic myeloid metaplasia, and myelofibrosis account for 30% to 50% of BCS cases in Western countries (*8*). Recently, a particular somatic mutation (V617F) in the Janus tyrosine kinase-2 (JAK2) gene in myeloid cells was identified in a study of chronic MPD (*14*). This mutation has been detected in around 40% of patients with primary BCS (*15-17*). Other causes of BCS such as Behcet's disease (*18,19*), antiphospholipid syndrome (*20*), and oral contraceptives (*21*) have been reported.

In Western countries, a prothrombotic condition leading to hepatic venous thrombosis is most often the cause of BCS, while in Eastern countries BCS is most often caused by the membranous obstruction of the vena cava (MOVC) or primary IVC thrombosis (*6,22*). Reports from China (*23*), Nepal (*24*), India (*25*), and South Africa (*26*) have revealed that almost all cases of BCS were caused by MOVC. This has also been the case in Japan, although hypercoagulable patients with hepatic vein thrombosis appear to be increasing (*27*). The reason for these epidemiological discrepancies has yet to be fully elucidated, but a possible explanation may be that thrombophilic genetic changes are more common among Caucasians and thus result mostly in hepatic venous thrombosis, while some infections are more common in Eastern countries that predispose Asians to IVC thrombosis (*6,28,29*).

3. Clinical presentation, diagnosis, and prognosis

The clinical presentation of BCS may be fulminant (5%), acute (20%), or subacute/chronic (60%) (*5*), though 15-20% of patients with BCS may be asymptomatic (*30*). Although nonspecific, the triad of hepatomegaly, abdominal pain, and ascites is typically present in patients with BCS. Lower extremity edema is also frequently encountered. Nausea, vomiting, and jaundice can develop more often in patients with fulminant or acute forms of BCS. In contrast, findings associated with portal hypertension such as splenomegaly, esophageal varices, encephalopathy, and gastrointestinal bleeding are more commonly seen in the chronic form. In some patients, the hepatic sinusoids may be completely decompressed *via* large portosystemic and intrahepatic collaterals so that they are completely asymptomatic (*31*).

Fulminant BCS: Fulminant BCS develops within a few days, presenting as acute liver failure with elevated liver enzymes, hyperbilirubinemia, coagulopathy, and encephalopathy (*32*). The liver is severely enlarged, with massive ascites and acute renal failure. This is an absolute indication for liver transplantation.

Acute BCS: Acute BCS develops usually within 1 month and is characterized by intractable ascites, abdominal pain, liver enlargement, renal failure, elevated liver function test results, and coagulopathy (*33*).

Subacute BCS: Subacute BCS is the most common clinical form of BCS in prothrombotic patients. This form of BCS has an insidious onset and may take as long as 3 months to become asymptomatic with the development of decompressive collaterals. Treatment should be started during the subacute phase before BCS becomes chronic (*33*).

Chronic BCS: This form is characterized by the development of portal hypertension. There is marked abdominal distension due to massive ascites, while liver function test results are minimally affected or normal. Renal failure is seen in 50% of patients and esophageal variceal bleeding is encountered in 15-20% of these patients (*33*).

3.1. *Diagnosis*

Physicians should always keep BCS in mind when seeing patients with acute or chronic liver disease. BCS is more likely when there is no other, more common, cause of liver disease or when there is a known underlying prothrombotic condition. The essential point is to consider BCS in patients with known prothrombotic states who present with abnormal liver function test results, upper abdominal pain, and ascites.

Definitive diagnosis is based on evidence of an obstructed hepatic outflow including that in the hepatic veins or suprahepatic IVC, and in principle, is reached based on findings of dilation of the hepatic veins upstream of an obstacle, the presence of a thrombus in the hepatic veins or IVC, the transformation of the veins into a cord devoid of flow signal, and venous collaterals depicted as an abnormally enhanced vessels branching to or from the hepatic veins or IVC. Doppler ultrasonography, contrast-enhanced triphasic computed tomography, and magnetic resonance imaging are sufficient to reveal these diagnostic features of BCS (*31,34*). Nowadays, there is no call for direct or retrograde venography solely to diagnose BCS, but patients with BCS are usually referred to an interventional radiologist to confirm the diagnosis and for therapeutic interventions (*35*). Catheter venography is considered the reference standard for the diagnosis of BCS. It provides anatomical information by directly depicting venous problems, hemodynamic and venous pressure measurements, and histologic information by facilitating a transjugular liver biopsy and it allows the possibility of endovascular management of the outflow obstruction.

3.2. *Prognosis*

Incidentally found, asymptomatic forms of BCS have

a good prognosis (*30*), but symptomatic forms have a poor spontaneous course; an estimated 90% of untreated patients die within three years (*36*). Circumstances have changed with advances in the management of BCS, allowing a 5-year survival rate on the order of 90% (*4*). A step-wise management strategy aimed at minimal invasiveness is recommended by expert panels is based on the response to previous therapy rather than on the actual severity of the condition, and this strategy has considerably improved the survival expectancy and the quality of life for patients with BCS (*2,37,38*). Several prognostic indices (PI) have been evaluated to predict outcomes for patients with BCS such as the Child-Pugh score (*39*), model for end-stage liver disease (MELD) score (*40*), Clichy PI (*41,42*), Rotterdam BCS PI (*43*), and BCS-TIPS PI (*44*). The BCS-specific PI, Rotterdam PI, and BCS-TIPS PI appear to be preferable for clinical studies but insufficient for individual management (*37,45*).

4. Treatment

Therapeutic approaches to treat BCS are diverse and should be adapted depending on disease severity. Recently, an expert panel advocated a step-wise approach to treating BCS; 1. Anticoagulation, 2. Angioplasty and stenting, 3. TIPS or surgical shunt, and 4. Liver transplantation (*2*). A recent report from Europe (*37*) revealed that this step-wise management has improved the 5-year patient survival to 72% and transplant-free survival to 72%.

4.1. *Anticoagulation therapy*

Logically, prompt recognition and initiation of treatment of the underlying disease is recommended when BCS is diagnosed, and anticoagulation therapy should immediately be started even for asymptomatic patients (*46*). Although specific therapy for underlying prothrombotic disease is crucial, this aspect is beyond the scope of this article. Low molecular weight heparin for the initiation of anticoagulation therapy and subsequent long-term anticoagulation with warfarin to achieve an international normalized ratio for prothrombin time of 2.0 to 2.5 are recommended (*2*).

4.2. *Angioplasty and stenting*

Catheter-directed thrombolytic therapy, angioplasty, and stent placement may be effective in treating acute BCS. Thrombolytic therapy is considered for patients with the acute form of BCS and especially in rare situations where angiography reveals a fresh thrombus. Urokinase (240,000U per hour for 2 hours, followed by 60,000U per hour) or tissue plasminogen activator (0.5 to 1mg per hour) is infused directly into the thrombosed hepatic vein for about 24 hours *via* an inserted catheter (*1,47*). Percutaneous or transhepatic angioplasty of localized segments of the narrowed hepatic vein or IVC membranous obstruction may relieve symptoms in more than 70 percent of patients (*48,49*). Short stenosis either of the hepatic veins or of the IVC is found in about a third of patients, and the restoration of outflow through just one of the three main hepatic veins is usually sufficient to relieve symptoms (*50*) . Stent insertion may be considered if there is an inadequate response to balloon angioplasty or it may be reserved for cases of recurrent stenosis or occlusion. Unfortunately, however, angioplasty in combination with anticoagulation therapy has been reported to successfully control BCS in only 20-30% of patients, at least in reports from Western countries where hepatic vein thrombosis predominates (*38*). In contrast, a Chinese report of 115 patients noted success rates of 94% and 87% for stents placed in the IVC and hepatic veins, respectively (*51*).

4.3. *Transjugular intrahepatic portosystemic shunt (TIPS) and surgical shunts*

In patients with BCS that is not fully controlled by the aforementioned treatments, the next step is either TIPS or a surgical shunt.

For patients presenting weeks to months after hepatic vein thrombosis, the obstruction is generally no longer amenable to thrombolysis or angioplasty. TIPS is recommended as the next step in management. TIPS is useful in patients with an occluded IVC, those in whom the portal vein-IVC pressure gradient is less than 10 mmHg, and those with poor liver function reserve. TIPS is also recommended for those with the acute form of BCS who failed to respond to thrombolytic therapy (*2,37*). TIPS is the most common intervention for BCS in Europe, and many studies have reported its high success rate and relatively low rate of complications (*52-55*). Compared to an open surgical shunt, TIPS is associated with lower morbidity and mortality (*56,57*), but its drawback is frequent shunt occlusions requiring repeated interventions. The development of covered stents, however, has significantly improved the patency of TIPS in BCS (*53,54*). Rossle *et al*. (*58*) achieved initial success in 33 of 35 patients with 1- and 5-year transplant-free survival rates of 93% and 74%, respectively. In another study, TIPS was successfully performed in 124 of 133 patients with a clinical success rate of 84% (*44*). A recent European multicenter study reported a 5-year transplant-free survival rate of 72% in 157 patients with BCS who were treated with TIPS (*37*).

A surgical portosystemic shunt is recommended for patients with the subacute form of BCS when the underlying disease is associated with a favorable long-term outcome, patients have preserved liver function (Child-Pugh class A), and a liver biopsy reveals ongoing hepatic necrosis (*59*). A pressure gradient between the portal vein and IVC of more than 10 mmHg is

associated with a successful long-term outcome. Surgical shunts include a side-to-side portocaval shunt, a central splenorenal shunt, and a mesocaval shunt. The 5-year survival rate after surgical shunting ranges from 75% to 94%, with the higher end of the range being achieved when the IVC is not occluded (*60,61*). The essential aspect is to use a side-to-side portocaval shunt in the early stage of BCS to achieve an excellent outcome for patients with BCS (*59,62*). The rationale for surgical portosystemic shunting is to convert the portal flow into an outflow tract of the liver, and some patients with the severe form of BCS may potentially benefit from this procedure. However, no studies have described the survival benefit of surgical shunts (*42*). In light of advances in the TIPS procedure and accumulated evidence showing the impact of TIPS on patient survival, TIPS is preferred as the first choice for safe and optimal decompression.

5. Liver transplantation for BCS

In the remaining 10% to 20% of patients with BCS treated with a step-wise management strategy, anticoagulation, angioplasty, and TIPS fail either due to technical failure or to poor clinical results of a technically successful procedure resulting in the need for rescue transplantaion. Liver transplantation may also be the treatment of choice in patients with fulminant liver failure and those with highly advanced liver cirrhosis (*3*).

5.1. *Deceased donor liver transplantation for BCS in Western countries*

A search of the literature indicated that more than 1000 patients with BCS have undergone liver transplantation. Table 1 summarizes recent reports of liver transplant for a considerable number (> 10) of patients with BCS

Table 1. Deceased donor liver transplantation for BCS in Western countries

Author	Year	Country	Period studied	*n*	Etiology (*n*)	Indication (*n*)	Shunt *n* (%)	Follow-up	90-day mortality	Survival
Srinvasan *et al*. (*63*)	2002	UK	1988-1999	19	MPD (11) PD (2) Others (6)	Advanced cirrhosis (13) Acute liver failure (6)	5 (26%)	1-119 months (median 89)	5%	95% (1 year) 95% (3 years) 95% (5 years)
Cruz *et al*. (*64*)	2005	US	1988-2002	11	MPD (8) PD (1) Others (2)	Advanced cirrhosis (8) Acute liver failure (3)	5 (45%)	1-132 months (median 56)	11%	81% (1 year) 65% (5 years) 65% (10 years)
Ulrich *et al*. (*65*)	2008	Germany	1988-2006	42	MPD (13) PD (11) Others (18)	Advanced cirrhosis (22) Acute liver failure (20)	10 (24%)	1-203 months (median 96)	7%	92% (1 year) 89% (5 years) 84% (10 years)
Chinnakotla *et al*. (*66*)	2011	US	1987-2007	25	MPD (15) PD (6) Others (4)	Advanced cirrhosis (23) Acute liver failure (2)	3 (12%)	7-264 months (median 96)	4%	92% (1 year) 88% (5 years) 72% (10 years)
Mackiewicz *et al*. (*67*)	2012	Poland	2000-2009	24	MPD (3) PD (7) Others (14)	The Advanced cirrhosis (18) Acute liver failure (6)	6 (25%)	NA	13%	80% (1 year) 80% (3 years) 80% (5 years)
Seijo *et al*. (*37*)	2013	Europe Multicenter	2003-2009	20	NA	Advanced cirrhosis (6) Acute liver failure (14)	6 (30%)	0.1-74 months (median 50)	NA	95% (1 year) 89% (3 years) 78% (5 years)
Registry study										
Mentha *et al*. (*68*)	2006	Europe Registry	1988-1999	248	MPD (116) PD (71) Others (61)	Advanced cirrhosis (136) Acute liver failure (50)	57 (23%)	Median 48 months	21%	76% (1 year) 72% (5 years) 68% (10 years)
Segev *et al*. (*69*)	2007	US Registry	1987-2006	510	NA	Unknown (62) NA	NA	NA	15%	82% (1 year) 76% (3 years)

MPD, myeloproliferative disorder; PD, Prothrombotic disease; NA, not available

Table 2. Living donor liver transplantation for BCS in Asian countries

Author	Year	Country	Type of report	Age/ Gender	Etiology	Indication	Shunt	Graft type	Outflow reconstruction	Outcome
Haberal *et al.* (*74*)	1999	Turkey	Case report	17/F	Unknown	AC	No	Left lobe	Auxiliary heterotopic partial liver transplantation	Alive, 4 months
Nezakatgoo *et al.* (*75*)	1999	Iran	Case report	14/M	Unknown	AC	No	Left lobe	NA	Alive, 2 months
Yamada *et al.* (*71*)	2006	Japan	Case series	3/M	MOVC	AC	No	Left lateral sector	IVC-Left hepatic vein, piggy-back	Alive, 15 years
				10/M	MOVC	AC	No	Left lobe	IVC patch plasty, piggy back	Alive, 7 years
				11/M	MOVC	AC	No	Left lobe	IVC-Left hepatic vein, piggy-back	Dead, 1 month
				11/M	PD	AC	No	Left lobe	IVC patch plasty, piggy back	Alive, 7 years
				26/M	MOVC	AC	Yes	Right lobe	IVC patch plasty, piggy back	Dead, 17 months
				27/M	MOVC	AC	No	Right lobe	IVC patch plasty, piggy back	Alive, 16 months
				39/M	MOVC	ALF	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 6 years
				32/F	MPD	AC	No	Right lobe	IVC patch plasty, piggy back	Alive, 4 years
				17/M	PD	AC	No	Left lobe	IVC-Left hepatic vein, piggy-back	Alive, 10 months
Yan *et al.* (*76*)	2006	China	Case report	35/M	MOVC	AC	Yes	Right lobe	IVC interposition with cryopreserved homologous IVC	Alive, 3 months
Shimoda *et al.* (*77*)	2007	Japan	Case report	40/F	MOVC	AC	Yes	Right lobe	IVC interposition with autologous veins	Alive,17 months
Liu *et al.* (*78*)	2008	Taiwan	Case report	11/M	MOVC	AC	No	Left lobe	IVC patch plasty, piggy back	Alive, 2 years
Sasaki *et al.* (*79*)	2009	Japan	Case report	10/M	MOVC	AC	Yes	Left lateral sector	IVC interposition with cryopreserved homologous IVC	Alive, 2 months
Kawaguchi *et al.* (*80*)	2009	Japan	Case report	20/F	MPD	AC	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 1 month
Kazimi *et al.* (*81*)	2009	Turkey	Case report	29/M	MOVC	AC	No	Right lobe	Direct anastomosis right atrium and right hepatic vein, end-to-end	Alive, 3 months
Choi *et al.* (*72*)	2010	Korea	Case series	44/F	MOVC	AC	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 2 years
				45/M	MOVC	AC	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 18 months
				50/F	MOVC	AC	No	Right lobe	IVC interposition with cryopreserved homologous IVC	Alive, 13 months
				41/F	MOVC	AC	Yes	Right lobe	PTFE graft interposition between right atrium and right hepatic vein	Alive, 2 years
Shirai *et al.* (*82*)	2011	Japan	Case report	26/M	MOVC	ALF	No	Left lobe	IVC-Left hepatic vein, piggy-back	Alive, 1 year
Ogura *et al.* (*83*)	2011	Japan	Case report	36/M	MOVC	AC	Yes	Right lobe	IVC interposition with PTFE graft	Alive, 2 years
Soyama *et al.* (*84*)	2011	Japan	Case report	63/M	Unknown	ALF	No	Right lobe	Thrombectomy, IVC-Right hepatic vein, piggy-back	Alive, 1 month
Iwasaki *et al.* (*85*)	2012	Japan	Case report	22/F	MPD suspected	AC	No	NA	NA	Alive, 4 years
Sakcak *et al.* (*86*)	2012	Turkey	Case report	12/F	Iatrogenic	AC	Yes	Left lateral sector	IVC interposition with cryopreserved homologous aorta	Alive, 4 months
Bas *et al.* (*73*)	2012	Turkey	Case series	34/M	MPD	AC	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 30 months
				27/F	MPD	AC	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 18 months
				25/F	MPD	AC	No	Right lobe	IVC-Right hepatic vein, piggy-back	Alive, 6 months
Fukuda *et al.* (*87*)	2013	Japan	Case report	34/F	MOVC	AC	Yes	Left lobe	Supraphrenic vena cava-Left hepatic vein, end-to-end	Alive, 5 years
The present report		Japan	Personal experience	43/M	Unknown	ALF	No	Right lobe	IVC patch plasty, piggy back	Alive, 10 years
				52/M	MOVC	AC	No	Right lobe	Interposition with cryopreserved homologous IVC between right atrium and right hepatic vein	Alive, 14 months

IVC, inferior vena cava; PTFE, polytetrafluoroethylene; MOVC, membranous obstruction of the vena cava; MPD, myeloproliferative disorder; PD, Prothrombotic disease; AC, Advanced cirrhosis; ALF, Acute liver failure; NA, not available.

