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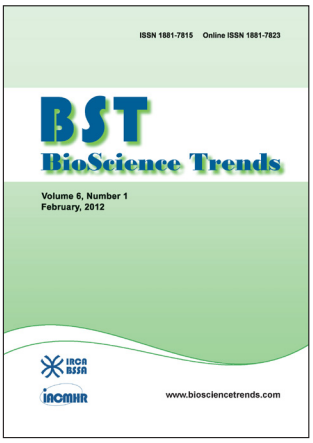

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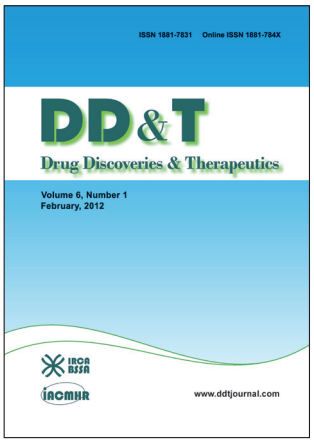

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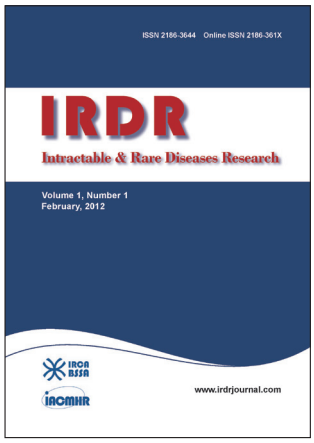

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A basic understanding of congenital extrahepatic portosystemic shunt: incidence, mechanism, complications, diagnosis, and treatment

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SUMMARY Extrahepatic portosystemic shunt belongs to a family of rare vascular abnormalities. The clinical importance and manifestations of this vascular abnormality range from asymptomatic cases to liver or metabolic dysfunctions of various degrees. Congenital extrahepatic portosystemic shunt, also termed as Abernethy malformation, is a very rare congenital vascular malformation in which splenomesenteric blood drains into a systemic vein, bypassing the liver through a complete or partial extrahepatic shunt. So far, limited cases of congenital extrahepatic portosystemic shunt have been reported. In this review, incidence, mechanisms, complications, diagnoses and treatments of congenital extrahepatic portosystemic shunt are described.

Keywords Congenital extrahepatic portosystemic shunt, incidence, mechanism, complications, diagnosis, treatment, review

1. Introduction

Extrahepatic portosystemic shunt belongs to a family of rare vascular abnormalities. Clinical importance of this vascular abnormality lies in its broad spectrum of symptoms and complications, ranging from incidentally discovered asymptomatic cases to liver or metabolic dysfunctions of various degrees and even severe clinical scenarios, which are caused by variations in sites or types of shunt (*1*). Congenital extrahepatic portosystemic shunt (CEPS), also termed as Abernethy malformation, is a very rare congenital vascular malformation in which splenomesenteric blood drains directly into a systemic vein, bypassing the liver through a complete or partial extrahepatic shunt. Since it was first documented in 1793 by John Abernethy (*2*), the number of reported cases has progressively increased (*3*). As is proposed by Morgan and Superina, CEPS is classified into two variants based on absence (type 1) or presence (type 2) of intrahepatic portal supply (*4,5*). Type 1 is described as complete portosystemic shunts (Figure A, end-to-side, a complete extrahepatic shunt) and the liver is not perfused with portal blood; whereas type 2 is defined as a certain proportion of portal blood perfused to the liver and the remaining portal flow bypasses the liver and is diverted into a systemic vein through a partial shunt (Figure B, side-to-side, a partial extrahepatic shunt) (*4,6*). This classification has been widely recognized and referred by a lot of studies because of its practical understanding of pathophysiologic implications and managements of included cases. In this review, we mainly present incidence, mechanisms, complications, diagnoses and treatments of CEPS.

2. Incidence

Knowledge about CEPS is scarce given that the low incidence of this malformation has prevented realization of large studies. Several issues stay unanswered, such as the actual incidence of CEPS. With limited sources, incidence of CEPS was once described close to 1/30,000 births and the prevalence proportion of permanent CEPS was 1/50,000, which came from results of a nationwide galactosemia survey. Because CEPS gave rise to increased levels of bile acids and galactoses, the incidence was extrapolated. Of note, only 7 CEPS cases were diagnosed and biases might remain in that survey (*7,8*). To a certain degree, the assumption that

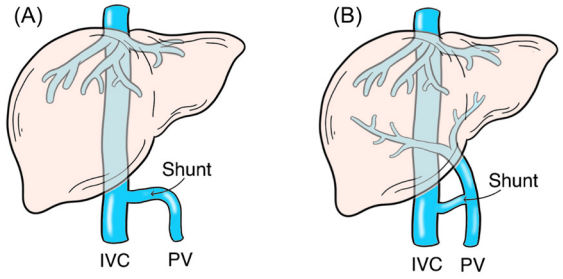

Figure 1. Classification of congenital extrahepatic portosystemic shunt. (**A**), Type 1 is described as complete portosystemic shunts (end-to-side) and the liver is not perfused with portal blood; (**B**), Type 2 is defined as a certain proportion of portal blood perfused to the liver and the remaining portal flow bypassed the liver and diverted into a systemic vein through a partial shunt (side-to-side). IVC, inferior vena cava; PV, portal vein.

CEPS could be diagnosed through increases in blood galactoses after birth yet requires further validations, and this incidence ought to be accepted with suspicions of a certain degree (*7*). No remarkable gender predominance was found in CEPS (*9*); however, it has been described in certain researches that type 1 shunts feature a significant female predilection of 61-78%, while no clear explanation is given (*7,10*). Thus, future studies to systematically evaluate presence of CEPS are warranted.

3. Mechanism

Rather limited researches have explored etiology mechanisms of CEPS. Two possible explanations have dominated literatures on development of CEPS: *i*) A genetic origin and complex congenital malformation processes: such malformations are considered to originate from an insult occurring in week 4-10 of embryological development, which is a crucial stage for hepatic and systemic vessel formations. And this to certain degree give evidence to the fair association with cardiac and other vascular anomalies (*7*); *ii*) The absence of ductus venosus during fetal stage: abnormal vessels may develop in association with occlusions or agenesis of ductus venosus. Through abnormal vessels, oxygenated blood from the umbilical vein flows into the fetal heart. Afterwards, certain vessels may persist and develop into anabnormal shunt, resulting in hypoplasia of the portal venous system (*11,12*). As is shown in literatures, absence of ductus venosus has been documented among some CEPS children (*13,14*).

Etiology mechanisms of CEPS differ significantly from that of acquired extrahepatic portosystemic shunts. As to acquired extrahepatic portosystemic shunts, due to liver cirrhosis, collateral vessels would generate or reopen to compensate for blocked or narrowed portal veins, so as to lower pressures on the portal tree. Gradually, this will result in an acquired extrahepatic portosystemic shunt (*15,16*). Hence, variations of therapeutic strategies should be noted for CEPS and acquired extrahepatic portosystemic shunts.

4. Complications

Spectrums of clinical variants of CEPS ranges from completely asymptomatic forms to severe forms of hepatic encephalopathy, hepatopulmonary syndromes (HPS) and pulmonary arterial hypertension (PaHT).

4.1. Hepatic encephalopathy

Hepatic encephalopathy is a common clinical manifestation in symptomatic patients, with a reported incidence of as high as 17-30% (*7,14,17,18*). Blood ammonia produced by gastrointestinal tracts directly bypasses the liver and flows into inferior vena cava, then it is metabolized in astrocytes to glutamine, which in turn has deleterious effects on the brain. This phenomenon is more frequently seen among older patients. Most children with CEPS are asymptomatic, which proves that children are more resistant to hepatic encephalopathies than adults. Partially, this might be caused by ageing of brain, decreased blood-brain barriers and losses of brain reserves, all of which make patients more vulnerable to harmful metabolic products such as ammonia and facilitate the revelations of hepatic encephalopathies (*19-21*). In a previous international observational study, Baiges, A. described that 29% of patients with CEPS presented hepatic encephalopathies at a certain time during the study. Median age at hepatic encephalopathy onset or diagnosis was 12 years (range, 5-65). Different patterns of hepatic encephalopathy were documented with a fair predominance of chronic hepatic encephalopathy: 73.7% featured persistent hepatic encephalopathy with permanent cognitive impairment, 11.0% featured recurrent hepatic encephalopathy, and 15.3% featured episodic hepatic encephalopathy. In most cases, in accordance with West-Haven standard, episodes of hepatic encephalopathies were of moderate intensities (*20*).

4.2. Pulmonary complications

Patients with CEPS will be frequently associated with severe pulmonary complications, such as HPS (*7,22*) and PaHT (*14,23*). Developments of HPS and PaHT can be linked with vasoactive mediators from intestines (*22,24,25*). Such mediators skip hepatic circulations and directly approach the pulmonary vascular bed, causing pulmonary circulation imbalances and inducing a long-term pulmonary vasoconstriction in PaHT (*9,24*) or, reversely, a pulmonary vasodilation in HPS (*26*).

HPS is a clinical relationship between hepatic diseases and existence of pulmonary vascular dilatations, which is characterized by presence of dyspnea and hypoxia and can result in arterial oxygenation abnormalities of a certain range. Prevalence of HPS

among patients with CEPS is still not clear, because mild HPS are often asymptomatic and a comprehensive study on lungs cannot always be carried out on all patients. Hence, an actual prevalence cannot be accurately predicted. In addition, it is recognized that CEPS should be considered as one of the etiologies of HPS (*27*). Since the first case report about CEPS with HPS by Papagiannis (*28*), there have been more than 20 similar cases described in literatures (*22,29,30*). A baseline comprehensive assessment on pulmonary complications is suggested to be undergone. In follow-up durations, transcutaneous oxygen saturation measurements can be of help in early and primary detections of HPS.

It has been demonstrated that PaHT is a considerable issue among CEPS patients, inducing symptomatic dyspnea in about 80% of cases; it has been shown by data that PaHT occurred in 13-66% of children with CEPS (*17,23*). When carefully screened, PaHT is also manifested on nearly 11% of asymptomatic patients (*20*). Rarity of such disease and the small amount of patient series have led to a rather broad percentage range. Despite virous hypotheses and reports, PaHT mechanisms stay controversial. From histological perspective, the lungs present microthrombotic lesions, and it is considered that PaHT resulted from recurrent microemboli originating in the mesenteric circulation and travelling through a portosystemic shunt directly to the lungs (*23*). Symptoms of PaHT vary from disturbed consciousness to syncope. Right ventricular hypertrophies, decompressions of left ventricle or an increased estimated right ventricular systolic pressure are vital signs of an echocardiography. Notably, PaHT is not seemingly related to severity or degree of a shunt, while it features a decreased outcome, with a reported morality of nearly 50% (*7*). As PaHT can occur in a broad range of age, periodic and regular surveillances for PaHT may be of a great significance during follow-ups.

4.3. Liver nodules

An abnormal shunt of blood leads to non-specific liver disturbances because of uneven portal flow perfusions and arterialisations (compensatory increases in hepatic arterial flows). In ischemic liver parenchyma, absent or reduced portal flows result in lacks of nutrition and fatty degenerations in hepatocytes; thusly, liver dysfunctions occur, certain normal hepatocytes diminish, and hepatic atrophy follows; in well perfused areas, regenerative nodule generated. Altogether, these elements contribute to abnormal hepatic developments and functions, which will bring an incentive to nodule generations (*21,31,32*).

Nodular liver tumors are commonly seen in as many as 40-65% of patients with CEPS. Although most of such tumors are benign, among other neoplastic lesions, malignancies such as hepatocellular carcinomas and adenomas have also been reported (*20,21,33,34*). In a case series involving 26 CEPS patients with liver nodules, 70% of them had focal nodular hyperplasia or regenerative nodular hyperplasia, 20% had hepatocellular carcinoma, and 10% had adenomas; also, it has been shown in researches that 21% of patients had single nodules and 79% had multiple nodules (*9,14,20,21,31*). Hence, it should be noted that performances of a rigorous and periodic screening of liver nodules for patients with CEPS is of significant needs and values. Furthermore, researchers have also advocated that it is sensible to term cirrhosis strategies of hepatocellular carcinoma surveillance every half year (*20*).

5. Diagnosis

CEPS can be diagnosed at any age, which is often in childhood during the setting of neonatal cholestasis, hypergalactosemia, failures to thrive, psychomotor delays or other congenital defects. Prenatal diagnoses will be considered through ultrasound detections of abnormal communication vessels between portal and peripheral venous systems or an enlarged umbilical vein (*9,14,35,36*). In adulthood, CEPS could be incidentally diagnosed through abdominal ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) in the screening of unexplained abdominal pains, abnormal liver functions and hepatic nodules, or *etc*. (*37-39*). Of note, for accurate diagnoses of CEPS, one should first excluded potential acquired portosystemic shunts, such as those patients with hepatic cirrhosis, with or without concomitant presentations of portal hypertension (*40*), and surgically created portosystemic shunts (*41*). Generally, non-congenital or acquired shunts are small, tortuous peripheral vessels, and CEPS patients will not present hepatic cirrhosis or portal hypertension imaging features (ascites, varices, or splenomegaly) (*42,43*).

Ultrasound is widely utilized in abdominal diseases for its safe and fast imaging properties. Primarily, it can be a useful diagnostic tool to identify presence of portosystemic shunts. Doppler ultrasound is of special diagnostic values for its abilities to assess vessel flow directions. As is reported previously (*35*), clinically asymptomatic shunts have been occasionally diagnosed in children through ultrasound during galactosaemia test. Ponziani FR, *et al*. have summarized concomitant presence of five ultrasound signs to strengthen the suspicion of CEPS (*39*), including: *i*) Solid focal lesions in liver parenchyma; *ii*) Portal trunk absence/hypoplasia; *iii*) Deficiencies of intrahepatic portal vessels and flow signals; *iv*) Existence of porto/systemic shunts; *v*) Hepatic artery hypertrophies. However, ultrasound might fail to accurately detect inapparent or small shunts. Hence, anomalies identified by ultrasound should be further confirmed through other imaging methods (*44*).

Radiological methods like CT or MRI are preferred investigations to confirm a portosystemic shunt. Asymptomatic CEPS are often diagnosed through incidental presentations on CT or MRI scans in

abdominal imaging. CT and MRI are also of great values in further classifications of shunts and measurements of accompanying anomalies (*10*). Postprocessing technologies, such as multiplanar reformations, have supplied additional information about diagnoses and managements.

A Contrast-enhanced CT can document anatomies, locations and sizes of abnormal shunts. With techniques like maximum intensity projection and multiplanar reconstruction, much information will be provided by CT, including shunt courses, sizes and orientations, which help both radiologists and surgeons to make or choose suitable treatment regimens.

Furthermore, MR can be used to visualize shunts and avoid radiation exposures for both radiologists and patients. Particularly, in assessments of associated liver nodules, MRI includes diffusion sequences and shows unique advantages over CT (*10*). Compared to CT, MRI would be preferred as the first-choice test. A research team from Canada analyzed 61 reported cases of CEPS and recommended MRI as a superior tool in diagnoses and classifications of CEPS as well as examinations of associated cardiovascular and hepatic abnormalities (*42*). Furthermore, MRI angiography serves as a reliable and noninvasive examination for hepatic vascular anatomies. Despite this, CT can be reserved for patients who are noncooperative or have specific contraindications. Traditionally, conventional angiography is regarded as a golden standard for detections of portomesenteric vasculatures. However, improvements in CT and MRI techniques have changed the situation. Currently, conventional angiography is not necessarily a must in CEPS diagnoses, for CT and MRI can provide accurate diagnoses in most cases.

According to existing findings on nuclear medicine researches, iodine-123 iodoamphetamine could also be used to measure shunt dynamics (*10,19*).

Furthermore, serum ammonia level is useful, although it is a non-specific and investigative adjunct. In 66-100% of CEPS cases, a heightened level of serum ammonia is found (*7*). Hence, concentrations of ammonia without known hepatic cirrhosis or portal hypertensions ought to imply subsequent examinations for CEPS (*45*). In certain cases, liver biopsies may illustrate small portal venules within portal triads, which is indicative of type 2 shunt (*42*).

6. Treatment

Currently, there is no standard therapeutic approach to treat extrahepatic portosystemic shunts due to rarity of such diseases (*46*). Several approaches including shunt closures through surgical or radiological interventions and liver transplantations have been proposed, but clear comparisons among different treatment strategies are still unavailable. Treatment strategies are decided according to shunt types, locations, symptoms severities and related complications. There still remains debate regarding therapeutic strategies for asymptomatic cases (*1,46*). Conservative managements including lifestyle changes, such as protein restrictions as well as lactulose and non-absorbable antibiotics administrations, may be recommended in asymptomatic shunts. Yet, presence of shunts could potentially develop clinical implications, such as supplying a route for intestinal toxic materials to bypass hepatic circulations, immune surveillances as well as offering a possible route for lung tumor metastases of gastrointestinal tumors (*1,19*). A Japanese research team has previously observed natural courses of 51 patients with CEPS and found that spontaneous shunt closures were never evident. Hence, early detections and suitable therapies are vital for a good prognosis (*32*).

6.1. Shunt closure for type 2 CEPS

Discrepancies regarding management of patients with asymptomatic type 2 did exist. Researchers from University of Catania chose active surveillances (*46*). However, more experts urge early and active interventions to be mandatory (*9,47*). When a clear diagnosis of extrahepatic shunt is made, it is important to remark that shunt closures facilitating progressive redirections of portal blood flowing to livers are possible and essential for such patients. As is proved in treatments of Abernethy malformations, shunt closures are especially useful in improving hepatic encephalopathies. Baiges A, *et al*. suggested that shunt closures had a huge efficacy in managing most shunt complications and, most interestingly, preventing their occurrences (*3*); this is in agreement with findings of Papamichail M. *et al*., who proved that early occlusions would reverse associated complications (*48*). Sanada Y, *et al*. and Pathak A, *et al*. had also demonstrated that patients with CEPS and hepatic encephalopathies can benefit by early shunt occlusion surgeries (*49,50*).

Therefore, shunt closures must always be considered for symptomatic patients and should also be regarded as a prophylactic treatment early in evolutions of the disease to prevent developments of severe complications. Earlier studies have shown that for shunt occlusions, either interventional embolization or surgical ligations (open or laparoscopic surgical techniques) can be choices of treatment that lead to rapid ameliorations of symptoms and normalizations of ammonia levels (*25,51*). Likewise, multiple results have proved that the associated HPS could also be resolved by shunt closures (*25,26,52,53*). The choice from an interventional embolization or a surgical ligation depends upon medical expertise, shunt vessel anatomies and sizes as well as induvial general conditions (*48,54,55*). For patients with wide and short shunt vessels or those who fail to receive embolization, a surgical ligation will be preferred (*54,56*).

6.2. Liver transplantation for type 1 CEPS

Shunt closures are not a feasible option for patients with type 1 CEPS, as the shunt stands for the only drainage path of mesenteric and splenic venous blood. Thusly, liver transplantation serves as effective therapeutic approaches for both liver and pulmonary complications. Literatures have described the successful application of liver transplantation for CEPS patients with severe complications (including refractory encephalopathy, CEPS associated with biliary atresia, or patients with severe HPS) (*37,57,58*).

Timing of liver transplantations for type 1 CEPS is still a matter of debate. Results from Japan show that prophylactic liver transplantations should be justified prior to occurrences of severe pulmonary complications (HPS or PaHT), because such complications would complicate or even preclude liver transplantations (*57*). However, Guerin F, *et al*. found that asymptomatic patients with type 1 CEPS ought not to receive liver transplantations as a prophylactic option, which would make them experience prolonged periods of immunosuppression (*7*). Sakamoto S, *et al*. reviewed a collection of 34 transplantation cases of CEPS and concluded that presence of pulmonary complications was an early indication of liver transplantations; in the review, 30 out of 34 CEPS cases with liver transplantations stayed alive after a median follow-up period of one and a half years, indicating an encouraging outcome (*6*). Sanada Y, *et al*. also revealed that liver transplantations could be potentially curative for patients with symptomatic type 1 CEPS (*49*). In most reported cases, liver transplantations provide a complete resolution for associated complications. Technical difficulties of portal system anatomies and reconstructions are main challenges (*39*).

6.3. Management of liver nodules

When liver nodules are identified in patients with CEPS, differential diagnoses of malignant and benign tumors will be crucial for determination of following treatments. For benign tumors, conservative treatments and regular follow-ups should be suggested. In cases of malignant tumors, subsequent surgical interventions like biopsies will be adequate.

At last, a close surveillance is indicated for patients with such vascular malformations. Long-term follow-ups will create a good clinical compliance and provide a comprehensive understanding and management of disease processes.

7. Conclusion

Differential diagnoses between CEPS and acquired portosystemic shunts are of much importance. When a clear diagnosis of CEPS is made, it is important to remark that active interventions are possible and essential for such patients. As to type 1 CEPS, liver transplantation serves as an effective therapeutic approach for both liver and pulmonary complications; as with type 2 CEPS, shunt closures must always be considered for symptomatic patients and should also be regarded as a prophylactic treatment early in evolutions of the disease, so as to prevent developments of severe complications. Yet, knowledge about CEPS is scarce due to its low incidence. Future studies on systematical explorations on CEPS are warranted.

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Review

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Update on cystine stones: current and future concepts in treatment

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SUMMARY Cystine stones are relatively uncommon compared with other stone compositions, constituting just 1% to 2% of adult urinary tract stone diseases, and accounting for up to 10% of pediatric stone diseases. Two responsible genes of cystinuria have been identified, the SLC3A1 and the SLC7A9. Cystinuria is diagnosed by family history, stone analysis, or by measurement of urine cystine excretion. Current treatments for cystinuria include increased fluid intake to increase cystine solubility by maintaining daily urine volume of greater than 3 Liter (L). Limiting sodium and protein intake can decrease cystine excretion. When conservative therapy fails, then pharmacologic therapy may be effective. Alkaline urine pH in the 7.0-7.5 range will reduce cystine solubility and can be achieved by the addition of alkali therapy. If these measures fail, cystine-binding thiol drugs such as tiopronin and D-penicillamine are considered. These compounds bind cysteine and prevent the formation of less soluble cystine. These drugs, however, have poor patient compliance due to adverse effects. Captopril can be useful in the treatment of cystine stones but the drug has not been tested in rigorous clinical trials. Novel potential therapies such as alpha-lipoic acid and crystal growth inhibitors (L-cystine dimethyl ester (L-CDME) and L-cystine methyl ester (L-CME)) were developed and tested in animals. Those therapies showed promising results. Compliance with treatment was associated with a lower rate of cystine stone formation.