(*37,63-69*). The early mortality rate in these reports ranged from 4% to 21%, and the 1- and 5-year survival rates ranged from 80% to 95% and from 65% to 95%, respectively. All of these outcomes seem acceptable in comparison to those for other diseases requiring liver transplantation. Recently, large retrospective database studies were reported in Europe (European Liver Transplantation Registry [ELTR]) and the United States (United Network for Organ Sharing [UNOS] registry). Segev *et al*. (*69*) examined the UNOS database of recipients who underwent liver transplantation for BCS (n = 510) from 1987 to 2006 and found that 1- and 3-year patient survival rates were 82% and 76%, respectively. When stratified based on use of MELD, patients treated during the days of MELD (n = 100, 3-year patient survival of 85%) had significantly better survival than those treated prior to MELD (n = 168, 3-year patient survival of 73%). A longer cold ischemic time (> 12 hr), preoperative life support, and retransplantation were found to be independent risk factors for poor patient survival, while preceding TIPS appeared to have no impact on patient prognosis. Representing the ELTR, Mentha *et al*. (*68*) reviewed 248 patients who underwent liver transplantation for BCS from 1988 to 1999. They reported overall 1-, 3-, and 5-year patient survival rates of 76%, 75%, and 72%, respectively. They found that renal failure and the presence of a shunt were independent predictors for patient survival.

5.2. *Living donor liver transplantation for BCS in Asian countries*

Due to the severe scarcity of deceased-donor liver grafts, living-donor liver transplantation (LDLT) has been the mainstay for patients with end-stage liver disease and acute liver failure, including BCS, in most Asian countries (*70*). A search of the English literature yielded 30 patients with BCS who underwent LDLT; all were from Asian countries. This literature search included 3 case series reports (*71-73*) and 14 case reports (*74-87*) (Table 2). Additionally, the current authors encountered two cases of BCS out of 535 cases of LDLT at the University of Tokyo Hospital, and these cases are also shown in Table 2. Most cases (19/32) were from Japan, six cases were from Turkey, four cases were from South Korea, one was from Iran, one was from China, and one was from Taiwan. The etiology of eighteen cases (60%) were MOVC, which was as expected. This indicates the common difference in epidemiology in the East and the West (*6*). A point worth mentioning is outflow reconstruction during LDLT for recipients with BCS; the deceased donor graft includes the hepatic IVC and hepatic veins, and the removal and replacement of the native hepatic IVC with a cavo-caval anastomosis between the recipient's native superior/inferior IVC and the donor IVC is easily accomplished. In contrast, the piggy back technique for LDLT involves the preservation of the recipient's IVC, and anastomosis between the recipient's IVC and graft hepatic vein is mandatory in the absence of the donor IVC. Thus, LDLT presents substantial challenges in terms of treating BCS. The key consideration for using LDLT to treat BCS is the management of a stenotic or occluded native IVC and the choice of techniques used to reconstruct the hepatic outflow. Many of the reports listed in Table 2 described venous reconstruction in various fashions, as briefly explained in the table. As in the literature, one of cases of MOVC that the current authors encountered required difficult outflow reconstruction. This was the interposition of the cryopreserved homologous IVC between the right atrium and the right hepatic vein. Replacement and interpositioning of the IVC with the vascular graft was done in 7 cases (22%), direct reconstruction of the outflow to the atrium (or supraphrenic IVC) was done in 4 (13%), and patch plasty of the IVC was required in 7 (22%).

Although the long-term outcomes of LDLT for patients with BCS could not be determined from the case report literatures, the Japanese Liver Transplant Society recently published a report on the nationwide LDLT registry in Japan. That report identified 41 cases of BCS. According to that report, the 1-, 3-, 5-, and 10-year cumulative patient survival rates after LDLT for BCS were 89%, 84%, 81%, and 81%, respectively (*88*).

6. Conclusion

BCS should be treated with a step by step treatment strategy. Physicians should be aware of the diverse etiology of BCS, and first-line therapy should be anticoagulation with medical treatment of the underlying illness (if indicated). Interventional revascularization and TIPS are indicated in the event of a lack of response to medical therapy. Liver transplantation may be indicated as a rescue treatment or for fulminant cases with promising results. LDLT can also be used for patients with BCS, but it involves a difficult procedure particularly with regard to venous outflow reconstruction.

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Review

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Liver transplantation and autoimmune hepatitis

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Summary **Liver Transplantation (LT) is an effective treatment for patients with end-stage liver disease including autoimmune hepatitis (AIH). Indication for LT for AIH does not differ basically from other liver diseases including both acute and chronic types of disease progression, although it is reported to be an infrequent indication for LT worldwide due to the therapeutic advances of immunosuppression. The outcome following LT is feasible, with current patient and graft survival exceeding 75% at 5 years. Recurrent and *de-novo* AIH posttranslant has also been reported; and this seems to have important clinical implications because its management differs from the standard treatment for allograft rejection. In this review, we discuss the characteristics of AIH, focusing on the indication for LT and issues raised following LT.**

Keywords: Autoimmune hepatitis (AIH), liver transplantation, anti-nuclear antibody (ANA), rejection, *de-novo* AIH

1. Introduction

Autoimmune hepatitis (AIH) is a chronic or acute hepatitis which is characterized by hepatocyte injury by an autoimmune process (*1*). It generally includes the appearance of circulating autoantibodies such as anti-nuclear antibody (ANA) and anti-smooth muscle antibody (SMA), and high serum globulin concentrations mainly with elevation of IgG (*2*). AIH usually responds to immunosuppression (mainly corticosteroid with or without azathioprine), and its prognosis has been reported to be comparatively good (*2,3*). However, there are a group of patients which develop into decompensated liver cirrhosis or fulminant hepatic failure (FHF) despite aggressive medical treatment; liver transplantation (LT) is still a last resort for those with end-stage liver disease due to AIH for those refractory to such immunosuppressive therapy (*4-7*). Even though LT is considered to be the only therapeutic option for those with liver failure due to AIH, this disease can recur after LT, with the recurrence rate ranging from 17 to 41% (*8*). Posttransplant *de novo* autoimmune hepatitis has also been described, although it is still an unsolved question whether it is a true autoimmune disease or a type of rejection (*9*). The diagnosis of recurrent and *de novo* AIH is often challenging, and it is usually treated by increasing or re-introducing immunosuppressant (mainly corticosteroid) (*10*).

The scope of this review is to: (A) overview the indications and outcomes of patients with end-stage AIH; and (B) discuss the characteristics of its recurrence and *de novo* AIH in the allograft for better understanding of both improving liver transplantation in this setting and better understanding of the pathogenesis of the primary, recurrent and *de novo* AIH.

2. Indication of liver transplantation for chronic and acute liver failure due to autoimmune hepatitis

The majority of patients (more than 75%) with AIH are presented with chronic disease (*11-13*). Its diagnostic criteria have been standardized and validated by the International Autoimmune Hepatitis Group (IAIHG) and is widely used (*14*). On the other hand, although several useful prognostic models are proposed in other autoimmune liver diseases such as primary

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biliary cirrhosis and primary sclerosing cholangitis (*15*), there are no useful prognostic tools available in AIH. Thus, the indication for LT based on the prognosis of the native liver in the AIH setting should be evaluated similarly as other non-autoimmune liver diseases; LT is usually indicated in patients with chronic decompensated liver disease with a Model for End-Stage Liver Disease (MELD) score of ≥ 15 (*16*). Complications of hepatopulmonary syndrome, portopulmonary hypertension and hepatocellular carcinoma with or without an elevated Child-Pugh score can be other factors in consideration for the timing of LT and a MELD exceptional point (*17-21*).

In contrast, fewer cases with AIH are presented with acute hepatitis, and a subset of these meet the criteria for acute liver failure (ALF). Because the number of "acute AIH" cases are few, it is usually difficult to propose optimal and simplified diagnostic criteria in this setting (*22,23*). Further studies are strongly warranted to find reliable biomarkers capable of defining AIH as the pathogenic process related to the development of ALF. Indeed, diagnosis of AIH in acute phase is based on serological markers such as autoantibodies, absence of viral/alcoholic/drug-induced etiology, physician's (clinical) experience, and when possible histopathological features (*23*). Especially severe coagulopathy induced by ALF often makes the decision to perform liver biopsies difficult, but finding the characteristics of central zonal perivenular hepatitis, a feature infrequently observed in chronic AIH is reported to be useful in acute hepatitis by AIH (*24*). However, in real clinical situations, the rapid and significant deterioration of patients with ALF forces physicians to decide whether the immunosuppressive therapy or urgent LT should be considered, before sufficient information for the diagnosis of AIH is obtained (*7,11*).

The important issues in management of ALF by AIH are: (A) establishing an appropriate diagnosis; (B) evaluation of risks and potential benefits of immunosuppressive therapy; and (C) urgent consideration for LT (*25*). As per the currently available data, universal application of imunosupressant (mainly corticosteroids) in ALF by AIH should be cautiously considered or even avoided, because of the risk of active infection/sepsis that might deprive a chance for LT which often could be the only curative treatment for this population (*11*). The critical issues in this decision are the selection of candidates for steroid therapy and the timing of withdrawal of steroids in viewing the possibility for LT. There are reports mentioning that a higher MELD score (such as greater than 24 or 28 points) was associated with poor response to steroid therapy (*11,12*). In addition, those receiving but not responding to steroids immediately (within 3 days) following its initiation showed a poor outcome (*25*). Thus, it can be argued that patients with ALF caused by AIH and severe deterioration should not receive steroids, and that immunosuppression should be discontinued and urgent LT becomes crucial if responses to such immunosuppressants are not confirmed promptly after introduction (*7,25)*.

3. Incidence of liver transplantation and its outcome

AIH is a relatively rare indication for LT; around 4-6% of transplantations in United States have been for AIH (*26*). European Liver Transplant Registry (ELTR) reported that 991 cases (3%) of all the 39,196 liver transplants performed in Europe from 1988 to 2001 were for those with AIH (*27*). Sixty of 5,510 (1%) living-donor liver transplantations (LDLT) were performed for patients with AIH between 1989 and 2009 in Japan (*28*).

The outcome following LT has been reported to be successful, since the development of the modern regimen of immunosuppressants which consists of the combination of corticosteroid and tacrolimus/cyclosporine A with or without mycophenolate mofetil (MMF), with 1- and 5-year graft survival rates of 84% and 75%, respectively, and 5- and 10-year patient survival rates of 80-90% and 75%, respectively (*2,29*). Especially as an important prognostic factor, recurrent AIH following LT and *de novo* autoimmune hepatitis, which are further discussed below, should be paid attention to (*8,9*).

In recipients who received LT with decompensated cirrhosis, there should be awareness for the development of osteoporosis. As AIH is predominantly in postmenopausal women with long-term use of corticosteroids, its risk is considered to be enormously high. The measurement of bone mineral density as well as the early initiation of medications such as vitamin D, calcium preparations or bisphosphonate, even before LT, is essential (*30,31*).

4. Diagnosis of recurrent autoimmune hepatitis, its risk factors and management

Despite receiving successful LT, several LT centers

Table 1. Diagnostic criteria of recurrent autoimmune hepatitis (*34-36*)

- Liver transplantation for confirmed diagnosis of autoimmune hepatitis
- Elevated transaminases
- Hyper-gammaglobulinemia (elevation of IgG)
- Presence of autoantibodies (ANA, SMA and/or Anti-LKM1)
- Compatible histopathology(interface hepatitis, portal inflammation and/or lymphoplasmacytic inflammatory infiltrates)
- Response to corticosteroid
- Exclusion of differential diagnostic considerations

have reported recurrent AIH posttransplantation (*8,32*), since the first report by Neuberger *et al.* in 1984 (*33*). However, there are no specific biomarkers to diagnose recurrent AIH. Currently proposed diagnostic criteria for recurrent AIH are shown as Table 1; it is essential to distinguish from other etiologies causing liver damage such as rejection, drug induced liver injury, biliary problems and viral hepatitis (*34-36*).

Recurrence rate of AIH posttransplant has been reported to be 17-41% (Table 2) (*5,34,37-46*), but they might have been influenced by diagnostic criteria, immunosupressants, follow up period, and the timing of liver biopsy especially between biopsy per protocol versus when clinically indicated.

Several risk factors of recurrent AIH have been proposed, but the clinical validity is still controversial (*47*). Although this is still controversial, the status of human leukocyte antigen DR3 (HLA-DR3) or HLA-DR4 were associated with a risk of recurrence in some research (*33,40,48,49*). It has been reported that the incident rate of rejection following LT is higher in AIH patients than non-autoimmune disease, although the impact of rejection for recurrent AIH is not certain (*5,32*). Interestingly it is suggested that acute (fulminant) AIH is less likely to recur than chronic presentation following LT (*5*). Primarily the immunosuppressive regimen does not seem to have great impact on recurrence rate (*50*). However, caution should be exercised when tapering patients off immunosuppression, especially corticosteroids, because recurrence has been associated with its discontinuation (*29,30,51*).

For recurrent AIH, mostly a re-introduction or an increase in the dose of corticosteroids and azathioprine is applied, and the response to this treatment is usually reasonable (*40,52*). For those refractory to the treatment, an alternative attempt, such as conversion to cyclosporine from tacrolimus (*53*), conversion to sirolimus from cyclosporine (*54*) or the addition of MMF (*35*), should be applied. However, there have been cases that required re-transplantation due to recurrence of AIH (*38,39*).