Keywords cystinuria, urolithiasis, novel therapy, cystine stone, tiopronin, D-penicillamine

1. Introduction

Cystinuria is an inherited disorder of the dibasic amino acid transport system in the proximal tubule and the small intestine. Two responsible genes have been identified, the SLC3A1 on chromosome 2 and the SLC7A9 on chromosome 19. The inability of renal tubules to reabsorb cystine and the relative insolubility of cystine at physiological urine pH lead to stone formation (*1*). Children at risk for nephrolithiasis can be identified by the level of urinary cystine only after tubular transport has matured (age 2 years). Conservative therapy with high urine volume and urinary alkalinization is sufficient for some, but recurrent stone formation may cause renal damage (*2*). Cystinuria is a genetic disease that leads to the frequent formation of stones. In patients with recurrent stone formation, particularly patients < 30 years old or those who have siblings with stone disease, urologists should maintain a high index of suspicion for the diagnosis of cystinuria (*3*). Cystinuria is the cause of about 10% of all kidney stones observed in children. Without any preventive measures, the patients will suffer from recurrent stone formation throughout their life. Even with medical management, the long-term outcome is poor due to insufficient efficacy and low patient compliance. Many patients suffer from renal insufficiency as a result of recurrent stone formation and repeated interventions (*4*). Cystine stones may become very large, are often recurrent, and are difficult to fragment with extracorporeal shock wave lithotripsy (ESWL), so that preventive therapy is essential, and should be started as soon as the diagnosis is made. Cystinuria is diagnosed by family history, stone analysis, or by measurement of urine cystine excretion. When the stone type is unknown, patients should have one urine screened with a qualitative test for cysteine (*5*).

Therapy to reduce stone formation is directed towards lowering urine cystine concentration and increasing cystine solubility. Determining cystine capacity may be an effective tool to monitor the individual patient's response. Compliance in cystinuric patients concerning both dietary and pharmacological intervention is poor (*6*). Medical management is mainly based on hyperhydration and urine alkalinization. Long-term therapy with

sulfhydryl agents to prevent the formation of renal stones seems to be effective but adverse side effects are frequent, requiring the withdrawal of treatment (*7*). Prevention of stone formation is the primary goal of management and includes hydration, dietary restriction of salt and animal protein, urinary alkalinization, and cystine-binding thiol drugs (CBTD) (*8*). In mild cases of cystinuria, judicious urinary alkalinization and fluid may suffice but in more severe cases, a thiol agent, such as tiopronin or D-penicillamine, should be added (*9*). The durability of medically treating patients with cystinuria is limited with only a small percent able to achieve and maintain the goal of decreasing cystine below the saturation concentration. Greater physician vigilance in these complicated stone formers is required to achieve successful prophylactic management (10). Prompt referral for metabolic assessment, early multidisciplinary input, and total removal of the stone fragments are keys to preventing stone episodes and improving the long-term function of patients (*11*).

2. Etiology

Cystinuria is an inborn congenital disorder characterized by defective cystine metabolism resulting in the formation of cystine stones. Two genes responsible for cystinuria have been identified: SLC3A1 (chromosome 2p21) encodes the heavy subunit rBAT of a renal b(0,+) transporter while SLC7A9 (chromosome 19q12) encodes its interacting light subunit b(0,+)AT. Mutations in SLC3A1 are generally associated with an autosomal-recessive mode of inheritance whereas SLC7A9 variants result in a broad clinical variability even within the same family. The detection rate for mutations in these genes is larger than 85%, but it is influenced by the ethnic origin of a patient and the pathophysiological significance of the mutations (*12*). In cystinuria, the kidney, due to a genetic defect in the cystine transporter, is unable to reabsorb cystine in the proximal tubule, resulting in urinary hyperexcretion of amino acids cysteine, ornithine, lysine, and arginine (COLA). Of these, only cysteine is relatively insoluble at normal urinary pH, leading to stone formation when cystine concentration rises above the solubility limit (*13*).

In homozygotes or compound/mixed heterozygotes, the mutation of SLC3A1 or SLC7A9 is associated with increased urinary cystine excretion and kidney stone formation in 100% and 94% of cases respectively. For SLC7A9 heterozygotes, an increased urinary cystine excretion can be observed in 86-90% of cases and kidney stone formation in 2-18% of cases. Polymorphisms of the SLC7A9 gene probably affect the clinical course in SLC7A9 mutation carriers (*14*). Cystinuria is also observed in patients with the cystinuria-hypotonia syndrome, which is due to the microdeletion of part of the SLC3A1 and PREPL genes on chromosome 2p21 (*15*).

3. Classification

Traditionally, cystinuria has been divided into three subtypes: types I, II, and III based on the excretion of cystine and dibasic amino acids by the obligate heterozygous parents of the affected children. Type I heterozygotes show a normal amino acid urinary pattern, whereas type II and III are characterized by an increase of cystine, lysine, ornithine, and arginine urinary excretion (*16*).

A new classification based on the genetic findings was implemented. Patients were classified as type A, type B, and type AB based on the genetic findings. Type A cystinuria is the result of mutations in both *SCL3A1* genes and type B results from mutations in both *SCL7A9* genes. Type AB Individuals have one mutation in SLC3A1 and one in SLC7A9. Probands with more than two mutated alleles were classified as AA(B) or BB(A), depending on the distribution of mutations in the two genes. None of the individual's AB had cystine urolithiasis. Obligate type AB (double heterozygous) individuals who develop cystinuria have not been found. The digenic inheritance of cystinuria was ruled out (*17*). Type AB patients may suffer from a mild phenotype and therefore, in most cases, escape detection. Alternatively, these patients may actually represent type B disease (two mutations in *SLC7A9*, one of which was detected, the other yet to be defined) and a coincidental carrier state for a *SLC3A1* mutation. Reliable classification of cystinuria requires the identification of the mutations in both alleles (*18*).

Multiple studies examining genotype-phenotype correlations in cystinuria did not show any correlation between patients with type A genotype and patients with non-A genotypes (*19,20*). In a study, where 37 different mutant variant alleles were identified, including 12 novel mutations; 22% of mutations were caused by large gene rearrangements. No genotype-phenotype association was detected (*19*). The lack of detectable mutations in many patients indicates the possibility of other yet unidentified genes involved in cystinuria. The severity of the disease to the type of cystinuria in pediatric patients cannot be correlated (*21*).

4. Prevalence

Cystinuria is a global disorder with population-specific prevalence, its overall prevalence has been estimated at 1:7,000 in neonates. It varies between different populations: the highest frequency has been observed among Libyan Jews with a rate of 1:2500 (*12*). Other population-specific rates are 1:17,000 in the United States, 1:18,000 in Japan, and 1:100,000 in Sweden. The mean age at which urolithiasis including cystinuria is diagnosed is reported to be 5.59 years. Of these patients, 41.4% were below the age of 1 year and 60.5% were below the age of 5 years (*22*).

5. Evaluation

The diagnosis of cystinuria is easily made by stone analysis, microscopic examination of the urine, and 24-hour urine testing. Sodium cyanide nitroprusside is a suitable screening test that should identify homozygous stone formers but will not detect all heterozygotes. A positive screening test should be followed by quantitation of urinary amino acids. A homozygous patient can be functionally defined as one who excretes 250 mg or more of cystine/g of creatinine in a 24-hour urine collection (*23*). A positive nitroprusside test is followed by a quantitative analysis of urine cysteine and homocysteine to differentiate between cystinuria and homocystinuria. A sensitive and reproducible assay for total urine cysteine and homocysteine has been developed (*24*). One problem is that the measurement of cystine excretion is complicated by artifactually low values when cystine solubility is poor. Cystine is least soluble at pH 5-7, a range frequently found in human urine. Another problem is that many cystine assays do not reliably distinguish cystine from soluble thiol drug-cysteine complexes. Colorimetric reactions measure the amount of free sulfhydryl group. In the presence of thiol-containing drugs, this no longer remains an accurate estimate of cystine concentration. A solid-phase assay of urinary cysteine is applied, which leads to direct measures of urinary cystine supersaturation and cystine capacity. It is reliable in the presence of cystine-binding thiol drugs. It should be useful in monitoring patients' responses to dietary interventions and administration of fluid, citrate, and CBTD (*25*).

Based on previous observations of the diurnal variation of urinary cystine excretion, the use of separate day and night urine collections was proposed. Analyses of separate day and night urine samples can be used advantageously to reveal episodes of high supersaturation with cystine not detected in 24-h urine samples. Such a procedure might be useful for optimizing the treatment of patients with cystinuria (*26*).

Cystine stones are yellowish with a waxy appearance macroscopically and are characterized by a flat hexagonal crystal microscopically. The stone analysis provides definitive proof of the composition. Radiographically, cystine stones appear lightly opaque (due to the sulphur content) with homogeneous density, typically a "ground glass" appearance (*27*). The classification of cystine stones into rough and smooth varieties has been suggested as an aid to choosing treatment for these difficult stones. The surface morphology of cystine stones correlates with their internal structure, as viewed by helical computerized tomography (CT). Rough cystine stones can be distinguished from smooth stones using helical CT *in vitro*, suggesting that it may be possible to distinguish these stones preoperatively (*28*). Cystine stones often are poorly visible on KUB radiography (*29*).

6. Clinical Presentation

Recurrent urinary tract stone disease is the only clinical manifestation of cystinuria in childhood. Cystinuria can also result in chronic kidney disease (CKD) due to recurrent stones, obstructive uropathy, and repeated urologic interventions. Most patients with cystinuria present in childhood with stone formation. The average age of detection of a first renal stone is about 12-13 years, with 50% forming a first stone in the first decade of life and another 25% in teenage years. Males and females have a similar age of onset but more male patients than female patients present in the first 3 years of life and males tend to have new stones more frequently than females (*30*). CKD and high blood pressure occur frequently in patients with cystinuria and should be routinely screened. A retrospective study of 442 cystinuric patients was conducted. Results showed that among the 314 patients aged ≥ 16 years, using the last available plasma creatinine, only 22.5% had an eGFR ≥ 90 mL/min per 1.73 m^2 (*31*).

Urolithiasis may be the cause of acute renal failure in young children, since urolithiasis may only cause nonspecific symptoms in this population. Those patients should be tested for cystinuria (*32*). Hypotonia-cystinuria syndrome is a recessive disorder caused by microdeletions of SLC3A1 and PREPL on chromosome 2p21. Patients present with generalized hypotonia at birth, failure to thrive, growth retardation, and cystinuria type I (*33*).

7. Management

The first approach to treatment of cystinuria is a conservative program that includes initiation of therapeutic lifestyle changes involving increased fluid intake and restriction of sodium and protein, as well as urinary alkalinization therapy (*34*). If conservative therapy fails to reduce urinary cystine concentrations to less than 250 mg/L or stones recur despite therapy, CBTD is the next step in treatment (*35*).

7.1. Hydration

Hydration is the mainstay of the treatment. Patients are advised to wake up at night to drink water in addition to their daytime intake. Therefore, maintaining urine output to keep up with cystine excretion helps prevent stone formation. To prevent nocturnal aggregation of crystals, 500 mL of water intake at bedtime, and another 300 mL overnight is advocated (*27*). The single most important intervention in patients with cystine stones is to increase cystine solubility by increasing fluid intake. Adults with stones should have a target urine output of at least 3 L daily and less than 200 mg of cystine/L of urine (*36*). A high fluid intake of around 4-5 liters a day is required, and to drink before going to bed and during

the night to maintain dilute urine overnight (*37*).

7.2. Dietary modifications

Despite the low level of scientific evidence, a low-protein (< 20 g/day), low-salt (< 2 g/day) diet with high hydration (> 3 liters/day) is strongly advised in children with cystinuria. Dietary restriction of sodium should be an important component of the therapeutic strategy of patients with cystinuria (*38*). There is little evidence to support dietary restriction of protein, although reducing animal protein will be beneficial to increase urinary pH. Restriction of methionine-containing foods like peanuts, pistachio, popcorn, broccoli, mushroom, cauliflower, avocado, bean sprouts, potatoes, spinach, green peas, tofu, kidney beans, black beans, and tempeh may prevent cystine crystal formation (*39*). Ingestion of vegetables high in organic anion content, such as citrate and malate, should be associated with higher urine pH and fewer stones because the amino acid cystine is soluble in more alkaline urine (*40*). Like all stone formers, cystinuric patients are advised to limit their sodium intake to less than 2,300 mg/day (100 mEq/day) (*41*).