Table 2. Published series of recurrent autoimmune hepatitis following liver transplantation

Authors	Year	Cases (*n*)	Recurrence rate (%)	Time to recurrence (median, mo)
Prados *et al.*(*37*)	1998	27	33	30
Milkiewicz *et al.*(*38*)	1999	47	28	29
Ratziu *et al.*(*39*)	1999	25	20	24
Reich *et al.*(*5*)	2000	32	25	15
Gonzales-Koch *et al.*(*40*)	2001	41	24	52
Yusoff *et al.*(*41*)	2002	12	17	n/a
Heffron *et al.*(*42*)	2002	52	17	39
Molmenti *et al.*(*34*)	2002	55	20	n/a
Renz *et al.*(*43*)	2002	37	32	24
Duclos-Vallee *et al.*(*44*)	2003	17	41	30
Vogel *et al.*(*45*)	2004	28	32	12
Montano-Loza *et al.*(*46*)	2009	46	24	30

Table 3. Published series of *de novo* autoimmune hepatitis following liver transplantation

Authors	Year	Cases (*n*)	Frequency (%)	Median time to *de novo* AIH (mo)
Kerkar *et al.*(*55*)	1998	180	4	24
Gupta *et al.*(*56*)	2001	115	5	102
Hernandez *et al.*(*57*)	2001	155	2.5	61
Miyagawa-Hayashino *et al.*(*58*)	2004	633	2.1	37
Venick *et al.*(*59*)	2007	619	6.6	84
Eguchi *et al.*(*60*)	2008	72	5.6	18 (mean)
Cho *et al.*(*61*)	2011	149	2.7	78 (mean)

5. *De-novo* autoimmune hepatitis

A clinical entity with clinical, serologic, and histologic features resembling AIH may develop in adults and children undergoing LT for end-stage liver disease other than AIH, which is called *de novo* AIH (*9*). *De novo* AIH was first reported in pediatric cases in 1998 (*55*), followed by several adult cases shown in table 3, with frequencies ranging 2.1-6.6% (*55-61*). Clinical manifestations of *de novo* AIH are usually similar to those of recurrent AIH, namely characterized by an infiltrate rich in plasma cells with interface hepatitis and perivenular necro-inflammation as well as elevated serum gammaglobulin (high IgG) and positive autoantibodies (*62*). In 2006, Banff working group proposed the diagnostic criteria for *de novo* AIH (Table 4) (*36*). However in some cases, serum IgG or autoantibodies can be normal (*61*), and such variations make the understanding and the diagnosis of *de novo* hepatitis challenging.

As a risk factor developing *de novo* AIH, the appearance of autoantibodies post-LT (*63*), repeated cellular rejection (*58,59*), positive HLA DRB1*03 (*64*), positive anti-GSTT1 (*65*), and cyclosporine compared to tacrolimus (*66*) have been reported. Importantly, there have been several publications regarding *de novo* hepatitis during or after interferon-based anti-HCV treatment for recurrent hepatitis C posttransplantation (*67-69*). However, its pathophysiology is still uncertain, and it is still controversial whether *de novo* AIH represents a specific type of rejection or a form of

Table 4. Diagnostic criteria of *de novo* autoimmune hepatitis by Banff Working Group (*36*)

- Interface hepatitis with portal lymphocytic infiltrates
- Significant titers (> 1:160) of ANA, SMA, or Anti-LKM1
- Hyper-gammaglobulinemia
- Exclusion of virus-induced or drug-related hepatitis and late acute or chronic rejection

hepatitis related to auto- or allo-immunity (*9*).

Once diagnosed as *de novo* AIH, treatment with corticosteroids alone or in combination with azathioprine or MMF should be considered in addition to the basic immunosuppressive regimen (*55,64,70*). Development of cirrhosis and either death or requirement for retransplantation have been observed without successful immunosuppressive treatment for *de novo* AIH; however, well treated patients seem to be spared from progressive disease (*56,64,70*).

6. Conclusion

The indication for LT in patients with end-stage liver disease due to AIH is similar to those other than AIH, and its outcome seems reasonable. However, recurrent and *de novo* AIH have been a growing concern; further studies are strongly awaited to reveal their clinical characteristics and pathophysiology.

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Review

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Fragile X syndrome as a rare disease in China – Therapeutic challenges and opportunities

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Summary **Recognized as the most common inherited from of intellectual disability (ID) and the most common known monogenic cause of autism spectrum disorders (ASD), Fragile X syndrome (FXS) is identified as an unmet medical need for the development of personalized medicine and targeted therapeutics for neurodevelopment disorders as a result of improved understanding of the genetic and cellular mechanisms. Consequently promising pharmacological targets have emerged from basic and translational research, are now being pursued by global pharmaceutical and biotech companies in early proof-of-concept clinical trials. With the world's largest rare disease population, China potentially has a large number of FXS patients, many of whom are under-diagnosed or even misdiagnosed, barely with any treatment. In spite of improved awareness of FXS in recent years, big gaps still exist between China and developed countries in multiple aspects. With increased public awareness, strong government support and investment, coupled with an increasingly large number of Western-trained experienced researchers engaging in new drug discovery and development, China has the potential to become an important player in the discovery of effective diagnostics and treatments for a rare disease like FXS.**

Keywords: Fragile X syndrome, *FMR1*, Drug development, mGluR5, Translational science

1. Introduction

Neurodevelopment disorders such as autism or ASDs are life-long conditions which historically have been viewed as intractable to pharmacological interventions. Fragile X syndrome (FXS) is the leading inherited cause of intellectual disability and autism that affects all major ethnic groups and races (*1*). FXS and the related autism spectrum disorders (ASDs) now represent an urgent unmet need for effective treatment due to the rapidly growing patient population and the consequent huge burden on affected individuals, their families and care givers, and society as a whole (*2*).

Following the discovery of the genetic mutation of *FMR1* gene for FXS in 1991 (*3*), major advances have been made in the understanding of the underlying neurobiology and pathophysiology of FXS. The generation and analysis of FXS animal models, in particular the development of *FMR1* KO mice, have paved the way for testing targeted therapeutic agents based on the underlying mechanisms, many of which have in recent years advanced into further assessment in human patients (*4*). Clinical investigation of these novel therapies has subsequently enhanced our understanding of the challenges involved in the development of therapeutic treatment for this monogenic yet complex disorder (*5*). Despite tremendous progress made in bench-to-bedside translation, discovering novel treatments for FXS remains a daunting task.

Compared with the more established infrastructure for translational research in the US and many Western countries, there is a relatively weak basis for both basic science and clinical research in China, which is mainly reflected in the following areas: scattered distribution of resources, lack of expertise and severe shortage of resource for clinical investigations. Furthermore, there is rather limited awareness of FXS among the general population, medical professionals as well as healthcare policy makers. Although the first reported case of autism in mainland China was made over 30 years ago

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by the pediatric psychiatrist Dr. Tao Kuo-Tai in 1982 (*6*), autism is a relatively new area for researchers in mainland China. At present, ASDs including FXS have not been included in medical school training. As indicated in a recent survey by Duan *et al*., no more than 30% of the medical students have ever heard of FXS (*7*). Furthermore, there are very few qualified hospitals, healthcare centers and research institutes in China where FXS genetic testing can be reliably conducted. Consequently, there have been no clinical studies ever conducted on human FXS or ASDs patients in mainland China. Thus there is a need to establish a network of scientists and clinicians for sharing resources and forging collaborative relationships to support the direction and outgrowth of translational research with emphasis on human patients.

There is also an urgent need for establishment of standardized clinical practice. Currently in mainland China the branch of developmental psychiatry has not been established as an independent department. So far there has been no focused research team on autism or ASDs, therefore there is no unified department for the clinical diagnosis of autism and ASDs, including FXS, unlike the Western clinical settings, where there is generally a multidisciplinary team for the diagnosis and subsequent management of ASDs. In China, diagnosis of ASDs heavily depends on the judgment of the clinicians to whom the patient was referred. In addition, there are only a limited number of psychiatrists or pediatricians who are specialized in ASDs. In recent years, there has been increased awareness about autism and ASDs as a result of media publicity and the dissemination of related information *via* the internet. Usually when parents become concerned that their child may have autism, they normally try first to locate information online prior to seeking diagnosis. In sharp contrast to autism or ASDs, currently there is rather limited awareness of FXS in China among the general public as well as medical professionals, largely due to the shortage of an effective diagnosis of FXS in the clinic.

2. Epidemiology, diagnosis and current management of FXS

Fragile X, or Martin-Bell, syndrome was reported in 1943 (*8*) in the first pedigree with mental retardation linked to the X-chromosome. FXS is the most common single-gene cause of autism and inherited cause of intellectual disabilities especially among boys. It is a genetic disorder that manifests itself through a complex spectrum of intellectual disabilities ranging from mild to severe, as well as physical characteristics such as an elongated face, large or protruding ears, and large testes (macrooorchidism), and behavioral characteristics, such as stereotypic movement (*e.g*. hand-flapping), and social anxiety (*5*).

2.1. *Prevalence of FXS*

FXS is a genetic disorder that occurs as a result of a mutation of the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome, most commonly an increase in the number of CGG trinucleotide repeats in the 5'untranslationed region of *FMR1*. Although this accounts for over 98% of cases, FXS also occurs as a result of point mutations affecting *FMR1*. The general prevalence of males with a full mutation is estimated as ~1 in 4,000. The female prevalence is presumed to be approximately one-half of the male rate, or ~ 1 in 5,000-8,000. All major ethnic groups and races appear to be susceptible to expansion of the *FMR1* CGG region (*2*).

In China, the first documented report on FXS was published in 1986 by Zhou *et al*. (*9*), whereas 6 FXS families were identified with a total of 15 male FXS patients and 13 female carriers. There has been very limited number of FXS-related studies published to date by Chinese researchers. A literature search has indicated a steady increase in the number of published studies in autism or ASDs since the first case report in mainland China in 1982, accumulating in the thousands as listed in the CNKI database. Of particular note, there is a huge surge in the amount of research work in autism and ASDs since 2005 after the Chinese government issued the Five-year 11-5 (2006-2010) Outline of Development Plans for the Chinese Disabled Persons' Federation, wherein autism was for the first time specifically listed as an important area for government funding and support (*10*). However a serious gap still exists between China and the Western countries in this field, especially in translational science. So far there have been no clinical studies conducted in autism or ASDs. This is in stark contrast to Western countries, where there are over 500 total registered clinical studies focused on ASDs and more than 50 clinical studies specifically on FXS, according to the data provided by ClinicalTrials.gov (*11*).

China has the world's largest patient population of ASD, and presumably FXS as well. Due to the lack of full awareness and limited access to diagnostic tools and standards, epidemiology data on FXS remains scarce, and thus well-organized epidemiological studies are yet to be conducted to get an accurate account (*12*).

2.2. *Diagnosis of FXS*

Early diagnosis of FXS or carrier status is important for providing early therapeutic intervention including speech therapy, occupational therapy, psychotherapy and special education that can considerably improve the quality of the patient's life. It also allows for genetic counseling in regard to the potential implications for the parents and their extended families.

As a monogenetic disease, FXS is mainly caused by a CGG-repeat expansion that triggers hypermethylation

of this region and silences the *FMR1* gene which leads to FMR protein (FMRP) production deficiency. In general, the severity of the FXS physical phenotype and intellectual impairment is correlated with the magnitude of the FMRP deficit. The diagnosis of FXS is now performed through the detection of genetic mutations in the *FMR1* gene. Available tests used for diagnosis include both chromosome DNA analysis and various protein tests, although protein-based analysis has not been recommended at this time due to its limitations such as sensitivity, technical challenge and suitability for prenatal testing. At present, the DNA-based test has been predominantly utilized and can be administered with two different lab procedures, *i.e.*, the polymerase chain reaction (PCR) method and Southern blot analysis. The Southern blot analysis test determines if the gene has a full mutation, its approximate size as well as methylation status, while the PCR analysis can determine the actual number of "CGG repeats". Historically PCR has been not the test of choice to diagnose a full mutation, in particular when the CGG repeat size is over 250, due to limitations in terms of accuracy, although PCR is quite accurate in determining premutation and normal gene repeat numbers. In case of a full mutation, laboratories have to use more than one method because no single method can characterize all aspects of the *FMR1* full mutation. On the other hand, PCR is less expensive and quicker than Southern blot analysis. A major effort has been made to advance the PCR-based technology, resulting in improved ability to identify full mutations with a large CGG repeat size. Currently, as the best practice to determine full mutation, laboratories have to use both PCR and Southern blot analysis. Consequently, in their most updated policy statement, the American College of Medical Genetics and Genomics (ACMG) recommends that Southern blot analysis always be performed along with traditional PCR (*13*).

It is worth noting that since the current DNA analyses only tests for expansion of the CGG repeat, individuals with FXS due to missense mutations or deletions involving *FMR1* have to undergo sequencing of the *FMR1* gene in order to be properly diagnosed. Recent technical advances in prenatal testing have enabled reliable diagnosis of *FMR1* mutation while the fetus is in utero.

From a regulatory perspective, even in the US or Europe, there is currently no genetic test for FXS which has been officially approved by the regulatory agencies. Thus, all FXS genetic testing is now offered as a laboratory-developed test for clinical research as well as for diagnosis (*13*).

Prenatal testing for FXS is also available in Western countries using chorionic villi and/or culture, amniotic fluid cells, and/or culture or blood samples, although the use of a methylation-sensitive method is not suitable for early prenatal diagnosis because the methylation of a full mutation is not always present in DNA from chorionic villi, whereas it is established after the 14th week of pregnancy. In addition, in contrast to lymphocytes and amniocytes, the *FMR1* gene is not methylated on the inactive X chromosome in the chorionic villi of female fetuses (*13*).

Due to technical difficulties associated with accurate assessment of the large CGG expansion, only a handful of genetic testing laboratories in China, mostly academic institutes, are capable of performing comprehensive FXS testing, including prenatal diagnosis. Recently some commercial FXS testing trial packs or kits such as those from Asuragen, Abbott and Perkin Elmer, have been introduced in China (*7*). All these commercial kits adopt PCR-based methodologies, however, due to a lack of appropriate instruments, and more importantly, severe shortage of well-trained technicians in addition to the associated large cost which is not covered by health insurance, application of these kits is limited to a few research institutions. On top of all these, there is also an uneven distribution of knowledge of FXS across different regions in China. All these factors have hindered the accurate and timely diagnosis of FXS in China.

2.3. *Management of FXS*

There are currently no FDA approved drugs addressing the core symptoms of FXS, leaving patients and their caregivers with limited treatment options. Nevertheless several drugs that alleviate various aspects of behavioral symptoms are available. Current trends in managing and treating FXS include early intervention with both speech and language therapy, and occupational therapy with sensory integration techniques are also given to children with FXS as early as possible. Medical treatment of FXS is largely symptom-based. For example, it is a common practice to treat FXS patients with stimulants and selective serotonin reuptake inhibitors (SSRIs) for anxiety and obsessive-compulsive behaviors; and an atypical antipsychotic agent for self-injury, aggressive behaviors and autism. It is recommended to combine the pharmacotherapies with speech and sensory integration occupational therapy, together with individualized educational plans, and behavioral interventions in order to achieve the most optimal outcome (*14*).

New targeted medicine for FXS including mGluR5 antagonists, GABA A and B agonists, minocycline, and lithium *etc*., are now undergoing human clinical studies. Early reports are promising for some of these novel pharmacotherapies, which have disease modifying potential. It is noteworthy to mention that many of these targeted investigational drugs are often mixed with maintenance drug treatments outlined above for an optimal efficacy for FXS patients (*15*).

In China, due to its limited awareness, there have

been no guidelines or policies regarding the diagnosis and management of FXS. Currently Chinese doctors generally prescribe medications according to the clinical presentation of the patients, and the diagnosis of FXS normally has no impact on this pragmatic approach (*7*).

3. Therapeutic development for FXS

3.1. *Scientific rationale for development of personalized pharmacotherapies for FXS*

Our knowledge of FXS and related ASDs has advanced substantially over the past two decades. FXS is caused by expansion of an unstable CGG repeat region in the 5' untranslated region of the *FMR1* gene. The full mutation (>200 CGG repeats) is often accompanied by extensive methylation of the *FMR1* promoter, leading to transcriptional silencing, resulting in reduced expression or entire absence of FMRP protein which plays essential roles in neural development. Lack of FMPR expression appears to be at the core of the intellectual disability and other features characteristic of FXS. FMRP is a repressor of mRNA translation that is particularly important for the regulation of activity-dependent protein synthesis in neurons. The absence of FMRP leads to significant alteration in cognitive functions, dendritic spine morphology and intracellular signaling (*15*).

Much of our understanding of the neurobiology of FXS has been gained from studies of the *FMR1* knockout (KO) mouse, which shares many anatomic and behavioral phenotypes with human FXS (*16*). These KO mice recapitulate many symptoms observed in human patients, with defects in neuronal development, dendritic spine morphology, synaptic plasticity, pain processing, and behavior (*16*). By definition, the *FMR1* KO mouse model does not have perfect construct validity because genetically it does not exactly mimic the initial pathological lesion underlying human FXS, which is due to the CGG trinucleotide expansion. As such they are not a perfect model to test all therapeutics targeted in human FXS. For instance, some potential therapeutic targets cannot be tested using these models, including those related to DNA methylation. Nevertheless this KO mouse recapitulates the human protein abnormality, *i.e.*, loss of FMRP protein expression, thereby giving it high face validity. Indeed this animal model has tremendously advanced our understanding of the underlying mechanisms of FXS and provided valuable insights for testing novel therapeutic approaches in human patients with FXS and potentially ASDs.

Based on accumulating evidence from early preliminary clinical studies, and/or studies in FXS animal models, many promising therapeutic targets have been identified or proposed. These targets can be further grouped into the following three major groups: (*i*) transmembrane receptors such as metabotropic glutamate mGlu1/5, dopamine D1/5, and GABA receptors; (*ii*) intracellular central signal transduction molecules including ERK1/2 or PI3K; and (*iii*) further downstream signaling molecules or proteins such as MMP-9, mTOR, GSK3β, or PAK (*15,16*). The ultimate validation of these novel therapeutic strategies will strongly depend on the availability of target-specific drugs that are safe for use and effective for long-term in patients. Currently several of these potential targets have been validated in early proof-of-concept clinical studies in FXS patients, although further studies are needed to investigate which of these therapeutic strategies might be the most beneficial for each distinct sub-population of FXS patients with a different clinical phenotype, though reliable biomarkers will be needed for this personalized or stratified medicine approach.

Based on numerous elegant studies using the *FMR1* KO mouse, mGluR5 was identified as one of the key players upstream of the FMRP-mediated pathways and activation of mGluR5 leads to protein translation. A landmark study by Huber *et al.* (*17*) reported that mGluR1/5-medited long-term depression (LTD) is elevated in *FMR1* KO mice. This work laid the foundation for the mGluR theory of FXS, which hypothesized that dysregulated mGluR1/5-mediated protein synthesis resulted in abnormal plasticity thus contributing to the pathology of FXS. Further genetic validation of the mGluR theory was provided by Dolen *et al.* (*18*) by genetic rescue of FXS phenotypes in *FMR1* KO mice that were crossed into an mGluR5 heterozygous background. It is now widely accepted that the loss of FMRP allows for excessive mGluR5 signaling, which in turn results in excessive protein translation and synthesis. Continuous treatment of FMRP mutant mice with mGluR5 inhibitors commencing early in life eliminate seizure and other phenotype abnormalities (*19,20*). These findings have provided a foundation and led to development of potent and selective mGluR5 inhibitors, including AFQ056, RG7090 and STX209, which have advanced into human clinical studies (*21-23*).

Although there is compelling evidence for the involvement of the mGluR pathways in the development of FXS, it is unknown to what extent dysregulation of these pathways contributes to the overall disease severity in FXS patients. FMRP is widely expressed throughout the brain and has recently been proposed to regulate approximately half of the known genes associated with ASDs (*24,25*). In light of this, it is not surprising that some observed phenotypes in the *FMR1* KO mouse model cannot be explained simply by dysregulation of mGluRs. One implication is that in order to test the clinical effectiveness of mGluR5 antagonists in human FXS patients, we need to choose mGluR5-specific abnormal phenotypes as

outcome measures. Besides serving as an excellent translation model for subsequent testing of a mGluR5 based therapeutic approach in human FXS patients, the *FMR1* KO mouse may in fact represent an ideal model for teasing out those abnormal phenotypes mostly likely mediated by excessive activity of mGluR5, and identifying potential biomarkers that can help us in patient stratification and subsequent selection of the right subgroup of FXS patients who are most likely responsive to mGluR5 antagonist treatment. Furthermore, the *FMR1* KO mouse model may also provide insight into the selection of specific outcome measures that are most relevant and robust for human clinical studies.

3.2. *Challenges in discovery of novel treatment for FXS: a drug development perspective*

CNS drug discovery has long been regarded as one of the most challenging areas in the pharmaceutical industry with disproportionally lower chances of success compared with other therapeutic areas. As a result, several major global pharmaceutical companies like GSK and AstraZeneca have in recent years decided to dramatically reduce their R&D investment in the CNS area, in particular, in the area of neurological diseases including mental disorders such as ASDs and FXS.

Researchers embarked on the search for novel targeted FXS therapies based on a solid understanding of the underlying mechanism as well as a well-characterized highly disease-relevant mouse model. Over the past two decades, tremendous progress has been made which has provided valuable insights for more future translational studies. Some major lessons the field has learned thus far over the past 20 years:

Lesson 1: Preclinical challenge – target selection is a critical first step toward a safe and effective treatment for neurodevelopmental disorders including FXS

In general, one of the crucial considerations in the target selection for a drug discovery program is whether such targets are tractable for drug development. A druggable target often refers to a protein which can be modulated with drug-like molecules, which often times for CNS indications, are small molecule drugs that can easily cross the blood-brain barrier. In this respect, transmembrane receptors or enzymes have historically been one of the most popular classes of investigational drug targets, especially for CNS disorders. An in-depth understanding of the underlying disease biology provides the foundation for the selection of a druggable target, which consists of identifying molecular processes that can be enhanced or inhibited in order to restore homeostasis disrupted under the disease condition. In many cases such as FXS and ASDs, instead of working directly on the genetic defect of the *FMR1* gene or the FMRP protein deficit, drugs work by acting on proximal or distal processes through compensation for the functional defect caused by the genetic mutation. Therefore, based on their key roles in the underlying pathophysiology elucidated preclinically, mGlu5 and GABA-A receptor, are ideally suited as drug targets for pharmacological treatment of FXS.

Even after the selection of a good druggable target, the ultimate creation of a drug for a CNS disorder normally requires a highly rigorous medicinal chemistry effort. A unique challenge for indications like FXS and ASDs, the prospect of life-long treatment and likely optimal starting at a young age, also puts stringent requirements on the drug safety with no side-effects or long-term toxicity. These important factors would exclude potential drug targets such as GSK3β and mTOR as these proteins have pleiotropic functions and are essential for body growth, even though there appears to be solid scientific basis for involvement of these proteins in the disease pathology of FXS (*15,16*). Furthermore, the possibility for transgenera-tional toxicity should also be evaluated in appropriate species. Another important factor to consider is that many FXS patients are already receiving other pharmacological treatments for symptomatic management, thus a novel treatment should be compatible with an ongoing treatment regimen with minimal drug-drug interaction. Overall, pediatric drug development poses additional challenges in terms of safety and tolerability. Drug metabolism is another important yet complicated factor since drug metabolism is more variable and less well characterized for pediatric patients. As such, the translation from *in vitro* testing to *in vivo* pharmacodynamic and pharmacokinetic predictions is much less established in young children than in adults.

Lesson 2: Preclinical challenge – FXS animal models need to be used wisely in order to realize its full translational power

One of the essential steps in the drug discovery and translational research process is the evaluation of pharmacological effects of prospective therapeutics using appropriate and/or validated animal models that can be reflected on the endpoint of its clinical treatment. For CNS disorders like FXS and ASDs, this step often relies on a broad repertoire of behavior tests related to the core and co-morbid symptoms. Relevant information gathered in animal studies should be translated into clinical relevance and vice versa, this 2-way communication between clinical and preclinical discovery scientists during the drug development process are likely to help in the development of more relevant, predictive animal models as well as biomarkers providing conceptual basis for development of effective treatment. The identification of the disease-causing mutation in the *FMR1* gene more than 20 year ago enabled the generation of genetically engineered mice carrying a similar defect, *i.e.*, loss of FMRP protein. As discussed above, this FXS mouse model

in many aspects recapitulates disease symptoms and pathophysiology present in human FXS patients, including behavioral, cognitive, neurochemical and physiological abnormalities.

Drug discovery and development belongs to translational science, therefore should be conducted as patient-oriented research. Convincing evidence has shown that FXS patients demonstrate abnormalities in sensory processing and communication. Clinical, behavioral, and electrophysiological studies consistently show auditory hypersensitivity in humans with FXS. One important characteristic of FXS is the co-occurrence of seizures in 10-40% of individuals with full mutations, which normally begin at a very young age, mostly between ages 4 and 10 years. Although seizures in FXS patients are infrequent and easily managed, there appears to be a strong correlation between seizure and overall disease severity, in particular comorbidity including autism and anxiety that impacts FXS patients' function and quality of life (*26,27*).

Interestingly, the most promising behavioral assays established thus far using FXS mouse models are those based on neuronal hyperexcitability, which have strong correlates in FXS patients, such as epilepsy and hyperactivity. In fact, one of the most robust assays tests the susceptibility to audiogenic seizures (AGS). Higher susceptibility to AGS has been reproducibly observed in *FMR1* KO mice of various genetic backgrounds and has become very useful in evaluating potential therapeutic strategies (*28,29*). Recently, a kindling paradigm also demonstrated higher seizure susceptibility in *FMR1* KO mice compared to wild-type controls, and thus potentially can serve as an additional tool to assess hyperexcitability using FXS mouse models (*30*). Numerous preclinical studies have suggested that auditory hyper-excitability can serve as a robust and reliable endpoint, and potentially as a translatable biomarker from *FMR1* KO mice to FXS patients.

Hence auditory hypersensitivity indeed provides a unique opportunity to integrate molecular, cellular, circuit level studies with behavioral outcomes in the search for therapeutics for FXS and ASDs. Theoretically this type of auditory deficits observed in FXS patients as well as FXS animal models should serve as relatively more tractable and more objective outcome measures than more complex and subjective social behaviors that are typically studied in FXS and ASD patients currently.

It is noteworthy that mGluR5 appears to play an essential role in mediating the hyperexcitability seen in FXS patients, as demonstrated in the *FMR1* KO mouse model (*18,19,29*). It is conceivable that this particular FXS model may represent a sub-population of FXS patients who exhibited significant dysregulation of the mGluR5-mediated pathways leading to the manifestation of FXS symptoms including susceptibility to seizure and epilepsy. Besides the convincing support from the seminal work by Dolen (*18*) showing that genetic knockdown of mGluR5 resulted in almost complete rescue of the AGS susceptible phenotype in *FMR1* KO mice (*18*), several mGluR5 negative allosteric modulators (NAMs), including MPEP, CTEP, RG7090 (Roche's clinical-stage drug that completed Phase II trial and achieved proof of concept status in treatment resistant depression patients) (*19,29,31*) have all been shown to be effective in ameliorating many defects including the susceptibility to AGS in FXS mice. Using the same study protocol, we tested HME01, one of the lead mGluR5 NAMs developed at Hua Medicine (*32*). Our data further confirmed that AGS is a robust objective endpoint for testing the efficacy of mGluR5 NAMs in FXS (Figure 1). One challenge of this AGS testing is that it has to be conducted on mice as young as 21-days old. This in fact reflects a reality with respect to the therapeutic window of this approach. In order to demonstrate the clinical effectiveness of an mGluR5 NAM with a more objective outcome measure

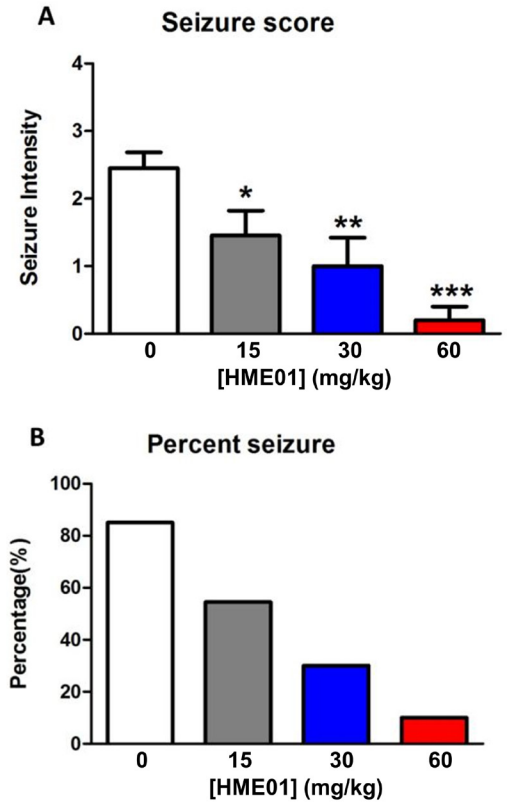

Figure 1. Audiogenic seizure (AGS) susceptibility was reduced in *FMR1* KO mice by the mGluR5 NAM HME01. (A) HME01 reduced severity of seizure as measured by intensity score in *FMR1* KO mice. Seizure intensity score 0 = no response; 1 = wild running and jumping; 2 = clonic seizures; 3 = tonic seizures; 4 = respiratory arrest. **(B)** HME01 decreased incidence of seizure as measured by percentage of *FMR1* KO mice exhibiting each phase of the sequential seizure response. Single injections of 0, 15, 30, or 60 mg/kg HME01 were administered 15 minutes before the test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

based on the hyperexcitability phenotype, it will be desirable for the therapeutic engagement to start earlier, no later than an age of 5 to 14 when seizure co-occurs at the highest frequency among FXS patients. Of course this argument requires further validation in basic and clinical research before it can be translated into clinical practice.

In terms of clinical translation, it appears to be rather challenging to utilize this endpoint as a viable outcome measure in the current setting of clinical studies, which in fact reflect the current gap between basic and clinical science in bringing novel neuroscience discovery into new treatment for FXS.

Lesson 3: Clinical Challenge – therapeutic development for a complex condition like FXS, predicting the outcome that will improve during a short trial is essentially guesswork without objective measures

The appropriate selection of patients is crucial for the success of any clinical research. Although a monogenic condition, clinical heterogeneity of FXS patients have demonstrated various severities with a wide range of co morbidities (such as mood and anxiety disorders, epilepsy and other behavioral problems) in addition to the core symptoms, which include physical impairment, cognitive, emotional and behavioral deficits. Results from recently completed clinical trials of novel FXS therapies have indeed highlighted several challenges including patient stratification with possible differential responses, the need for FXS-specific more objective outcome measures, and the lack of biomarkers for predicting a patient's response to a specific treatment.

With respect to patient stratification, drug development for childhood FXS poses unique challenges due to the compounded challenges associated with studying both a rare disease and pediatric population. Unique considerations include potential age-based differences in drug metabolism and toxicities, the long term consequences of a drug's effect on a growing child's physiology and development, *etc*. Based on convincing preclinical data, the optimal efficacy for pharmacological treatment of FXS and ASDs, most likely comes from early intervention in childhood, which theoretically offers the prospect of disease modification or correction of a developmental trajectory. On the other hand, traditional drug development and regulatory pathways require demonstration of safety and potential prospects of direct benefit in adult populations before pediatric studies can be conducted. While it is understandable that stringent safety requirements should be met before younger populations are exposed to experimental medicines, there is reason to believe that efficacy in adult patients might not be achievable in this case, or might not be fully predictive of the potential therapeutic efficacy in younger patients.