7.3. Urinary alkalinization

Urinary pH has a crucial role in prevention of stone formation. Therefore, cystine stone formation can be reduced by increasing the urinary pH level. The solubility of cystine does not increase significantly until a urine pH level above 7-7.5 is reached. Urine alkalinization up to pH 7.5 using sodium bicarbonate and/or potassium citrate is used (*42*). Because of the relationship found between the excretion of urinary sodium and cystine, potassium citrate has emerged as the preferred sodium-free alkalizing agent (*43*).

A reasonable goal is to keep the cystine concentration under about 240 mg/L and urine pH about 7, in order to maintain solubility. If the urine pH is below 7, potassium alkali in doses of 10-20 meq three times daily can be used to raise it (*5*). While urinary alkalinization for cystine calculi is an integral part of medical management, the effect of oral alkalinizing agents is limited because of the high pKa (8.3) of cysteine (*44*). Acetazolamide was effective in increasing the urinary pH in patients with cystine stone formation who were already taking potassium citrate. Caution must be taken when prescribing acetazolamide because it could be poorly tolerated and can induce calcium phosphate stone formation (*45*).

7.4. Cystine-binding thiol drugs (CBTD)

In patients who are refractory to increased fluid intake, urinary alkalinization, and dietary restriction of protein and salt, CBTDs are recommended (*42*). Agents most commonly used include α-mercaptopropionyl glycine (tiopronin) and D-penicillamine. Thiol compounds contain sulfhydryl groups that undergo a disulfide exchange reaction with cysteine to produce two molecules of cysteine bound to the CBTD, a complex that is 50 times more soluble than cystine. The effect of the drugs is dose-dependent (*46*). Twenty-four-hour urine cystine measurements are used to guide therapy: if 24-hour urine cystine concentration remains > 2,000 micromols(μmol)/L, chelation therapy is usually necessary, given as D-penicillamine or Tiopronin, to reduce free cystine concentration to < 1,000 μmol/L (ideally < 500 μmol/L) (*36*).

7.4.1. D-penicillamine

The most effective therapy for cystinuria is oral administration of thiol-containing compounds like penicillamine, which form mixed-disulfides with urinary cystine, reducing crystallization. Penicillamine's effectiveness in reducing stone formation and dissolving pre-existing stones in cystinuria has been well-documented (*47*). D-penicillamine is effective in decreasing the rate of stone formation in patients in whom hydration and alkalization failed (*48*). In adults, penicillamine reduces stone formation but has a high incidence of dose-limiting toxicity. A study was implemented to evaluate the effects and toxicity of penicillamine in pediatric patients. 11 children with cystinuria treated using a gradual dose escalation of penicillamine were included. During the gradual escalation of penicillamine to the target dose, none of the 11 patients experienced toxicity and all had improved urinary cystine concentration. Patients were followed for a total of 1,203 months. During this time only 2 patients experienced significant side effects and no patient had stones or stone crises while compliant with treatment (*49*).

The dosage of cystine-binding drugs required to achieve a free urine cystine level below 100 μmol/mmol creatinine was closely correlated with patient body weight: older children required a lower dose. Medical management of cystinuria is feasible. The treatment must be personalized in children, as the amount of drug required is strictly dependent on body size (*50*). A retrospective study was done to assess the efficacy and untoward reactions of D-penicillamine in the management of cystinuria. The incidence of acute drug sensitivity reactions (rash, fever, and/or arthropathy) was over 40 percent. Delayed drug-induced proteinuria occurred in 34 percent of treated patients (*51*).

The cutaneous side effects of penicillamine include acute hypersensitivity reactions and abnormalities of elastic fibers-elastosis perforans serpiginosa (EPS) and pseudo-pseudoxanthoma elasticum, autoimmune disorders such as pemphigus and a penicillamine-induced lupus erythematosus-like syndrome. These cutaneous adverse effects may correlate with the dosage

and duration of penicillamine therapy (*52*). Clinically, EPS presented with serpiginous or annular patterned lesions up to several centimeters with or without pruritus. The treatment of EPS primarily consists of oral isotretinoin, intralesional injections of triamcinolone acetonide, topical application of tazarotene, or allium cepa-allantoin-pentaglycan gel or cryotherapy (*53*).

7.4.2. Tiopronin

Oral administration of alpha-mercaptopropionylglycine (MPG) or tiopronin for cysteine stone dissolution and/or prevention of recurrence has proved its efficacy. It was associated with fewer side effects than are reported generally with D-penicillamine (*54*). The effect of long-term treatment with tiopronin was examined in 66 patients with cystinuria. Of the patients, 49 took D-penicillamine before therapy, whereas 17 did not. Tiopronin was equally as effective as D-penicillamine in reducing cystine excretion. During long-term treatment with MPG (average dose 1,193 mg per day), urinary cystine levels were maintained at 350 to 560 mg per day and urinary cystine was kept at undersaturated levels. Commensurate with these changes, tiopronin produced remission of stone formation in 63 to 71 percent of patients and reduced individual stone formation rate in 81 to 94 percent (*55*).

Thirty-two patients with cystinuria were enrolled in a long-term study where 16 patients were treated with tiopronin for 24 weeks. Tiopronin reduced daily urinary cystine excretion from 901.48 mg (before treatment) to 488.60 mg (on the average of 12th week and 24th week after tiopronin administration) successfully. Tiopronin therapy was tolerated well, but side effects were observed in 13 events in 6 patients. Thus tiopronin was expected to be effective in preventing cystine stone formation and tolerated well (*56*). Another study was done, where forty patients, belonging to six cystinuric families, were identified. These patients were excreting 3.1 ± 1.7 mmol/24 h of cystine in their urine. All patients were treated by oral administration of MPG in daily doses of 400-1,200 mg/24 h. During a 4 ± 2 years of follow-up of these patients. It was concluded that treatment with MPG is very effective with minimal side effects in patients suffering from cystinuria or cystine urinary calculi (*57*).

CBTDs lower the urinary supersaturation of cystine, as shown by a less-negative or more-positive cystine capacity. Cystine capacity can be measured directly, even in the presence of CBTDs including tiopronin. The value of this measurement lies in the potential to monitor the response to the drug, prescribe the minimum effective dose, and potentially decrease adverse effects often associated with CBTDs (*58*).

The recommended initial dosage in adult patients is 800 mg/day. For pediatric patients, the recommended initial dosage in pediatric patients weighing 20 kg and greater is 15 mg/kg/day. The dosage should be readjusted depending on the urinary cystine value to achieve a urine cystine concentration of less than 250 mg/L. The most common adverse reactions (≥ 10%) are nausea, diarrhea or soft stools, oral ulcers, rash, fatigue, fever, arthralgia, proteinuria, and emesis (*59*).

7.4.3. Captopril

Formation of captopril–cysteine disulfide accounts for part of the reduction in cystine excretion. Captopril-cysteine disulfide is 200 times more soluble than cystine. Sloand *et al*. reported the first clinical use of captopril in the treatment of homozygous cystinuria in two siblings. In the first patient, a 70% reduction in cystine excretion was observed after 26 weeks of therapy with 150 mg/d of captopril. In the second patient, cystine excretion was reduced by 93% after nine weeks of therapy with 75 mg/day of captopril. No adverse side effects were observed in either patient (*60*). Perazella *et al*. reported a marked decline in urinary cystine excretion of two cystinuric patients treated with captopril for one year. The two cases were intolerant of traditional therapy (tiopronin and D-penicillamine) (*61*). Another study was implemented to determine the clinical efficacy of captopril for the prevention of new or stone growth in patients with homozygous cystinuria. Nine patients with a history of multiple cystine stones despite standard fluid and alkalization therapy received 50 mg of captopril, 3 times daily in addition to the standard therapy. Findings suggest that captopril may be clinically efficacious in at least some patients with difficult to control cystinuria (*62*). Captopril should be considered an alternative to traditional drug management of cystinuria.

7.4.4. Bucillamine

Bucillamine is a drug developed from tiopronin, currently used as an antirheumatic agent and, acting as a thiol donor, which might be capable of binding cysteine from urine and thus reducing the risk of stone formation. A currently recruiting phase II trial is investigating the safety and effectiveness of bucillamine on urinary cystine excretion (*63*).

7.5. New therapies

7.5.1. L-cystine dimethyl ester (L-CDME) and L-cystine methyl ester (L-CME)

A new alternative approach for the prevention of recurrent nephrolithiasis is based on crystal growth inhibition. A group at New York University is using atomic force microscopy (AFM) to visualize the early stages of crystal formation in liquids. Real-time in situ atomic force microscopy reveals that L-cystine dimethylester (L-CDME) and L-cystine methylester

(L-CME) dramatically reduce the growth velocity of L-cystine molecules. This is a new pathway to the prevention of L-cystine stones by rational design of crystal growth inhibitors (*64*). CDME's efficacy in inhibiting L-cystine crystal growth *in vivo* utilizing a murine model of cystinuria was demonstrated (*65*). A study was done to assess the effectiveness of L-CDME, an inhibitor of cystine crystal growth, for the treatment of cystine urolithiasis in a Slc3a1 knockout mouse model of cystinuria. Treatment with L-CDME led to a significant decrease in stone size compared with that of the water group (p = 0.0002), but the number of stones was greater (p = 0.005). The data demonstrate that L-CDME promotes formation of small stones but does not prevent stone formation, consistent with the hypothesis that L-CDME inhibits cystine crystal growth (*66*).

To overcome the chemical and metabolic stability issues of L-CDME and L-CME, a series of l-cystine diamides with or without Nα-methylation was designed, synthesized, and evaluated for their inhibitory activity of l-cystine crystallization. Among the l-cystine diamides 2a-i, l-cystine bismorpholide (CDMOR, LH707, 2g) and l-cystine bis(N'-methylpiperazide) (CDNMP, LH708, 2h) are the most potent inhibitors of l-cystine crystallization (*67*).

One potential limitation of the molecules is the potential for toxicity. Incubation of noncystinotic renal epithelial cells (LLC-PK1) cells, a model for proximal tubular function, with CDME resulted in time- and dose-dependent accumulation of cystine, with 80% of the cystine in the lysosomal fraction. The accumulation of cystine in the lysosomes caused dose- and time-dependent cell mortality (*68*).

7.5.2. α-Lipoic acid

In a mouse model of cystinuria, it was reported that the nutritional supplement α-lipoic acid (α-LA) inhibits cystine stone formation in the *Slc3a1*$^{-/-}$ mouse model of cystinuria by increasing the solubility of urinary cystine. The pro-antioxidant compound α-LA was a strong suppressor of stone growth as mice treated with α-LA had lower stone formation growth compared to untreated mice (*69*). Exploring the mechanism of action of α-LA, the researchers found that treatment with the compound did not alter urinary cystine concentrations and that its effect was independent of Nrf2-mediated antioxidant responses (*via* increased cystine import for glutathione synthesis). Instead, it was observed that cystine was considerably more soluble in the urine of α-LA-treated mice than in that of untreated mice (*70*). These findings identify a novel therapeutic strategy for the clinical treatment of cystinuria. Implementation of clinical trials of α-LA treatment of cystinuria is needed before further conclusions can be made.

7.5.3. Tolvaptan

Tolvaptan, an arginine vasopressin receptor antagonist, decreases urinary supersaturation in kidney stone formers by considerably increasing diuresis. Patients had a significant increase in daily urine volume and a resultant decrease in urinary cystine concentration when taking 15 mg tolvaptan daily for 5 days (*71*). A study was implemented to evaluate the effect of tolvaptan, on cystine stone volume in mice with cystinuria. After treatment, mice treated with tolvaptan had significantly delayed stone growth, and exhibited lower overall stone volume accumulation, compared with the control group. The present study indicated that tolvaptan's efficacy in preventing L-cystine stone growth through an increased liquid intake and urine volume in cystinuric mice (*72*). These findings identify a novel therapeutic strategy for the clinical treatment of cystinuria.