Currently there are no FXS-specific outcome measures that have been established and validated in the clinic. The widely used Aberrant Behavior Checklist – Community Edition (ABC-C) was developed to assess problem behaviors in children and adults with intellectual disability and has been effectively employed in trials for ASD treatments. Nonetheless it was unclear whether the ABC-C is specific and sensitive enough to detect disease modification by targeted novel therapies in all FXS patients. Although based on the extensive preclinical mechanistic studies, many novel targeted therapies including mGluR5 NAMs and GABA-A agonists, have the potential to normalize or partially normalize the core mechanism underlying FXS, which translate into a stabilization or improvement in symptoms. A key challenge to assess disease modifying therapies is identifying an appropriate outcome measure, considering the wide range of symptoms and the big variations in the severity of each individual symptom observed in FXS patients.

Successful development of effective FXS treatments in the future will most likely rely heavily on the availability of a robust biomarker. The availability of the well-characterized highly relevant FXS mouse model has provided an excellent opportunity to identify a reliable biomarker which potentially correlates with clinical improvement. For example, auditory hypersensitivity including seizure susceptibility may actually represent one of the most promising candidates as a biomarker for patient stratification, in particular for targeted therapies that have demonstrated robust efficacy in the FXS mouse models, such as mGluR5 NAMs and GABA-A agonists, as discussed above.

Even though China potentially has a large number of FXS patients who urgently need medical care, conducting clinical trials of novel therapies for FXS patients in China has to face additional challenges. Historically in mainland China, the diagnosis of autism or ASD is only given by pediatrician or pediatric neurologists, and there is essentially no diagnosis for adult autism patients. At present, autistic individuals including FXS patients who are seeking professional help are almost exclusively young children under intensive parental care. Adult autistic patients have historically been a neglected population; many of them are no longer under the proper medical care and therefore not registered under the right category. Furthermore many adult autistic patients are often misdiagnosed as other mental disorders such as mania or schizophrenia. Therefore one major obstacle in conducting FXS clinical trials in China is the identification and recruitment of adult FXS patients.

4. Conclusions

The monogenic cause of FXS leads to a relatively genetically homogeneous patient population, and offers a unique and favorable opportunity for drug development of effective therapies which has been

facilitated by the availability of a variety of transgenic animal models mimicking the FXS phenotype. Despite a common genetic etiology, individuals with FXS display significant heterogeneity in clinical phenotype and drug response.

Because current clinical testing of targeted novel therapies are mostly conducted as a monotherapy, patients will display optimal responses to different targeted treatments based on individualized complex interactions between genetic variability, neuronal pathways and synaptic function. It is also very likely that target-based monotherapy may not be sufficient as a successful 'cure' for FXS, rather, a combination therapy will be more efficacious for long-term treatment. Thus, it is likely that different patients will function best with certain pathway targets or certain combinations of treatments. In regard to this, a personalized approach seems ideally suited for this complex condition.

There is hope that ongoing development of new targets will gradually build on previous knowledge to result in progressive improvement in treatments and ultimate reverse of core deficits in FXS patients in the future.

Clearly, there is overlap in molecular and synaptic pathways between FXS and autism. Thus targeted treatments for FXS will likely be effective in a subgroup of ASD patients who display dysregulated synaptic defects in the same pathways that are abnormal in FXS. The great progress made thus far holds a strong promise for continued development of target-based therapeutic strategies for FXS.

In lieu of the disconnection between the efficacy observed in the preclinical animal studies and the apparent lack of efficacy in the human clinical studies through statistics, as highlighted by the recent failed trials by Novartis and Roche, it has been proposed that future efforts are needed to help unravel the exact reasons for the lack of translation between preclinical data and clinical outcome. A well-defined clinical end point should be developed based on preclinical and clinical research on *FMR1* gene mutation and clinical symptoms, which will bridge the gap between the scientific and medical community and create a novel path to management of FXS. Furthermore, more coordinated efforts are needed to expand and refine the preclinical toolbox in order to increase confidence in predicating therapeutic benefits in FXS patients. In the future there is also a need to optimize behavioral assays in *FMR1* KO mice, including better standardization for experimental paradigms, age, and genetic background to provide a valuable and reproducible tool for the evaluation of novel therapeutic strategies in FXS.

5. Looking forward to the future in China

Despite abundant cases of this rare disease, the related work in China such as research, regulations and social support have only recently been initiated. In China, the level of care for common diseases such as tumors and cardiovascular disease has significantly improved. Now, the prevention and treatment of rare diseases including FXS is also drawing more public and government attention. At the moment, a program for collaboration on rare disease research is being implemented at the national level. This program is committed to promoting the study of rare diseases in China and will encourage international collaboration in this regard (*33*).

The past few years have seen great advancement in our understanding of the genetic and molecular basis for FXS and ASD, a gradually increased number of researchers in China have gained greater interest in FXS and ASDs, especially in basic science research. Furthermore, a growing number of clinicians, researchers, and government health officials began to become aware of the importance of resource allocation, epidemiological study, and clinical study of rare diseases in China. Furthermore, there have been an increasingly large number of Western-trained experienced researchers who are devoted and engaged in novel drug development in China and for those most afflicted.

Although novel targeted treatments for FXS are still in the research and development stage, many affected Chinese families have expressed their interest in participating in the clinical trials, though safety is their most important concern.

The prevention of a rare disease like FXS is a comprehensive work and requires a coordinated effort at various levels, including healthcare, medical insurance and civil affairs. In order to tackle complicated diseases such as FXS and ASDs, it is hoped that a specialized clinical and research consortium in China can be organized to provide the most up-to-date knowledge and recommendations for Chinese medical professionals and to provide optimal assistance to affected individuals and their families.

Policy wise, current nationwide insurance programs in China cover basic general health care expenses for 95% of the Chinese population. For example, general screening for Downs syndrome has been conducted as part of routine prenatal tests. Unfortunately, no major plans are available today to cover the relatively high cost of genetic testing for FXS which is unaffordable for many affected families, particular those in the underdeveloped rural areas.

Building upon the remarkable progress made over the past few years in the understanding of drug development for FXS and other neurodevelopmental disorders, with strong government support under the 12.5 plan, Hua Medicine, is currently taking a personalized medicine approach actively engaged in bringing new concepts and novel treatment to the FXS and autism patients in China. It can be anticipated that the path leading to an effective novel treatment in China

may be filled with even more challenges. As a pioneer pathfinder, we need to work with all relevant parties at various levels, both public and regulatory agencies. By focusing on the need of the patients it will be possible to dramatically change the therapeutic landscape for neurodevelopmental disorders including FXS, creating new medicines that will improve the lives of patients and their families.

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Original Article

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Heterozygous mutation of c.3521C>T in *COL1A1* may cause mild osteogenesis imperfecta/Ehlers-Danlos syndrome in a Chinese family

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Summary **Osteogenesis imperfecta (OI) is an inheritable connective tissue disorder with a broad clinical heterozygosis, which can be complicated by other connective tissue disorders like Ehlers-Danlos syndrome (EDS). OI/EDS are rarely documented. Most OI/EDS mutations are located in the N-anchor region of type I procollagen and predominated by glycine substitution. We identified a c.3521C>T (p.A1174V) heterozygous mutation in *COL1A1* gene in a four-generation pedigree with proposed mild OI/EDS phenotype. The affected individuals had blue sclera and dentinogenesis imperfecta (DI) was uniformly absent. The OI phenotype varied from mild to moderate, with the absence of scoliosis and increased skin extensibility. Easy bruising, joint dislocations and high Beighton score were present in some affected individuals. EDS phenotype is either mild or unremarkable in some individuals. The mutation is poorly conserved and *in silico* prediction support the relatively mild phenotype. The molecular mechanisms of the mutation that leads to the possible OI/EDS phenotype should be further identified by biochemical analysis of N-propeptide processing and steady state collagen analysis.**

Keywords: Osteogenesis imperfecta, Ehlers-Danlos syndrome, type I collagen, mutations, *in silico* prediction

1. Introduction

Mutations in the *COL1A1* or *COL1A2* genes that encode proα1 and proα2 chains of type I procollagen have been shown to be the main cause of osteogenesis imperfecta, a genetic connective tissue disorder characterized by brittleness of bones, blue sclerae, impediments of teeth, hearing and sight (*1,2*). *COL1A1* and *COL1A2* genes also lead to the arthrochalasis type of Ehlers-Danlos syndrome (EDS type VIIA and B). The clinical spectrum of OI is broad, ranging from mild, moderate, severe to lethal forms (*1*).

More than 1000 mutations in the *COL1A1* and *COL1A2* genes have been reported in OI mutation database and the largest majority of these mutations exist in the triple helix domain of procollagen type I genes (*3,4*). Glycine substitution is the most common mutation in the OI and glycine to serine substitution is the predominate one.

In comparison with mutations identified in OI, only

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a limited number of mutations have been reported in EDS (*3,4*). The arthrochalasis type of EDS is caused by mutations leading to skipping of exon 6 in type I procollagen, resulting in the removal of the cleavage site for N-proteinase (*5,6*). The combination of OI and EDS is very rare and only 26 cases have been recorded worldwidely (*3,4*). Most OI/EDS mutations are located at the N-terminal (exon 6 to 11) of the type I procollagen genes (*5,7-11*). C-propeptide mutation of c.3790A>G (p.M1264V) of *COL1A1* was recorded once (*12*). Arginine to cysteine substitution at position 1036 and 1066 in the helix exon 44 was also reported (*13,14*).

We describe here a Chinese pedigree from four generations with a tendency of mild OI/EDS phenotype, in which the symptoms of OI and EDS are both mild. Heterozygous mutation of c.3521C>T (A1174V) in *COL1A1* was identified and this mutation was once reported in one type III Chinese OI patient (*15*).

2. Materials and Methods

2.1. *Clinical findings of the probands and family members*

The study was performed in accordance with the tenets of the Declaration of Helsinki and approved by the Ethical Committee of Shandong Academy of Medical Sciences. Informed Consent was obtained from the proband who had OI family history (Figure 1). Clinical phenotypes and bone mineral density were described in Table 1.

2.2. *Identification of type I collagen gene mutations*

Genomic blood DNA was extracted using the E.Z.N.A.® Blood DNA Kit (Omega Bio-Tek, Georgia, USA). PCR reactions were performed according to previously described and PCR products were submitted for Sanger sequencing. Genetic variations were evaluated by both Mutation Surveyor 4.0 software (SoftGenetics LLC, Pennsylvania, USA) and human collagen mutation database (*http://www.le.ac.uk/ genetics/collagen*).

2.3. *In silico prediction of mutation effects and alignment analysis*

Polyphen (*http://genetics.bwh.harvard.edu/pph*) (*16*), Align GVGD (*http://agvgd.iarc.fr/agvgd_input.php*) (*17*) and SIFT human coding snps (*http://sift.jcvi.org/www/sift_chr_coords_submit.html*) (*18*) softwares were adopted to predict the mutation effects on function. ClustX2 were used for the conservation analysis.

3. Results

3.1. *Clinical descriptions*

The pedigree was a four-generation family with 46 family members, including seven affected males, four affected females and three dead fetuses. Spontaneous abortion occurred at 10th week of the pregnancy for the first fetus. Medical abortion was performed at week of 23 and 22 respectively for limb abnormality observed by 4D color Doppler ultrasound. Gracile ribs and a narrow chest apex were observed in the third dead fetus. A bilateral asymmetry in shortened and bent lower limbs was obvious (Figure 2). The length of bilateral femur was 27.4 mm and 32.3 mm respectively. The size of biparietal diameter (BPD), head circumference, and abdominal circumference, cerebellar diameter, posterior fossa pool and transparent compartment was 51 mm, 195 mm, 175 mm, 23.6 mm, 6.7 mm and 4.8 mm respectively when tested by 4D color Doppler ultrasound of the third fetus at 21 weeks and 5 days.

At the time of delivery, the proband was 52 cm tall and weighed 3.9 kg. No limb malformation was observed despite frequent fractures occurring before the age of twelve. The proband also had some clinical symptoms resembling a mild form of EDS, including easy bruising, smooth skin and joint laxity. Easy bruising and joint dislocations were also observed in the affected family member (PIII-5). Though there's no fracture history, family member PIII-3 had low bone mineral density (BMD) (Table 1) and he complained of tooth pain when eating hard food and had an ankle sprain history.

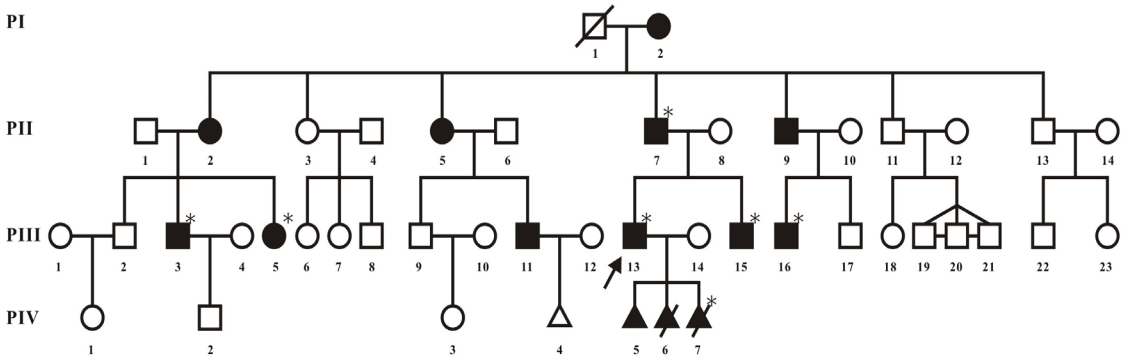

Figure 1. Pedigree map for the paiteint. "*" noted the patients tested by type I collagen gene analysis.

Table 1. Clinical characteristics of the proband and his affected family members

Items	PIII-3	PIII-5	PIII-13	PIII-15	PIII-16	PIV-7
Age (years) gender	27/M	23/F	26/M	22/M	23/M	21weeks/F
Height (cm)	170	148	173	169	155	28
Weight (kg)	75	42	80	57	65	0.463
Blue sclerae	slight	*+	+	+	+	N.A
Dentinogenesis imperfecta	**-	-	+	-	-	N.A
Spine deformities	-	-	-	-	-	-
Chest deformities	-	+	+	-	+	-
Limb deformities	-	+	-	-	+	+
Age (years) at first fracture	-	4 mon.	2 mon.	-	2 y	N.A
Number of fractures	0	5	10	1	3	N.A
Joint laxity	***N.A	+	+	+	+	N.A
Skin	normal	normal	Smooth, velvety skin	Smooth, velvety skin	N.A	N.A
Easy bruising	-	+	+	N.A	N.A	N.A
Joint dislocation	-	patella and finger	Shoulder, finger	N.A	left elbow joint, shoulder	N.A
Beighton score	2	3	5	5	0	N.A
other	Slight ptosis, flatfoot, have history of ankle sprain	Have history of ankle sprain	Congenital cataracts, thin corneal	-	Pinched nose, ptosis	-
DEXA z-score (L1-L4/Hip)	-1.6/-1.5	-2.5/-1.4	N.A	N.A	N.A	N.A

*+, presence of trait ; **-, absence of trait; ***N.A, not available

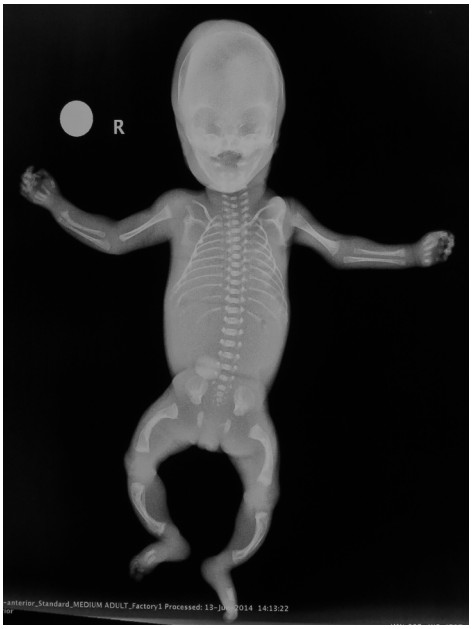

Figure 2. Radiographic examination of the patient PIV-7 at 22 weeks.