8. Conclusion

Cystinuria is a rare cause of urolithiasis. Affected patients have an earlier onset and more aggressive disease than patients with other stone types. Current treatment options of cystinuria are limited in their effectiveness at preventing stone recurrence and often poorly tolerated. Multiple studies suggest that L-CDME is as effective at inhibiting the growth of cystine crystals *in vitro* as well as *in vivo*. Also, the nutritional supplement α-LA prevents the formation of cystine stones. Thus this represents a potentially promising therapy for cystine stones. Clinical trials to support the use of those modalities are warranted.

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Performance of matching methods in studies of rare diseases: a simulation study

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SUMMARY Matching is a common method of adjusting for confounding in observational studies. Studies in rare diseases usually include small numbers of exposed subjects, but the performance of matching methods in such cases has not been evaluated thoroughly. In this study, we compare the performance of several matching methods when number of exposed subjects is small. We used Monte Carlo simulations to compare the following methods: Propensity score matching (PSM) with greedy or optimal algorithm, Mahalanobis distance matching, and mixture of PSM and exact matching. We performed the comparisons in datasets with six continuous and six binary variables, with varying effect size on group assignment and outcome. In each case, there were 1,500 unexposed subjects and a varying number of exposed: N = 25, 50, 100, 150, 200, 250, or 300. The probability of outcome in unexposed subjects was set to 5% (rare), 20% (common), or 50% (frequent). We compared the methods based on the bias of estimate of risk difference, coverage of 95% confidence intervals for risk difference, and balance of covariates. We observed a difference in performance of matching methods in very small samples (N = 25-50) and in moderately small samples (N = 100-300). Our study showed that PSM performs better than other matching methods when number of exposed subjects is small, but the matching algorithm and the matching ratio should be considered carefully. We recommend using PSM with optimal algorithm and one-to-five matching ratio in very small samples, and PSM matching with any algorithm and one-to-one matching in moderately small samples.

Keywords matching methods, propensity score matching, small samples, rare diseases

1. Introduction

The generation of an appropriate comparator group is a challenge for many studies related to epidemiology, health care, benefit assessment, and the cost-effectiveness of treatments. The potential exists to establish a comparator group from either secondary data, historical groups or meta analyses. The number of studies using propensity score matching (PSM) for this purpose is increasing, with the number of publications using these methods increasing from 432 in 2010 to 3,335 in 2018 (*1*).

Studies of rare diseases often include comparisons between two or more groups, at least one of which has a small sample size. Such comparisons are subject to confounding and the consequent biases that are generated. Confounding is present when subjects' characteristics, both observed and unobserved, are not randomly imbalanced between two comparison groups (*2*). In randomized controlled trials, the balance (or imbalance due only to chance) of characteristics is usually achieved in the study design stage, when the two groups are randomized before treatment assignment. In observational studies, it is impossible to achieve this balance before the group assignment, and accounting for it in the analysis stage is therefore necessary.

Confounding in comparison analyses can be reduced in a number of ways, such as by regression adjustment, stratification, or matching (*3*) or by a combination of two methods (*4*). Regression adjustment is a common method, which adjusts for confounding by directly including the confounding covariates in the regression model. Another method of confounding adjustment involves stratifying the observations into groups based on the fixed values of a confounder, so that the values of the confounder do not vary within each group. The analyses are performed separately in

each of the stratified groups, and the results are then combined. Lastly, adjusting for confounding in large observational studies can be achieved by matching, where similar exposed and unexposed subjects are matched, and the analysis is performed on the matched exposed and unexposed subjects only. One common method for matching the observations is PSM (*2*). In this method, exposed and unexposed subjects are matched on a similar propensity score, which is the probability of being exposed given the values of other covariates. As with all the methods for adjusting for confounding, PSM is based only on a subject's observed characteristics. That is, PSM can only adjust for characteristics included in the propensity score model, and it is sensitive to how the propensity score model is specified.

Various aspects of PSM have been studied in the past; however, most of the studies on and using the method have involved datasets with large samples (*5,6*). Studies have also been conducted to gain more specific details about matching, such as the advantages of one-to-many matching in PSM (*7*) or the ideal caliper widths in PSM (*8*). However, to our knowledge, the performance of PSM in small samples has only been scarcely studied (*6,9,10*). A recent study by Cottone (*11*) explored the advantages of combining propensity score methods (matching, stratification, and weighting) with regression adjustment. Austin (*12*) performed one of the most detailed studies on propensity-score-based matching in small samples, in which several propensity-score-based matching methods were compared to one another. None of the previous studies, however, including the studies by Cottone (*11*) and Austin (*12*), has compared the performance of PSM methods in small samples to matching methods that are not based on propensity scores, and no study has assessed the performance of matching methods across different outcome rates. In addition, no studies have been carried out with small samples examining the sensitivity of various matching models to unobserved confounding.

Statistical methods have been developed to provide unbiased results under certain assumptions, such as appropriate variable distribution or adequate sample size (*13*). Those assumptions are especially likely to be violated in studies with small sample sizes, and the methods used in such studies should be carefully considered (*14-17*). Matching observations from small samples can pose some unique problems that might not exist in matching larger datasets. First, because of the small sample size, building a propensity score model that takes into account all the relevant variables might be difficult. Furthermore, if a propensity score model is not specified correctly or does not include all the variables associated with the outcome, then the bias in the results might not be lessened using PSM. Second, there has recently been a discussion about whether PSM increases or decreases the balance in matching variables (*18,19*), but the balance in these variables has not been studied in small samples. Since PSM does not balance the sample on individual variables, but on an overall score, it is possible that in small samples, the overall balance of the samples will not be achieved. Finally, in small samples, the outcome rate should possibly influence the analysis method that is used. Outcomes with low rates of occurrence can result in a small number of outcomes observed, thus making the analysis highly sensitive to analysis methods.

In this study, we evaluate the performance of PSM when the number of exposed subjects is small, and we compare it to performance of matching methods that are not based on propensity scores or not based only on propensity scores. We examine the performance of each of the methods in several different scenarios in terms of sample size and outcome rate. Lastly, we examine the effect that unobserved confounding has on the results from each matching method.

2. Materials and Methods

We used a series of Monte Carlo simulations (*20*) to compare the performance of different matching methods when the number of exposed subjects is small. Each simulated dataset included one binary outcome and 12 covariates, 6 of which were continuous and 6 of which were binary. We evaluated the performance of seven matching methods, which are described in Table 1. Briefly, four of the matching methods were based on propensity score distance, with two of them using greedy and two of them using optimal matching algorithms. Two of the methods were based on the Mahalanobis distance. The last method was based on a mixture of PSM with a greedy algorithm for

Table 1. The list of matching methods evaluated in the study

Method	Distance Measure	Variables included	Matching Algorithm	Matching Ratio
1	Propensity Score	All	Greedy	one-to-one
2	Propensity Score	All	Greedy	one-to-five
3	Propensity Score	All	Optimal	one-to-one
4	Propensity Score	All	Optimal	one-to-five
5	Mahalanobis	All	N/A	one-to-one
6	Mahalanobis	All	N/A	one-to-five
7	Propensity Score	Continuous	greedy	one-to-one
	Exact	Binary	N/A	

continuous covariates and exact matching for binary variables. For three of the methods, we assessed both one-to-one matching, where each exposed subject is matched to only one unexposed subject, and one-to-five matching, where each exposed subject is matched to five unexposed subjects. All matching algorithms were assessed without replacement, meaning that each unexposed subject can be matched to only one exposed subject.

2.1. Description of the matching methods

Propensity score matching identifies pairs of exposed and unexposed subjects based on propensity score and not based on any specific variable value. Propensity score is the probability of a subject being exposed based on its characteristics (*2*). Two algorithms are commonly used to determine how exposed and unexposed subjects should be matched based on propensity score (*12*):

1). Greedy algorithm. Here, an exposed subject is matched to an unexposed subject with a propensity score closest to that of the exposed subject.

2). Optimal algorithm. Here, all matched pairs are formed by minimizing the average within-pair difference of propensity scores.

Mahalanobis distance matching (*3,21*) is another matching method that considers the overall distance between subjects, not the values of individual variables. This distance measurement is based on the Euclidean distance between two observations, and it takes into account the variance-covariance matrix (Σ). The Mahalanobis distance between two observations *i* and *j* with covariate vector x is defined as follows:

$$d^2(i,j) = (x_i - x_j)^T \Sigma^{-1} (x_i - x_j)$$

Exact matching (*3*) is a method that examines the value of each variable, and it matches subjects only when they have the exact same values for matching variables. This method is most commonly used for discrete variables.

2.2. Description of the Monte Carlo simulations

We based the design of our study on previous studies by Peter Austin (*8,12*); however, we adapted it to our research question. The specific details for covariates and outcome simulations are presented below.

2.2.1. Covariates

For each observation, we generated six continuous variables and six binary variables with values of either 0 or 1. For the unexposed subjects, each continuous variable was drawn from an $N(0,1)$ distribution. For the exposed subjects, variables X_1 - X_3 were drawn from an $N(0,1)$ distribution, and variables X_4 - X_6 were drawn from an $N(0.5,1)$ distribution. Furthermore, all the binary variables for the unexposed subjects were drawn from Bin(1,0.5) distribution, whereas for the exposed subjects, variables X_7 - X_9 were drawn from a Bin(1,0.5) distribution, and variables X_{10} - X_{12} were drawn from a Bin(1,0.75) distribution. The reason for choosing different variable distributions between exposed and unexposed subjects was to simulate confounding. If the distribution of a variable is different for exposed versus unexposed subjects, and if the variable is associated with the probability of the outcome, then such a variable is considered to be a confounder.

2.2.2. Outcome

We generated a binary outcome for each observation based on the values of the covariates. Binary outcomes are common outcomes in observational health-care research. Some examples of binary outcomes include mortality, the presence or development of a comorbidity (*e.g.* cancer or heart disease), the presence or development of a symptom (*e.g.* pain), and hospital readmission. We assumed the following logistic regression model that relates the probability of outcome (Y) to the covariates (X_1 - X_{12}) and the assignment variable (exposed: $T = 1$; unexposed: $T = 0$).

$$\begin{aligned} logit\,(p_{i,outcome}) = {} & \alpha_{0,outcome} + \beta T_i + \alpha_N X_{1,i} + \alpha_M X_{2,i} \\ & + \alpha_H X_{3,i} + \alpha_N X_{4,i} + \alpha_M X_{5,i} + \alpha_H X_{6,i} \\ & + \alpha_N X_{7,i} + \alpha_M X_{8,i} + \alpha_H X_{9,i} + \alpha_N X_{10,i} \\ & + \alpha_M X_{11,i} + \alpha_H X_{12,i} \end{aligned}$$

The regression coefficients were set to reflect no effect, a medium effect size, and a high effect size:

$$\alpha_N = \log(1),\ \alpha_M = \log(1.25),\ \alpha_H = \log(2).$$

Here, $\alpha_{0,outcome}$ was estimated three times using a Monte Carlo iterative process and bisection method (*22*), so that the probability of outcome in unexposed subjects was one of the following: 5% (rare outcome), 20% (common outcome), or 50% (frequent outcome). In addition, β was estimated using a separate Monte Carlo iterative process and bisection method (*22*), so that the risk difference between exposed and unexposed subjects equaled 0.1.

2.2.3. Monte Carlo simulations

We used a complete factorial design in which two factors are allowed to vary: the number of exposed subjects and the probability of outcome among the unexposed subjects. We randomly generated datasets with size 1,500+N. In each case, there were 1,500 unexposed subjects and a varying number (N) of exposed subjects: N = 25, 50, 100, 150, 200, 250, or 300. This led to final datasets (exposed and unexposed subjects combined)

with sample sizes varying from 1,525 to 1,800. The results from any method with a one-to-five matching ratio when N = 300 are equivalent to those without matching, since in this case, one-to-five matching will include all exposed and unexposed subjects. This scenario is included in the study to illustrate the general significance of matching. Next, we generated 1,000 samples from each of the 1,500+N observations, with error term ~N(0,1). We calculated the probability of outcome ($p_{i,outcome}$) using the logistic regression model above, and we then used the probability to generate the outcome for each observation from binary distribution Bin(1,$p_{i,outcome}$).

2.3. Analysis

Once the datasets were generated, we applied the seven matching methods described above in each of the 1,000 samples of each scenario. We used all 12 covariates in the estimation of the propensity score model and the Mahalanobis distance. In each matched sample, we estimated the following characteristics:

Bias of the estimate of risk difference. We estimated the prevalence of the outcome both in exposed and in unexposed subjects in each matched dataset and calculated the difference between them. The overall bias of the estimate was the difference of mean of this measure across the 1,000 samples and 0.1 (the risk difference set by simulations).

Balance. we calculated the mean of absolute values of standardized difference between exposed and unexposed subjects across all the variables. The overall balance was the mean of this measure across the 1,000 samples. This measure of balance was introduced by Austin (*23*) and is defined as follows:

$$\text{Continuous Variables: } d = \frac{(\bar{x}_{treatment} - \bar{x}_{control})}{\sqrt{\frac{s^2_{treatment} + s^2_{control}}{2}}}$$

$$\text{Binary Variables: } d = \frac{(\hat{p}_{treatment} - \hat{p}_{control})}{\sqrt{\frac{\hat{p}_{treatment}(1-\hat{p}_{treatment}) + \hat{p}_{control}(1-\hat{p}_{control})}{2}}}$$

Coverage. For each matched dataset, we determined whether the 95% confidence interval for risk difference contained the true risk difference (*24*) (0.1). The overall coverage was the percentage of 1,000 samples in which the 95% confidence interval included 0.1.

The simulations were conducted using SAS software (*25*), and the matching was performed using MatchIt (*26,27*) R package.

3. Results

3.1. Bias

We observed three noteworthy points when examining the bias of estimated risk difference across different outcome rates and sample sizes of the exposed subjects. First, the baseline outcome rate was a factor in how different matching methods performed when estimating the risk difference between exposed and unexposed subjects (Figure 1). When the outcome was rare (5%), the differences in the bias of estimated risk difference were smaller between the methods studied than when the outcome was frequent (20%) or common (50%). The outcome rate also seems to affect whether the methods lead to overestimation or underestimation of the risk difference. When the outcome was rare, all methods led to overestimation of the risk difference, and when the outcome was common or frequent, five of the matching methods (propensity score methods using one-to-five matching, Mahalanobis distance methods, and the mixture of propensity score and exact matching methods) also consistently overestimated the risk difference. While PSM methods with one-to-one matching did not necessarily estimate the risk difference exactly, they did not consistently overestimate or underestimate it.

Second, the more common the outcome, the largest the bias in risk difference for fully matched samples. That is, in one-to-five matching with 300 exposed subjects, which is equivalent to no matching at all, the bias was largest for the frequent outcomes and smallest for the rare outcomes (Figure 1).

Third, a difference in the performance of methods was found in very small samples (25-50 exposed subjects) and in moderately small samples (100-300 exposed subjects). In very small sample sizes, no method was found that clearly outperforms all others. Mahalanobis distance matching using one-to-five matching led to the largest bias in very small samples, independent of outcome rate. Moreover, all the propensity-score-only methods performed similarly in very small samples, and there is some indication that propensity score one-to-five matching with the optimal algorithm performed especially well in the smallest samples (N = 25) (Figures 1 and Figure 2). The mixture of propensity score and exact matching did not perform as well as propensity-score-only matching methods in very small samples.

In the moderately small samples, PSM with a one-to-one ratio, with either an optimal or a greedy matching algorithm, resulted in smaller bias than any other matching method. Furthermore, the mixture of propensity score and exact matching performed better than the Mahalanobis methods or any one-to-five matching methods, but not better than propensity-score-only one-to-one matching methods (Figures 1 and Figure 2). In addition, independent of outcome rate, the performance of propensity-score-only one-to-one matching methods and a mixture of propensity score and exact matching methods were better in moderately small samples than in very small samples, which was not true for other matching methods (Figure 1). Finally, similar to very small samples, the bias was also the largest for one-to-five Mahalanobis distance matching

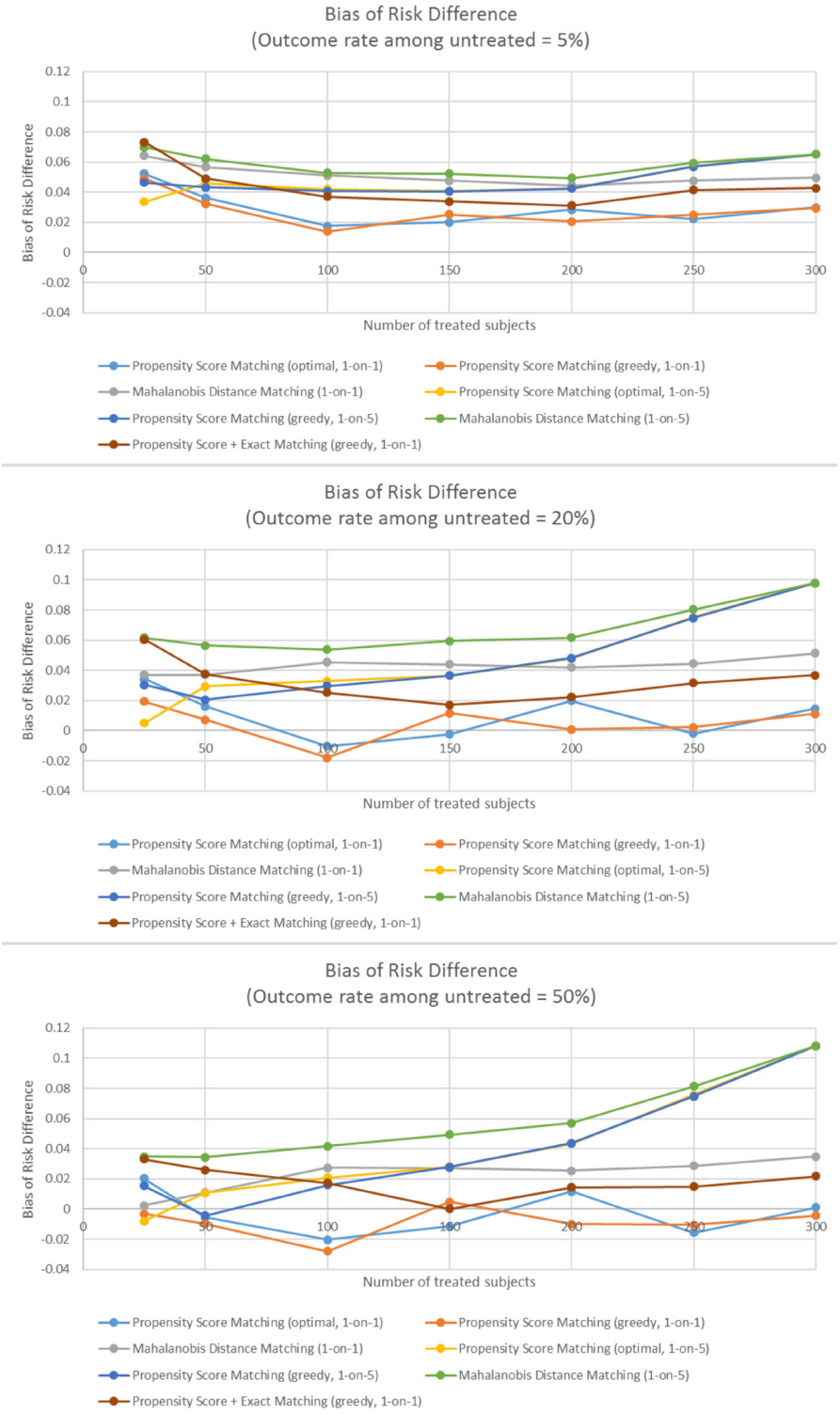

Figure 1. The bias of risk difference across different samples sizes of exposed subjects and different matching methods when outcome is rare (panel A), common (panel B), and frequent (panel C).

in moderately small samples.

3.2. Balance

As with the bias of estimated risk difference, the method that leads to the best balance differs between very small samples of exposed subjects (N = 25–50) and moderately small samples (N = 100–300). In very small samples, all propensity-score-based matching methods performed similarly well, with the exception of propensity score one-to-one matching with an optimal algorithm. In moderately small samples, similar to bias in most cases, propensity score one-to-one matching, with either optimal or greedy algorithm, lead to the best balance between exposed and unexposed subjects. All the matching methods with one-to-five matching result in worse balance, especially as the sample size increases (Figure 3).

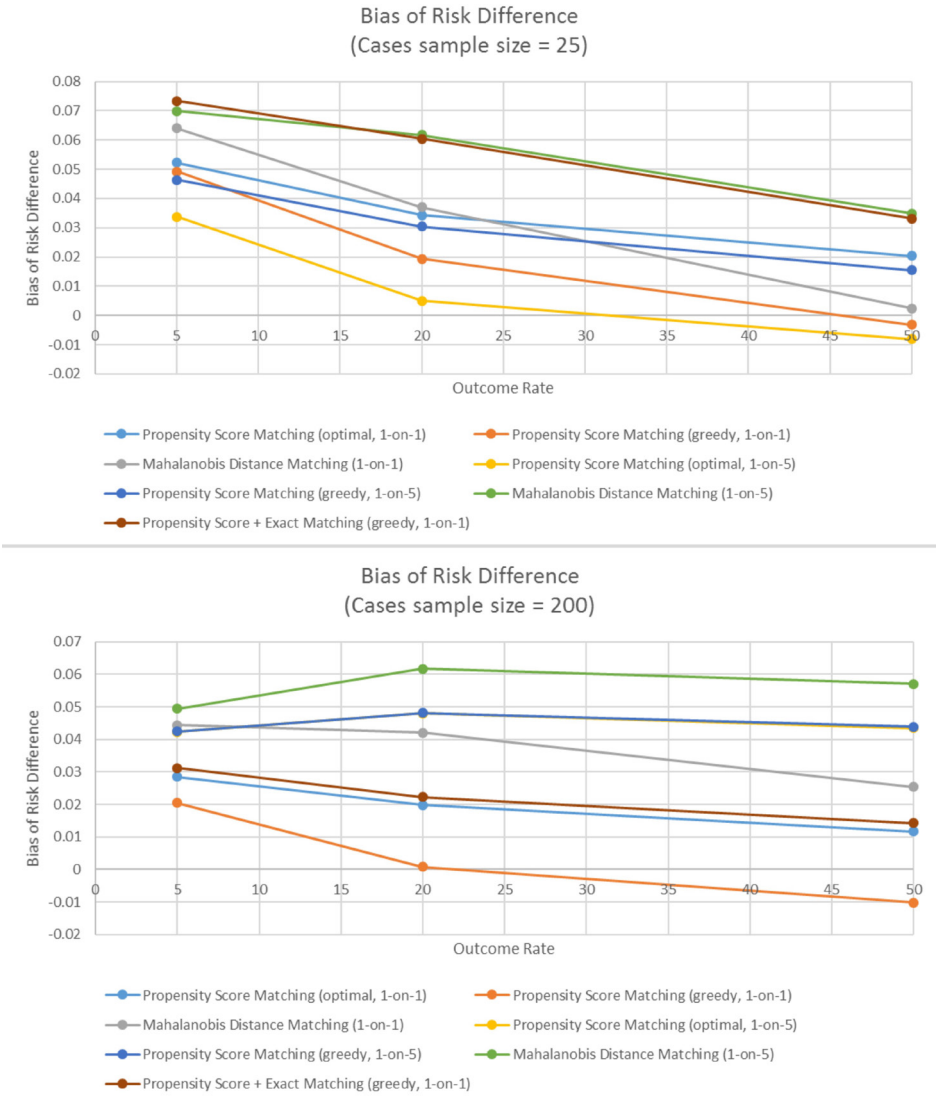

Figure 2. The bias of estimated risk difference when number of exposed subjects is 25 (Panel A), and when number of exposed subjects is 200 (Panel B). The bias is evaluated across different outcome rates and different matching methods.