The typical clinical symptoms of the pedigree are shown in Table 1, the phenotypes of affected family members vary from mild to moderate clinical severity. All affected members had blue sclera, with nearly normal dentition. No affected members of the pedigree have scoliosis. Skin extensibility, atrophic scars and increased transparency were unremarkable. In some affected family members, fractures and joint dislocations were noticed especially at early ages and became less frequent with age. High Beighton score existed in both the proband and his brother, but was not obvious in all other patients. Upper eyelid drooping was observed in two patients.

3.2. *Molecular analysis of COL1A1 and COL1A2 genes*

Heterozygous c.3521C>T mutation resulting in alanine to valine substitution at position 1174, was identified in all tested affected family members, but not healthy family members (Figure 3A).

3.3. *In silico prediction of mutation effects and alignment analysis*

Align GVGD predicted the high Class values of C65, which indicated that the mutation has a pathogenic effect on protein function. Polyphen and SIFT software predicted a benign and tolerated effect of the mutation. Multiple sequence alignment discovered the relative poorly conserved variation of residue 1174 of pro-α1(I) in different organisms and different types of collagen (Figure 3B).

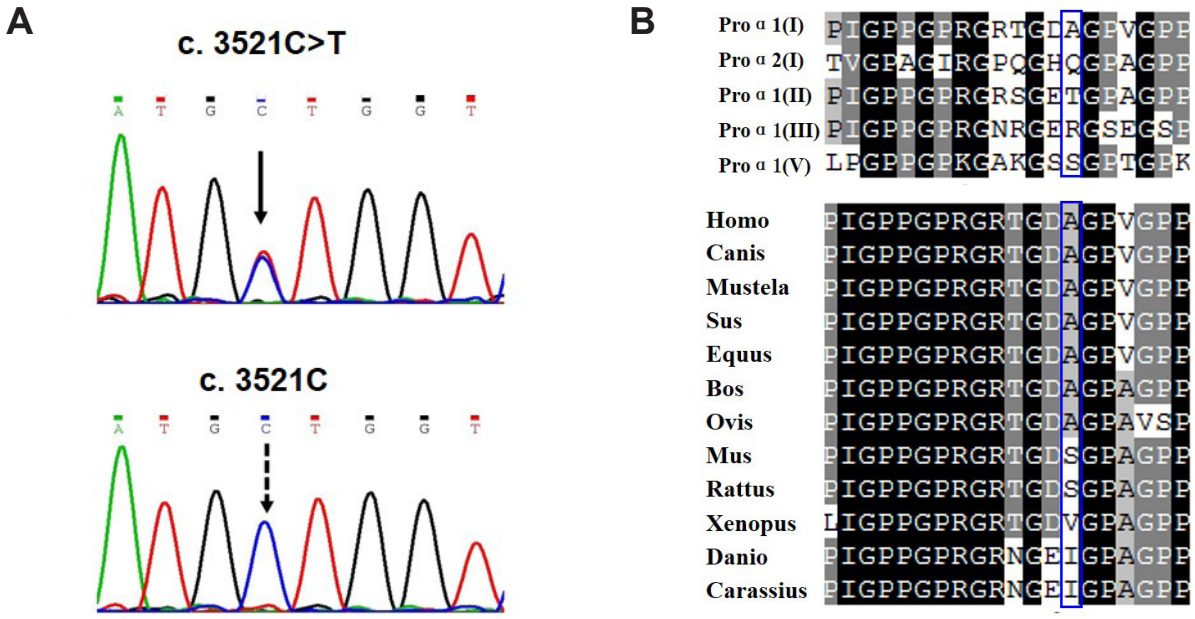

Figure 3. Molecular anlysis for type I collagen genes. **(A)** Heterozygous mutation of c.3521C>T was found in all tested affected patients, but not in the healthy family individuals **(B)** Alignment of the variant between different human fibrillar procollagen chains and different species.

4. Discussion

Substitution of alanine to valine at position 1174 was reported once in one Chinese type III OI patient (*15*). We identified this mutation in a second Chinese family with mild or moderate clinical phenotypes, which resembled OI type I and type IV. Besides the blue sclera and fracture history, joint dislocation, myopia, hypermobility value Beighton score and skin abnormities in the proband and his affected family members are highly suspected that the OI patient was complicated with Elhers-Danlos syndrome (EDS).

OI mutation severity is related with many factors, including location, mutation type and mutated residues. Heterozygous mutation of c.3521C>T (A1174V) located in exon 48 of *COL1A1* is near the lethal region (*2*). Substitution of glycine to serine/cystein at position 1175 and glycine to arginine or aspartic acid are related to severe OI type II or III clinical phenotypes(*3,4*). Residue 1174 is poorly conserved in different species and different types of collagen and it lies in the helix region of proα1(I), which binds interleukin 2 and amyloid precursor protein (*2*). *In silico* prediction of Align GVGD support that A1174V substitution is damaging while PolyPhen and SIFT predicted a benign and tolerated effect on protein.

Reduction of elastic modulus of tropocollagen is depicted as the severe OI (*19*). Alanine displayed a low value of Young's modulus for their softer tropocollagen mechanical properties than valine. Decrease of adhesion energy and increased equilibrium intermolecular spacing could describe the increase of severity of the OI mutation (*20*). All these values support that the severity of alanine to valine substitution is less severe than that of aspartate, glutamate and arginine.

OI/EDS patients are rarely reported and most of the mutations are located at the amino terminal N-anchor region of type I procollagen (*21*). The molecular mechanism of the mutation is related to the interference with N-propeptide processing and thus the defective procollagen and cross-linking are formed (*6,11*). M1264V substitution located at the proα1(I) of C-propeptide found in the OI/EDS patient was supposed to impede C-propeptide folding and chain association (*12,22,23*). R1066C non-glycine substitution near the C-terminal of type I procollagen helix region, could result in OI/EDS phenotype. The introduction of cysteine could cause helix kinking, resulting in the propagated register shift and delayed N-proteinase cleavage (*13*). This is the first time reporting alanine substitution at the end of the C-terminal procollagen helix region and it is unclear how this substitution leads to abnormal function of collagen I and OI/EDS phenotype still needs further study.

Acknowledgements

This work was supported by the grant from The International Science and Technology Cooperation Project of Shandong Province, the Project for Shandong Academy of Medical Sciences, The Development of Medical Science and Technology Project of Shandong Province (2013WS0374) and the National Key Technology R & D Program (No. 2013BAI07B02, 2013BAI07B01); We thank Dr. Joan Marini for discussion.

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Original Article

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Different types of androgen receptor mutations in patients with complete androgen insensitivity syndrome

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Summary **Mutations of androgen receptor (*AR*) are the most frequent cause of 46, XY disorders of sex development and associated with a variety of phenotypes, ranging from phenotypic women (complete androgen insensitivity syndrome (CAIS)) to milder degrees of undervirilization (partial form or PAIS) or men with only infertility (mild form or MAIS). From 2009 to 2012, two young Chinese female individuals with CAIS from two families were referred to our hospital due to primary amenorrhea. Defects in testosterone (T) and dihydrotestosterone (DHT) synthesis were excluded. Physical examination revealed that the patients have normal female external genitalia, normal breast development, vellus hair in the axilla and on the arms and legs, but absence of pubic hair, and a blind-ending vagina. Two different types of *AR* mutations have been detected by sequencing of genomic DNA: Family A showed deletion of exon 2 in AR gene; Family B showed a single nucleotide C-to-T transition in exon 8 of *AR* gene resulting in a proline 893-to-leucine substitution (Pro893Leu). Testicular histology showed developmental immaturity of seminiferous tubules with the absence of spermatogenic cells or spermatozoa. No *AR* immunoreactivity was observed in either case. Three adult patients recovered well from bilateral orchiectomy. The juvenile patient of family B was followed up. Our present study on these two families revealed two different types of *AR* mutation. The definitive diagnosis of AIS was based on clinical examination and genetic investigations. Our findings verified the mechanism of CAIS and also enriched *AR* Gene Mutation Database.**

Keywords: Complete androgen insensitivity syndrome, androgen receptor, disorder of sex development, AR domains, deletion and transition

1. Introduction

Androgen plays a key role in the control of male sexual differentiation and the maintenance of normal male reproductive function. Androgen actions are mediated by ligand-dependent transcription factors, and the androgen receptor, which is translocated into the nucleus and binds to the regulatory regions of specific chromosomal DNA sequences to activate androgen dependent genes. The androgen-AR complex functions in conjunction with co-regulatory proteins (*1-3*).

Inability to respond to circulating androgens named as androgen insensitivity syndrome (AIS), formerly known as "testicular feminization syndrome", was first described by Morris in 1953 (*4*). Androgen receptor (AR) gene mutations are the most frequent cause of 46, XY disorders of sex development and associated with a variety of phenotypes, ranging from phenotypic women (complete androgen insensitivity syndrome (CAIS)) to milder degrees of undervirilization (partial form or PAIS) or men with only infertility (mild form or MAIS) (*5*). The mutated *AR* gene products which lost androgen-binding ability and transcriptional activity abolished the target cells' response to testosterone and dihydrotestosterone (*6,7*).

The estimated prevalence of this disorder was 1:20,000 to 1:64,000 live male births (*5,8-10*). To date, over 500 unique mutations in the *AR* gene causing androgen insensitivity syndrome had been reported

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from more than 850 patients (*http://androgendb.mcgill.ca)* (*11*). Most cases of AR mutation were inherited and transmitted from parents to offspring generation (*9*).

We reported our experience in the diagnosis and treatment of four patients with CAIS from two unrelated Chinese families at Huashan Hospital of Fudan University. Diagnosis was made by physical examination, imaging examination (B-ultrasound, CT scan) and laboratory tests (including measurement of blood sexual hormones and karyotype analysis). Polymerase chain reaction (PCR) and DNA sequencing of *AR* gene were also carried out using peripheral blood leukocytes of the probands and siblings.

2. Materials and Methods

This study was approved by the institutional review board of Huashan Hospital, Fudan University. From 2009 to 2012, two young Chinese female individuals and their siblings with CAIS from two families were referred to our hospital.

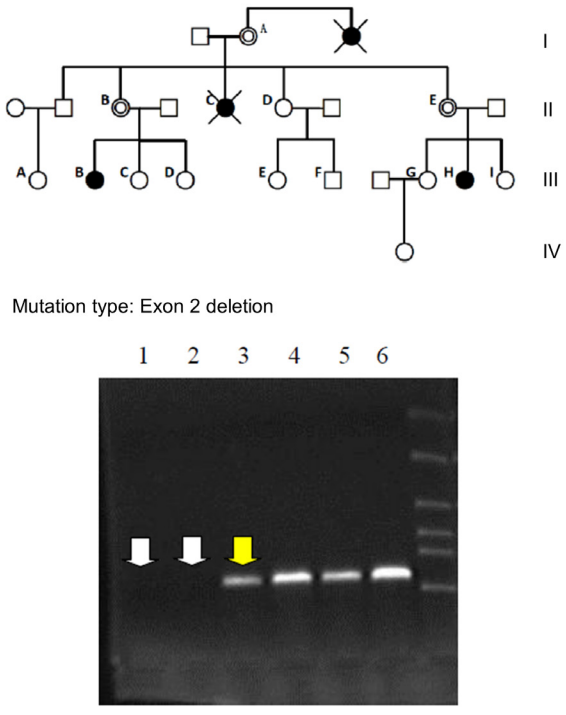

Figure 1. Amplification of the AR gene exon 2 in family members. Lanes 1 and 2 = No amplification in the CAIS patients (III-B and III-H). Lane 3 = Intermediate amplification in an obligate carrier mother of a CAIS patient (II-B).Lanes 4 to 6 = Strong amplification in three normal family members. Lane 5 is a normal male family member. PCR amplification and sequencing of *AR* gene exons in the probands showed a deletion of exon 2. DNA sequencing confirmed that there was no point mutation, except the deletion of exon 2, and showed that the remaining exons and introns were intact in the *AR* genes of the probands. (*Note*: The pedigrees with complete androgen insensitivity syndrome (CAIS); Black circles = CAIS 46, XY individuals; open circles = normal females; double open circles = obligate carrier women; open squares = normal males; crossed black circles = deceased individuals suspected of having CAIS.)

2.1. *Clinical features*

Family A: A 24-year-old female (III-B in the pedigrees of family A, Figure 1) was referred to our hospital due to primary amenorrhea. Physical examination revealed a 171 cm height, 55 kg weight patient with normal female external genitalia, normal breast development, but absence of pubic hair, and 4 cm deep blind-ending vagina. B-ultrasound showed one testicle located at right inguinal area accompanying right inguinal hernia, another testicle located in the left side of pelvic cavity, and without internal female genital organs (uterus and ovaries). A peripheral leukocyte chromosome analysis gave a 46, XY karyotype. The patient expressed satisfaction with her sexual life and accepted female gender very well. These data supported the diagnosis of CAIS, and the patient underwent bilateral orchiectomy without vaginal lengthening. Her 22-year-old cousin (III-H in the pedigrees of family A, Figure 1) with 46, XY karyotype was also diagnosed with CAIS. The presentation, diagnosis, and treatment were the same as that of the proband, except that testes were found in bilateral inguinal regions.

Family B: A 20-year-old female (III-B in the pedigrees of family B, Figure 2) was referred to our hospital due to primary amenorrhea with bilateral solid inguinal mass. Physical examination revealed 170 cm height, 50 kg weight young girl with normal female external genitalia, normal breast development, and an 8 cm deep blind-ending vagina (Figure 3A). Imaging examinations (computer tomography and B-ultrasound) showed testis-like gonad located at each side of inguinal

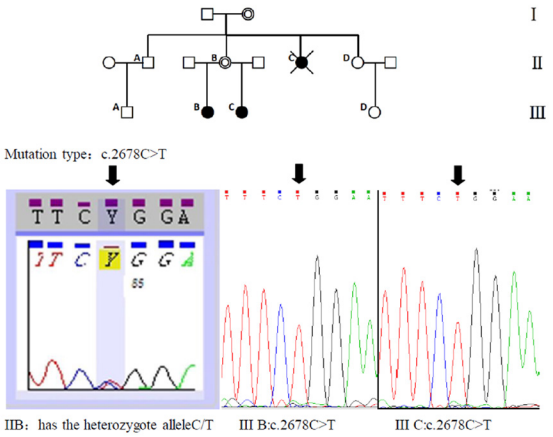

Figure 2. Direct sequencing analysis of PCR products revealed in the patients with the presence of a C to T transition in exon 8 resulting in the proline 893 leucine substitution (Pro893Leu) in CAIS patients of this family. Their mother was detected with the same mutation in heterozygous form. (*Note*: The pedigrees with complete androgen insensitivity syndrome (CAIS); Black circles = CAIS 46, XY individuals; open circles = normal females; double open circles = obligate carrier women; open squares = normal males; crossed black circles = deceased individuals suspected of having CAIS.)