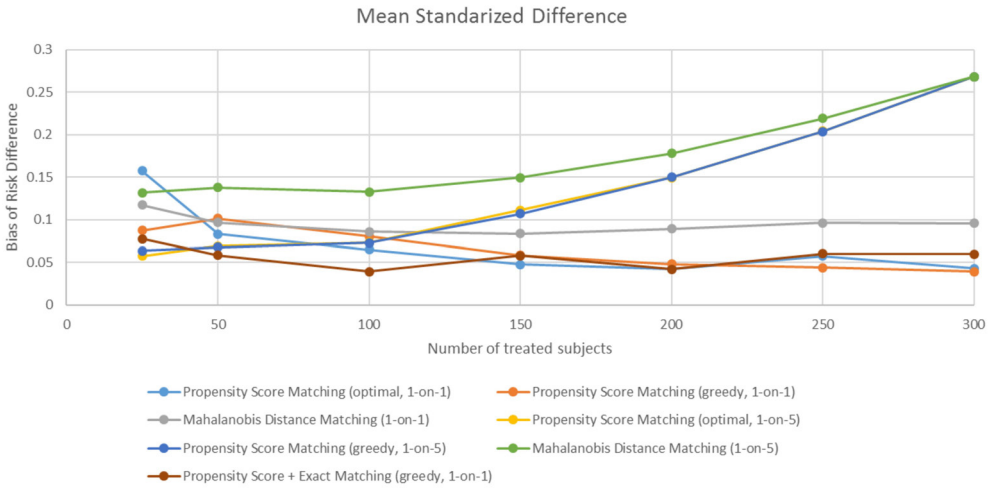

Figure 3. The balance of covariates in matched datasets, defined as mean standardized difference. The balance is evaluated across different sample sizes and different matching methods.

3.3. Coverage

In very small samples, all of the matching methods result in 95% confidence intervals that include the true risk difference between 83.2% and 91.8% of the time. Overall, methods based on propensity score only perform better than other methods, especially as the sample size increases. In very small samples, methods with one-to-five matching ratio outperformed methods with one-to-one matching ratios (Figure 4).

As the number of exposed subjects increased, increasing the matching ratio led to a loss of accuracy.

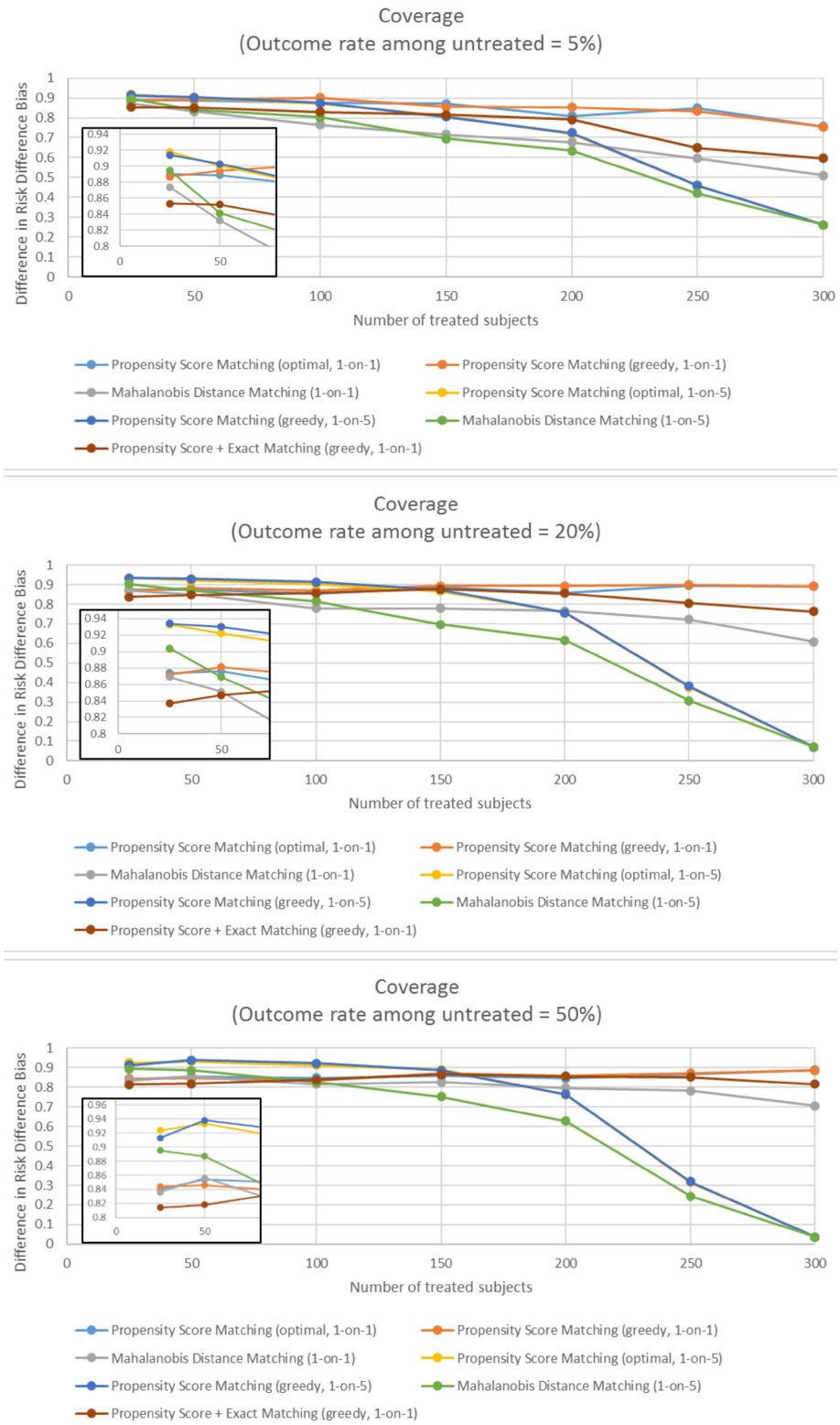

Figure 4. The coverage of matching methods, defined as percent of matched datasets in which the 95% confidence intervals contains the true risk difference, when outcome is rare (panel A), common (panel B), and frequent (panel C). The coverage is evaluated across different sample sizes and different matching methods.

Therefore, for the moderately small samples, the coverage was the best when using one-to-one PSM, in which case the 85% confidence intervals (CIs) included a true risk difference between 85% and 90%. Finally, across all sample sizes of exposed subjects, the mixture of propensity score and exact matching resulted in worse coverage than propensity-score-only matching methods (Figure 4).

4. Discussion

In this study, we used Monte Carlo simulations to evaluate and compare the performance of several matching methods for a small sample of exposed subjects. The methods we considered were based on propensity scores, the Mahalanobis distance, and a mixture of propensity score and exact matching. We considered a combination of continuous and binary variables, various baseline outcomes rates, and various numbers of exposed subjects in a dataset.

Our study demonstrates that differences exist in the performance of matching methods between very small samples (N = 25-50) and moderately small samples (N = 100-300). Based on the results of our study, we conclude that in very small samples, PSM still performs better than matching based on the Mahalanobis distance; however, there are still differences between PSM algorithms and ratios. Depending on the goal of the matching analysis, different methods might be considered. Both algorithms in one-to-one matching lead to a relatively small bias, and some evidence was found that in very small sample sizes, one-to-five matching with an optimal algorithm results in the smallest bias. The mixture of propensity score and exact matching does not perform as well as methods that include both continuous and binary variables in the estimation of propensity scores, and it should therefore not be used in studies with very small samples. Furthermore, propensity score one-to-one matching with an optimal algorithm results in a relatively high imbalance in the distribution of covariates, but the greedy algorithm in one-to-one matching performs better. Propensity score one-to-five matching results in the highest coverage when the number of exposed subjects is very small. Therefore, if the goals of the matching are to decrease the bias of estimated risk difference, increase the balance in covariates, and increase the coverage of 95% confidence intervals in very small samples, then propensity score one-to-five matching with an optimal algorithm should be used.

In moderately small samples, PSM with an optimal algorithm performs similarly to PSM with a greedy algorithm, and they both perform better than matching on the Mahalanobis distance. In addition, one-to-one matching leads to a smaller bias of estimated risk difference, better balance, and better coverage than one-to-five matching. The poor performance of one-to-five matching in moderately small samples is likely a result of the lack of high-quality matches once the sample size of exposed subjects increases. Nonetheless, propensity score one-to-one matching is the better method in moderately small samples.

Our study also demonstrates that the baseline outcome rate affects the size of the bias in estimated risk difference, but it does not necessarily influence which method performs best compared to others. The more important factor in distinguishing between the methods is the number of exposed subjects.

Our results are similar to other studies that have suggested that there is no clear best-matching method. Fullerton *et al.* assessed the performance of propensity-score-based methods and exact matching methods in terms of balance (*28*). While their study was not performed using small samples, it concluded that the best method is sensitive to the definition of balance, and they recommended that best practice would be to include the application of several matching methods. Moreover, a study by Baser examined the performance of different PSM methods (*29*) in a large sample; it also concluded that no superior method exists and that sensitivity analysis should be used. In one of the rare studies with small samples, Pirracchio found that PSM matching methods lead to unbiased results for estimating marginal odds ratios (*9*). Our study builds on these conclusions and demonstrates that there is no one superior propensity-score-based matching method in small samples, but that any such method is better than the Mahalanobis-distance-based method or a mixture of propensity score and exact matching methods.

One other important point in analyzing matching samples is the statistical significance. Our study suggests that, as expected, one-to-five matching can lead to a higher bias than one-to-one matching. However, the higher number of subjects in a comparison sample increases the accuracy of statistical comparisons, as revealed by the increased coverage of one-to-five matching in very small samples. This increase in coverage might be more important in studies with a small number of exposed subjects than in larger studies. In small samples, variability is a more significant problem; therefore, adding more observations to the analysis might have an important impact. Prior studies have found possible benefits of one-to-many matching (*7,30*) and the optimal matching ratio for decreasing bias but increasing power. Those studies, however, did not focus on scenarios when samples are very small, and our finding is thus significant.

While our study explored different scenarios in terms of sample size and outcome rates, it has several limitations, and other topics should be studied further. For example, the optimal caliper width was not studied in small samples. In such samples, the trade-off between the percentage of observations matched and the quality of matches might be different than in large samples. In

addition, in our study, we did not examine the effects of including in the propensity score model matching variables that are not associated with the exposure and/or outcome. The effect of including variables with little or no associations should be studied more closely, since inclusion of variables might have a major impact in small samples.

Our study has significance in several applications, such as health-care research; pharmacoepidemiology; clinical effectiveness studies; and health economics studies in rare diseases based on nonexperimental observational studies, secondary data, or registries. Studies in health-care research can be time and resource consuming, and often only samples of limited sizes can consequently be collected. It is important to ensure that the results and conclusions of those studies are accurate, as they might lead to significant policy changes. Similarly, pharmacoepidemiological studies and research in clinical effectiveness are also often performed using small groups of patients; therefore, appropriate methods are imperative to ensure drug safety. For example, the number of innovative treatments developed for rare diseases is growing each year, and an increasing number of them are licensed on the fast track (*31,32*). Studies that do not undergo a regular approval process require subsequent additional pharmacovigilance, relative effectiveness, and health economics studies (*33*). The validity of methods used in these studies is of high importance.