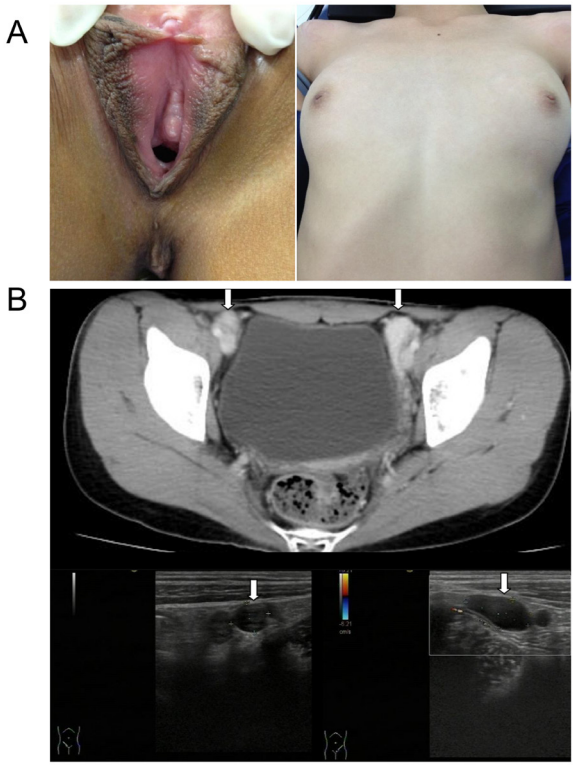

Figure 3. Patient III-B from Family B. (A), showed normal female external genitalia, normal breast development, absence of pubic hair and a blind-ending vagina. **(B)**, Imaging examinations (computer tomography and B-ultrasound) showed testis-like gonads located in the inguinal canals bilaterally.

canal (Figure 3B). Sex chromosome analysis reported 46, XY karyotype. This patient was also diagnosed with CAIS and underwent bilateral orchiectomy. Her 13-year-old half-sister (III-C in the pedigrees of family B, Figure 2) was also diagnosed with CAIS after clinical examination. The juvenile patient was followed up.

2.2. *Pedigree analysis*

Two pedigree analyses of these two unrelated families were performed. The confirmed CAIS patients were subjects III-B and III-H of family A; subjects III-B and III-C of family B. Subject I-B, subject II-C of family A and subject II-C of family B were reported infertile with female phenotype and were also suspected to have CAIS.

2.3. *Hormone assays*

Serum levels of testosterone (T), estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and dihydrotestosterone (DHT) were measured by radioimmunoassay for the probands and their siblings.

2.4. *DNA extraction and sequencing*

Genomic DNA was extracted from peripheral blood samples of family members using a Qiagen Pure Gene Blood Core Kit C according to the manufacturer's instructions (QIAGEN, Shanghai, China). The coding region of the *AR* gene was screened by polymerase chain reaction (PCR) amplification and direct sequencing was screened using the ABI 3730 XL DNA analyzer (Applied Biosystems). PCR primers were designed as reported before (*12*). The *AR* gene variations were identified between the patient with CAIS and the reference genome using the BLAT tool of the UCSC Genome Browser (available from: *http://genome.ucsc.edu*).

2.5. *Testicular histology*

Gonadal tissue of the adult patients (III-B and III-H of family A; subjects III-B of family B.) was fixed with 10% buffered neutral formalin solution. Histopathological change was observed by hematoxylin-eosin stain microscopically. Immunohistochemistry was conducted on paraffin-embedded tissue sections of the viable testicle using AR antibody (DAKO, Glostrup, Denmark).

3. Results

3.1. *Probands' clinical characteristics*

The results of hormone levels and physical examination are shown in Table 1. Elevated blood LH level was found in the probands and their siblings. Three adult CAIS patients revealed slightly increased E2 level, but T and DHT were within normal range. Detailed physical examination performed on our three adult CAIS patients didn't show any prominent difference compared to a normal woman. The external genitalia and blind-ending vagina didn't affect their sexual life.

3.2. *Identification of the genetic mutation*

Different types of AR mutations have been detected on genomic DNA. Family A: PCR amplification and sequencing of *AR* gene exons in the probands showed the deletion of exon 2. DNA sequencing confirmed that there was no point mutation, except for the deletion of exon 2, and showed that the remaining exons and introns were intact in the *AR* genes of the probands (Figure 1). Family B: Direct sequencing analysis of PCR products revealed the presence of a single nucleotide C-to-T transition in exon 8 resulting in a 893 proline-to-leucine substitution (Pro893Leu) (Figure 2) in CAIS patients of this family. Their mother has the same mutation in heterozygous form.

3.3. *Histology report*

The specimen from the gonadectomy was identified as testis with epididymis and vas deferens attached.

Table 1. Summary of clinical characteristics of CAIS patents

Items		Family A		Family B	
		Patient III-B	Patient III-H	Patient III-B	Patient III-C
Age		24	22	20	13
Height (cm)		171	172	170	155
Weight (kg)		55	52	50	40
Clitoral length (cm)		1.4	1.2	1.5	1.0
Clitoral to urethral length (cm)		2.0	2.2	2.0	1.5
vaginal depth (cm)		4	5.5	8	(not measured)
(Hormone analysis results)	(Normal male range)				
Testosterone	9.90-27.80 nM	23.5 nM	31.1 nM	40.57 nM	20.1 nM
Estradiol	28.00-156 pM	171.8 pM	143.3 pM	187.5 pM	121.3 pM
Luteinizing hormone	1.70-8.60 IU/L	45.81 IU/L	33.70 IU/L	47.04 IU/L	22.9 IU/L
Follicle-stimulating hormone	1.50-12.40 IU/L	2.35 IU/ L	13.80 IU/L	25.04 IU/L	7.30 IU/L
Dihydrotestosterone	55.10-386.5 ng/dL	51.0 ng/dL	49.1 ng/dL	60.4 ng/dL	55.4 ng/dL

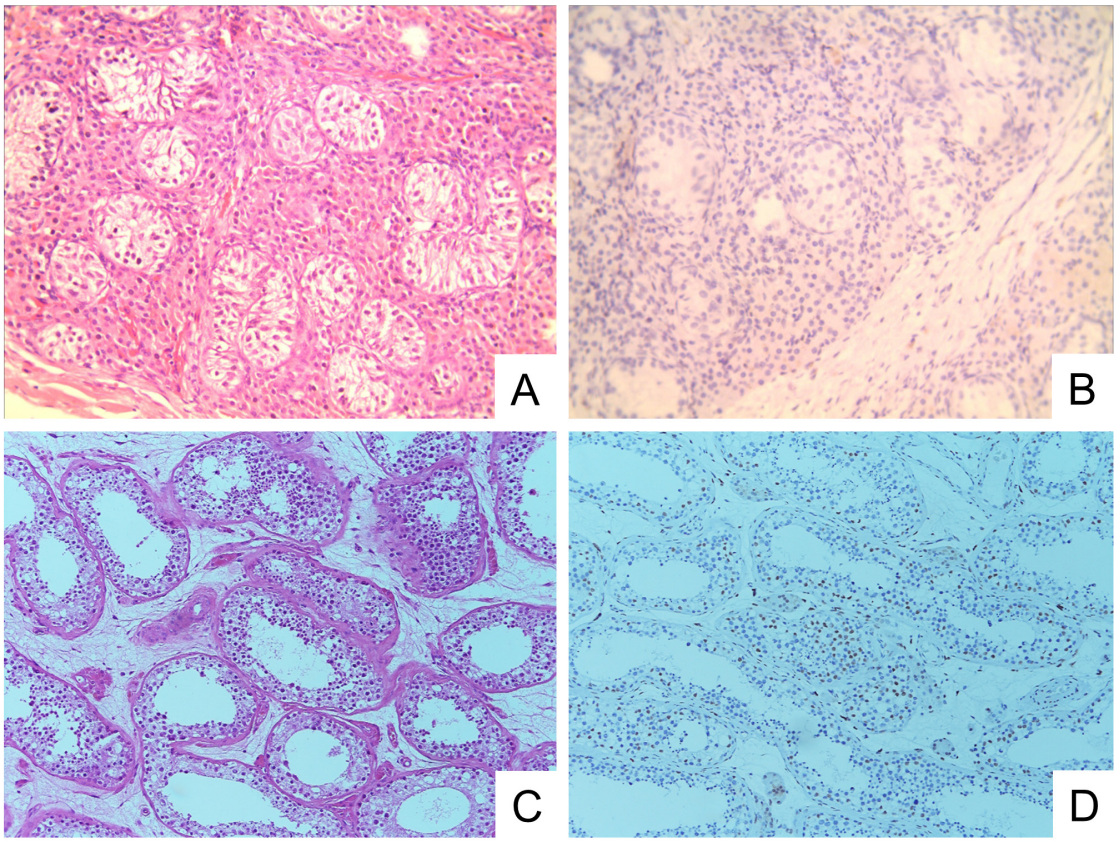

Figure 4. Patient III-B from family B: (A), showed developmental immaturity of seminiferous tubules containing monolayers of Sertoli cells without spermato¬genic cells or spermatozoa. **(B)**, showed no immunoreactivity for androgen receptors. **(C)**, showed normal seminiferous tubules with spermatogenic cells and spermatozoa (positive control). **(D)**, the seminiferous tubule normal testicular tissue demonstrates strong nuclear immunoreactivity for androgen receptors.

Testicular histology showed developmental immaturity of seminiferous tubules containing monolayers of Sertoli cells without spermatogenic cells or spermatozoa together with hyperplasia of mesenchymal cells and fibrous tissue (Figure 4A). No AR immunoreactivity was observed in all cases (Figure 4B). Negative AR immunostaining was attributed to the absence of AR production at the protein level. The absence of AR immunostaining in our cases could reflect that either Sertoli cell immaturity or *AR* gene mutation could result in no expression of AR protein at all.

4. Discussion

The androgen receptor gene is more than 90 kb long with 8 exons and located at Xq11–12 (*13*) The subsections, or domains, consist of the N-terminal domain (NTD, residues 1–534) harboring *AR* transcriptional activation function encoded by exon 1, a central DNA-binding domain (DBD, residues 559-624) encoded by exons 2 and 3, the "hinge" region which binds the NTD and DBD, and ligand binding domain (LBD, residues 664–919) encoded by exons 4-8 (*14,15*).

The DNA-binding domain (DBD) is the region of the protein that interacts with DNA. The androgen receptor DBD determines androgen selectivity of transcriptional response (*16*). The ligand binding domain (LBD) is the site of interaction of the ligand (the androgen hormone), binding of which will turn on the androgen function that will lead to a migration of the receptor to the cellular nucleus and the activation of the receptor's target genes (*17*).

According to the *AR* mutation database (ARDB *http://androgendb.mcgill.ca*), out of 314 unique *AR* mutations causing CAIS, 89 mutations were located at the NTD, 49 mutations located at the DBD, 158 mutations located in the LBD, and 18 mutations located in the intron and splice site. Not surprisingly, most mutations (207/314) are found in the DBD and the LBD. Most of mutations of these two domains would make the crystal structure change and cause the mutated *AR* to be completely inactive (*18*).

Our present study of these two families revealed two different types of *AR* mutation in CAIS patients: deletion of exon 2 and a single nucleotide mutation transition in exon 8. Although there were a few similar reports about these mutations, it was the first found in Chinese people.

In our study, the probands' mothers were carriers of the mutant allele and the patients' fathers exhibited the normal allele. *AR* mutation was inherited and transmitted from mother to the offspring generation. Our study provided useful information in prenatal diagnosis and recommendation of appropriate counseling for these two families.

In the female infant or toddler, no immediate therapy was needed for CAIS patients. These patients who had normal female hormonal levels would develop into phenotypically normal females. Once final height and breast development had been obtained, the gonads should be removed because of risk of testicular tumors (*19*). Our three adult CAIS patients accepted orchiectomy surgery and estrogen replacement therapy was applied afterwards.

It was reported that 90% of women with CAIS had sexual difficulties when compared to the general female population, including most commonly sexual infrequency and vaginal penetration difficulty (*20*). Women with CAIS may have vaginal hypoplasia, clitoral hypoplasia, and psychological problems that might contribute to sexual dysfunction. Detailed physical examination performed on our three adult CAIS patients didn't show a remarkable difference compared to a normal woman. We assumed that sexual difficulties were related to the degree of feminization.

After orchiectomy surgery, the regular follow-up included three aspects: sexual hormone levels, sexual function and psychological state. Our adult CAIS patients accepted their female gender with psychological gratification pre- and post-operatively. We were more willing to encourage our patients to accept their female gender because of the concern that the female-to-male sexual transition could be more challenging (*21,22*).

In conclusion, the definitive diagnosis of CAIS was based both on clinical examination and the results of appropriate investigations. Our clinical experience revealed that the mutated *AR* gene resulted in primary amenorrhea and absence of internal genitalia. Our findings of *AR* mutations verified the mechanism of CAIS and also enriched the *AR* Gene Mutations Database.

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Case Report

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Cardiac amyloidosis in a heart transplant patient - A case report and retrospective analysis of amyloidosis evolution

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Summary **Cardiac amyloidosis is a very rare cause of heart failure in heart transplant recipients but an important differential diagnosis in cases of progressive cardiac failure. We report a 72-year-old male patient with the diagnosis of senile systemic amyloidosis (SSA) in a transplanted heart 15 years after transplantation by the initial diagnosis of the dilated cardiomyopathy. Additionally performed immunohistochemical analysis with anti-transthyretin antibody of the cardiac biopsies of the last 15 years enabled the possibility to show the evolution of this disease with characteristic biphasic pattern.**

Keywords: ATTR amyloid, senile systemic amyloidosis, heart transplantation, cardiac biopsy

1. Introduction

Amyloidosis results from a systemic or localized accumulation of polypeptides and proteins, which are typically arranged in an anti-parallel β-sheet conformation, rendering them insolubly. Those amyloid deposits can affect diverse tissues and organs. Amyloidosis of the heart eventually leads to heart failure. Cardiac amyloidosis is most commonly of either ATTR- (SSA and hereditary), AL- or AANP-type. Other forms are exceptionally rare. The common types of cardiac amyloidosis have variability in their precursor protein, age of manifestation, extracardiac organ involvement, treatment and prognosis (*1*). Depending on the type of amyloid, supportive therapy and ultimately heart transplantation as therapeutic approach can be performed (AL amyloid with combined heart/bone marrow transplant, hereditary ATTR amyloid with orthotopic heart transplantation or combined heart/liver transplantation and SSA with orthotopic heart transplantation (*2*)).

Herein we report a case of a 72-year-old male patient with the diagnosis of isolated cardiac amyloidosis in a transplanted heart 15 years after transplantation. Subsequently performed retrospective immunohistochemical analysis of myocardial biopsies provided an insight in the time progression of this disease.

2. Case report

The now 72-year-old male Caucasian patient underwent heart transplantation in 1998 due to severe, idiopathic dilated cardiomyopathy (DCM). Following transplantation the patient was treated with immune suppressive therapy and underwent repeated diagnostic tests *i.e*. echocardiography, cardiac magnetic resonance imaging and a total of 42 repeated biopsies of the heart transplant (Figure 1) to evaluate signs of graft rejection. The post-transplant visits took place in the outpatient transplantation unit of the Aachen University Hospital RWTH. Due to low-grade graft vasculopathy the patient underwent percutaneous coronary intervention (PCI) of the left anterior descending (LAD) with implantation of a bare metal stent in the year 2000. In April 2001 a severe graft rejection (type A3) was diagnosed by biopsy. Consequently

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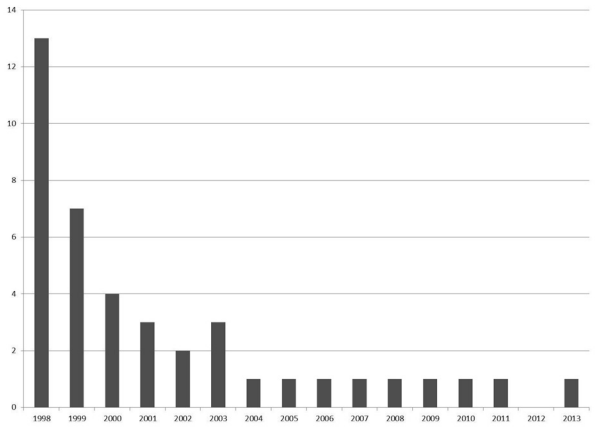

Figure 1. Numbers of biopsies per annum since 1998 (year of the heart transplantation).

the patient received a glucocorticoid pulse therapy of 1000 mg of methylprednisolone for three consecutive days. There were no signs of progression of the graft vasculopathy in follow-up angiographies. Two subsequent MRI studies (2011 and 2013) showed signs of intracardiac fibrotic remodelling mainly at the base of the interventricular septum and the inferio-lateral ventricle – which was clinically attributed to averted graft rejection - without overt aggravation. Repeated echocardiography revealed progressive lateral and septal wall thickening of the left ventricle and increasing diastolic dysfunction (Figure 2). To rule out storage diseases like amyloid deposition, a rectal biopsy was done in January 2013, which returned negative results. Furthermore, no λ- or κ- light chain proteins were detected by serum electrophoresis as possible sign of monoclonal gammopathy.

In spring 2013 the patient presented with an onset of new symptoms of dyspnoea on exertion (New York heart association, NYHA grade II) and paroxysmal palpitations especially during night time. NT-proBNP serum levels progressively increased. Electrocardiography revealed new onset of intermittent atrial fibrillation. Due to his history of graft vasculopathy, the patient underwent repeated angiography, this time revealing a severe vasculopathy with a three-vessel disease needing PCI with implantation of two drug-eluting stents into the circumflex artery. Immune suppression was changed from cyclosporine and mucofenolat-mofetil to cyclosporine and everolimus. In 2013 the state of health progressively deteriorated and dyspnoea worsened. The patient developed ankle oedema and signs of pulmonary venous congestion upon chest X-ray. Another graft biopsy was finally taken and revealed the presence of homogeneous eosinophilic material in a routine haematoxylin eosin (H&E) stain, suggestive of amyloidosis. This was confirmed by light microscopy on Congo red stain with characteristic green birefringence under polarized light (Figure 2). The immunohistochemical classification of the amyloid deposits revealed ATTR amyloidosis resulting in two differential diagnoses: *i*) hereditary ATTR amyloidosis and *ii*) senile systemic amyloidosis (SSA). Genetic testing unravelled wild type-*TTR*. Despite one single episode of syncope in 2013 as a possible sign of involvement of autonomic nervous system, clinically no other evidence of amyloid deposition was found. *E.g*. urinary protein excretion was minimal. Finally, a diagnosis of SSA was obtained. Typical for this type of amyloidosis are cardiac manifestations and involvement of the peripheral nervous system with symptoms such as carpal tunnel syndrome (*3*). The cardiac involvement - as seen in our patient - manifests itself with cardiac failure, due to disorders of transmission of electrical impulses and atrial fibrillation (*4*), whereas a carpal tunnel syndrome was not found.

Based on frequently performed cardiac biopsies during the 15 years after the heart transplantation we had an opportunity to analyse the evolution of amyloidosis with immunohistochemical methods and subsequent quantification of amyloid load.

3. Materials and Methods

For histological analysis H&E stained slides and paraffin blocks from the heart biopsies were retrieved from the archive of the Institute of Pathology of the University Hospital RWTH Aachen. 4 µm thick paraffin sections were stained with H&E. Amyloid was detected in Congo red-stained sections viewed under cross-polarized light. Immunostaining was carried out as described in detail elsewhere (*5*).

The immunohistochemically (anti-transthyretin-antibody) stained slides were scanned using a Leica SCN400 whole slide scanner (Leica Biosystems, Nussloch, Germany) with 40 times magnification. For image analysis the slide images were exported as overview and with 9 times magnification, corresponding to a pixel width of about 1.2 µm. The percentage of the amyloid area of each specimen was evaluated using ImageJ version 1.47v (National Institute of Health, USA) by counting the immunohistologically stained and non-stained pixels. In a prior step artifacts surrounding the specimen within the original image were removed manually using Adobe Photoshop CS4 Extended Version 11.0.2 (Adobe Systems Incorporated, San Jose, USA). Processing in ImageJ was done using the "Color Threshold" function to filter pixels based on ranges of hue, saturation and brightness values in the HSB color model. Background was detected by filtering pixels of high brightness and low saturation. The specimen's pixel count was calculated by subtracting the background's pixel count from the total pixel count of the image. Stained areas (red pixels) were detected by filtering the corresponding range of hue values in combination with a lower threshold of saturation. The threshold values were adjusted individually for each

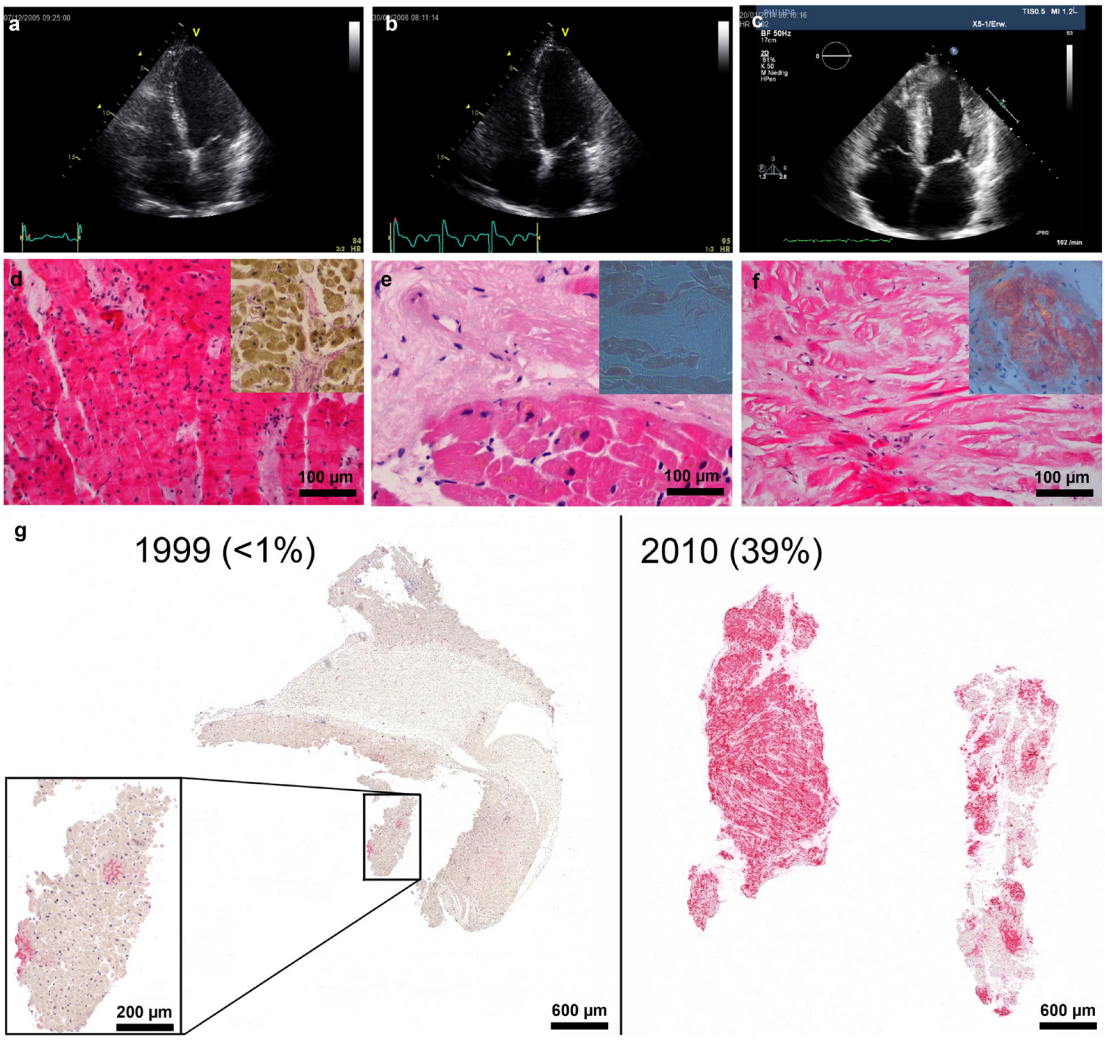

Figure 2. First row: Echocardiography: a: 4-chamber view from 2005 showing normal size of all four chambers without signs of left- or right ventricular hypertrophy, **b:** 4-chamber view from 2008 showing light left ventricular hypertrophy, **c:** 4-chamber view from 2014 now showing severe concentric left- and right ventricular hypertrophy as well as the typical sparkling pattern of storage diseases with myocardial affection. **Second row: Histology of heart biopsies: d** initial biopsy after heart transplantation without fibrosis or signs of eosinophilic deposits, hematoxilin and eosin-stain (H&E) and Elastica van Giesson (EvG) stain, **e** focal marked fibrosis 8 years after transplantation, H&E and Congo red stain under polarized light (insert) without green birefringence, **f** current biopsy, H&E and Congo red stain (insert) under polarized light with green birefringence indicating amyloid deposits. **Third row: Immunohistological staining with anti-transthyretin antibody: e** comparison shows the remarkable contrast of the amyloid load in 1999 and 2010.

image under supervision of a pathologist in order to compensate for variations of the staining process.

4. Results

Reassessment of the stained slides of the heart biopsies showed that the patient's own myocardium did not contain any amyloid. During the first 7 years after the transplantation three graft biopsies enclosed no amyloid. Nine out of 12 biopsies obtained before 2006 enclosed tiny (< 2%) amyloid deposits, which were unnoticeable by routine H&E-staining. Starting in 2006 the amyloid load increased gradually from 4% to 43% (Figure 2 and 3). Interestingly, a biopsy obtained in 2011 harboured only 12% amyloid load, while a biopsy obtained in 2010 and 2013 enclosed 39% and 43%, respectively, indicating that amyloid load is susceptible to sampling errors.

5. Discussion

Amyloidosis is a heterogeneous disease according to the diversity of proteins, which are able to form amyloid and the various different aetiologies. However, the clinical presentation of cardiac involvement is rather homogenous and may present as hypertrophy of the left ventricle, diastolic and/or systolic heart failure. Especially in SSA the heart involvement leads – with the exception of one reported case of a 77 years old patient with dilated cardiomyopathy and angina pectoris (*6*) – usually to congestive heart failure. Dyspnoea, oedema and reduced physical activity are typical

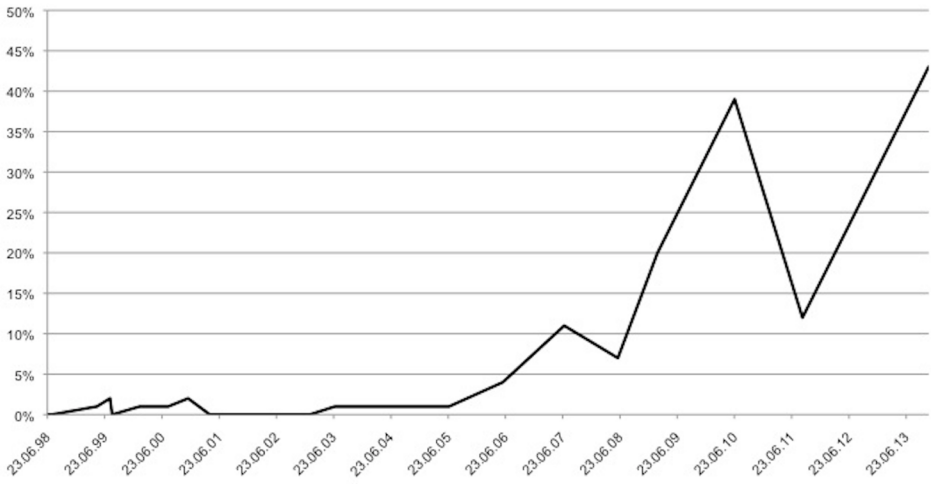

Figure 3. Changes of amyloid load in the heart biopsies during 15 years, starting in 1998, the year of transplantation. The first result from 23rd June of 1998 shows the amyloid load in patient's own heart, the other results are from the transplanted heart.

clinical signs.

Our case provides several interesting and partially novel findings:

i) SSA can affect cardiac grafts and should be considered in the differential diagnosis of graft failure. Thus, using Congo red as a routine stain in graft biopsies of elderly patients may be sensitive in order to reach an early diagnosis. However, minimal amyloid deposits can be missed and sensitivity maybe increased by using fluorescence microscopy and immunohistochemistry, as has been suggested before (*7*). In our case cardiac amyloidosis was eventually diagnosed 15 years after the heart transplantation, despite continuous close monitoring of graft function and repeated close-meshed biopsies (Figure 1), which previously did not show signs of amyloid deposition in H&E routine stains like homogenous eosinophilic low cellular areas, which would have led to additional histological investigations. Storage diseases were initially clinically considered when echocardiography showed increased thickening of the left ventricular wall and the septum (*8*). Usually, SSA becomes apparent beyond the age of 80 (*9*) despite isolated reports about early cardiac involvement in SSA as early as 67 years of age (*10*). Our patient was 72 years old at the time of the diagnosis.

ii) Interstitial deposits of cardiac ATTR-amyloidosis commonly show a patchy deposition pattern, which is different from the more uniform reticular deposition pattern of cardiac AL-amyloidosis (unpublished observation of Co-author Christoph Röcken). This carries the risk of a sampling error and a negative test results may not exclude the presence of amyloid. In our series, 3 out of 12 biopsies obtained before 2006 enclosed no amyloid, reaching a false negative rate of 25% at the early stage of the disease. Thus, amyloid should be sought even when previous graft biopsies failed to demonstrate amyloid. After diagnosis of graft amyloidosis the important question to clarify remained whether the initial heart failure before the patient was transplanted was associated with amyloidosis? This might at that time have fundamentally altered the diagnostic and therapeutic approach (*11*). But reassessment of the heart biopsies before transplantation did not reveal signs of amyloidosis.

iii) This study provides for the first time biopsy-proven insights into the natural course of SSA. The progression of SSA appears to follow a biphasic pattern with a relatively long lag-time without any obvious disease progression and minimal amounts of amyloid deposition followed by an acceleration phase with a steady incline of the amyloid load finally leading to clinically overt SSA. SSA is a disease of the elderly and factors contributing to this age-dependency are slowly unravelled. Amyloidosis is caused by misfolding and aggregation of polypeptides in a β-pleated sheet confirmation. A lack of protective chaperones, which are essential for the physiological folding of proteins, facilitates amyloid formation. The age-dependent disease manifestation may depend on the activity of stress-responsive signalling pathways. Their activity decreases with increasing age and enable the occurrence of misfolded, potentially amyloidogenic peptides and proteins (*12*). Heat shock proteins (HSP) are involved in these pathways and processes (*13*). The expression of HSP27 and HSP70 correlate with the presence of ATTR amyloid. Furthermore, the heat shock transcription factor-1 (HSF-1) is up-regulated in ATTR amyloidosis. HSF-1-deficient mice rapidly develop an amyloidosis (Almeida *et al*. 2012). Thus, the mere detection of amyloid in heart biopsies currently allows no prediction of disease progression. Amyloid load can be relatively static for a long period of time.

How is the prognosis of this disease? The outcome

of SSA is generally better compared to cardiac light chain AL amyloidosis. Median survival time of SSA is 7 - 8 years with supportive therapy and estimated survival time of cardiac light chain AL-amyloidosis is 48 months (*14*). It is fundamental to identify the subtype of the amyloid depositions (*15*) and to rule out any underlying and possibly treatable disease such as plasmacytoma for AL amyloidosis although in the case presented even earlier diagnosis of amyloidosis probably would have not altered the performed supportive therapy since there is no causative clinical approach. Due to the patient's age and comorbidities the patient presented cannot be considered for re-transplantation, although, his disease will probably progress. Destination therapy with ventricular assist devices may be a therapeutic option for patients as him.

In summary, amyloidosis (*16,17*) has to be added to the list of differential diagnoses of graft failure in a heart transplant recipients. The diagnosis is difficult to obtain and needs to be verified *e.g*. by Congo red-staining in combination with fluorescence microscopy and immunohistochemistry in order to detect the disease at an early stage.

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

Example 4 (Sample web page reference):
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