In conclusion, our study demonstrates that PSM performs better than other matching methods in terms of bias in estimating risk difference, coverage, and balance of covariates, when matching a small number of exposed subjects to a larger dataset of unexposed subjects. Based on the results of the study, we recommend that a higher matching ratio (*e.g.* one-to-five) be used in very small samples, and a lower matching ratio (*e.g.* one-to-one) be used as the sample size of exposed subjects increases. It is unclear whether a greedy or an optimal algorithm performs better in PSM, and our recommendation is that both algorithms be performed as sensitivity analyses.

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Original Article

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Association between antinuclear antibodies (ANA) patterns and extractable nuclear antigens (ENA) in HEp-2 cells in patients with autoimmune diseases in Riyadh, Saudi Arabia

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SUMMARY Antinuclear antibodies (ANA) and extractable nuclear antigens (ENA) are instrumental biomarkers crucial in the detection of autoimmune disorders (AID) such as systemic lupus erythematosus (SLE), Sjogren syndrome, *etc.*. In the present study, an assessment of the most frequent ANA patterns associated with most detectable ENA that could be used as efficient prognostic markers in the diagnosis of autoimmune diseases was conducted. Data was retrospectively analyzed from AID patients, retrieved from the medical records of King Fahad Medical City, Riyadh, KSA, from January 2016 to October 2018 who underwent ANA immunofluorescence of HEp-2 cells and their ENA detection was studied. Of the 453 total patients, 39/55 AID males (71%) and 332/398 AID females (83.4%) exhibited ANA positivity. The most common pattern was speckled S-ANA (32.4%) in females and homogenous H-ANA pattern (25.4%) in males. The histones were found at higher frequency in different ANA patterns. anti-Sjogren syndrome related antigen A (SSA), anti-ribonucleoprotein antibody (RNP-Sm), and histones were observed to be associated with homogenous and speckled nuclear patterns. Frequencies of ENA in all ANA patterns were found significant at $p < 0.05$ in males and $p < 0.001$ in females. Spearman's rank correlation of ENA within and among the ANA patterns was non-significant. SSA was significantly correlated with RNP-Sm and Sm at $p < 0.05$ and $p < 0.01$, respectively. The extractable nuclear antigens SSA, RNP-Sm, and histones were found associated with the S-ANA and H-ANA patterns. These correlations are of relevance for the accurate diagnosis of autoimmune diseases.

Keywords autoimmune diseases, extractable nuclear antigens, ANA immunofluorescence

1. Introduction

Research shows that autoimmune diseases (AIDs) are usually characterized by the auto-aggression of the body's immune system. Mainly, this is often against the self-antigens through the production of antibodies (*1*). Ideally, all AID comprises a strange etiology though it is clear that they share a fundamental mechanism in inflammation and destruction of a person's immunity. While at this, it suffices to observe that the antinuclear antibodies (ANA) consist of specific antibodies whose attack is directed at self-proteins in the cellular nucleus possessing features such as small nuclear ribonucleoproteins (snRNP) or deoxyribonucleic acid (DNA). It is observed that indirect immunofluorescence (IF), through human laryngeal carcinoma HEp-2 cells, is the prevalent technique detecting ANA (*2*). Essentially, this detection is crucial in the diagnostic process for systemic autoimmune rheumatic diseases (SARD). When positive results are observed, anti-ENA tests follow as a means of confirming the diagnosis. Ideally, the ANA are critical biomarkers during diagnosis for SARDs such as systemic sclerosis (SSc), Sjogren syndrome (SS), polymyositis/dermatomyositis, and mixed connective tissue disease (MCTD) (*3,4*).

Fundamentally, the ANA assay is vital in detection of a range of antibodies notable in their reaction with antigens within the nucleus and the nucleolus. Apart from these, few ANA are targeted to antigens in the cytoplasm, mitotic cellular apparatus, *etc.* (*5*).

Susceptible targets of ANA include histone proteins, ds-DNA, DNA/histone complexes (nucleosomes), various nuclear enzymes, other proteins, and RNPs. ANA plays a significant role as diagnostic and prognostic biomarkers or for monitoring of autoimmune diseases. ANA are present in various infectious, inflammatory, and neoplastic diseases, and low levels of ANA are detected in 30% of healthy individuals (*6*). Thus, this correlation between ANA and specific diseases is indicative of its usefulness in screening specifically as a prognostic marker, and eventually, providing crucial data on a disease's mechanism. Among the antinuclear staining patterns studied are, the speckled, centromere, homogenous, and the nucleolar patterns. Based on the intranuclear distributions of the antigen, the IF-ANA patterns are often subdivided into, nucleolar-(N-ANA), homogenous/chromosomal-(H-ANA), centromere-(C-ANA), speckled/extrachromosomal (S-ANA), nuclear dot, nuclear membrane patterns and mixed patterns (*7*). Association of H-ANA with normal cells or with AID is controversial.

Analysis of ENA assists in the diagnosis of the exact autoimmune disease. ANA positivity and its clinical significance in autoimmune diseases has been studied worldwide. As the ANA tests facilitate the determination of the presence or absence of autoantibodies, ENA profiling is crucial in the evaluation of the proteins within the nucleus recognized by the antibodies. Additionally, profiling is vital in the analysis of the progression of autoimmune diseases. There is no clear consensus on the prevalence of AID in Saudi Arabia, as the epidemiology of AID is not accurately documented in this region. For SLE, however, there are significant studies though few and inadequate. As such, it is crucial to conduct further research on the disease to accurately determine its epidemiology. In the above prospective, the present study reviewed data of AID patients tested at the Immunology Department, King Fahad Medical City, Riyadh, Kingdom of Saudi Arabia.

The study aim to investigate: *i*) the most prevalent form of IF-ANA staining pattern among AID patients; *ii*) evaluation of the correlation between IF patterns of ANA on the HEp-2 cells and the ENA frequencies in patients with autoimmune diseases.

2. Methods

This retrospective study was carried out in the Immunology and HLA Laboratory, Pathology and Clinical Laboratory of Medicine, King Fahad Medical City, Riyadh, KSA. Hospital ethics committee approved the study. Mainly, this involved documenting ANA patterns and the conduct of ENA tests among patients diagnosed with a myriad of autoimmune diseases. Of the 666 patients recorded between January 2016 and October 2018, 453 presented non-specific autoimmune diseases while 213 had SLE. Of the 453 patients, 398 were females and 55 males. For the patients with SLE, ENA profiles were lacking; hence that data was excluded.

2.1. ANA detection by immunofluorescence technique

ANA were directly analyzed by indirect IF microscopy using multispot slides with fixed HEp-2 cells as antigen substrate and fluorescein-isothiocyanate (FITC) conjugated γ-chain specific antihuman IgG as detection antibody (DAKO, Glostrup, Denmark). As per the staining of the nucleus and cytoplasm cells, different patterns were described. These were Homogenous (H-ANA), Speckled (S-ANA), Nucleolar (N-ANA), Cytoplasmic(C-ANA), Centromere (Cen-ANA), Mitochondrial (M-ANA), and some mixed patterns such as Speckled + Cytoplasmic (SC-ANA), Homogenous + Mitochondrial (HM-ANA), Homogenous + Nucleolar (HN-ANA).

2.2. ENA profiling

ENA profiling included detection of extractable nuclear antigens such as RNP; anti-ribonucleoprotein antibody, Sm; anti-Smith, Ro (SSA; anti-Sjogren syndrome related antigen A), La (SSB; anti-Sjogren syndrome-related antigen B), Scl-70; anti Scl 70 antibody, Jo-1; anti Jo1 antibody, CenpB and histones, by immune-enzymatic assays and Western blotting.

2.3. Statistical Analysis

The frequency of ANA patterns and ENA was expressed as a percentage. The non-parametric tests -Wilcoxon signed rank test and Kruskal test were used to check significance between ANA and ENA in both genders followed by Tukey's test. The correlation between ANA and ENA was studied using Spearman's rank correlation. $p < 0.05$ was considered statistically significant.

3. Results

A total of 453 AID patients, consisting of 55 males and 398 females, were assessed for ANA patterns. Of the total number of patients, 71% were ANA positive among males and 83.4% were positive among females. Demographic data of patients with percentages of ANA patterns are represented in Table 1. The highest frequency of participants was in the age group from 21-40 years. The frequency of ANA patterns scored in both genders and microphotographs of ANA immunofluorescence patterns are depicted in Figure 1 and Figure 2. The most prevalent pattern identified in males was H-ANA (31.4%), and S-ANA (32.41%) among females. For both genders, C-ANA was least frequent. As for the females, it was observed that there were varied patterns relative to the males. Mainly, this was inclusive of Homogenous + Mitochondrial (HM-ANA), Cen-ANA, and the

Table 1. Demographic data of the studied population

Total	Males	Females
Number	55	398
Age		
0-20	10	37
21-40	24	188
40-60	13	130
> 60	8	43
N-ANA	8	18
S-ANA	11	129
M-ANA	2	11
H-ANA	14	124
C-ANA	1	6
SC-ANA	3	11
Centromere-ANA	-	12
HN-ANA	-	12
HM-ANA	-	2
Negative	16	66

The different ANA patterns are abbreviated as follows: Nucleolar (N-ANA), Speckled (S-ANA), Mitochondrial (M-ANA), Homogenous (H-ANA), Cytoplasmic (C-ANA), Speckled + Cytoplasmic (SC-ANA), Centromere (Cen-ANA), Homogenous + Nucleolar (HN-ANA), and Homogenous + Mitochondrial (HM-ANA).

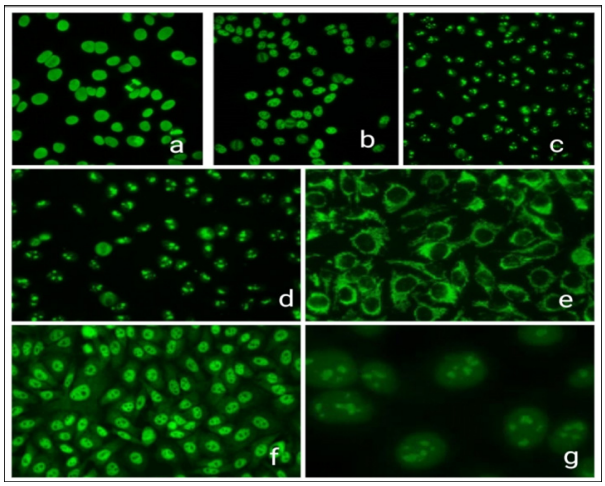

Figure 2. Immunofluorescence patterns in AID patients. (a), Homogenous pattern; **(b)**, Speckled; **(c)**, Nucleolar; **(d)**, Centromere; **(e)**, Mitochondrial; **(f)**, Mixed (Speckled + cytoplasmic); **(g)**, Mixed (Homogenous + Nucleolar).

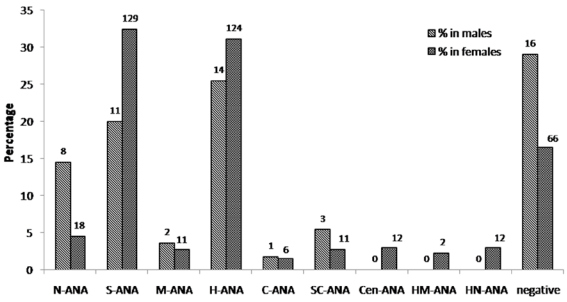

Figure 1. Frequency of antinuclear antibodies (ANA) patterns in male and female AID patients. Data labels represent the frequency (n) positive for the pattern. The different ANA patterns are abbreviated as follows: Nucleolar (N-ANA), Speckled (S-ANA), Mitochondrial (M-ANA), Homogenous (H-ANA), Cytoplasmic (C-ANA), Speckled + Cytoplasmic (SC-ANA), Centromere (Cen-ANA), Homogenous + Nucleolar (HN-ANA), and Homogenous + Mitochondrial (HM-ANA).

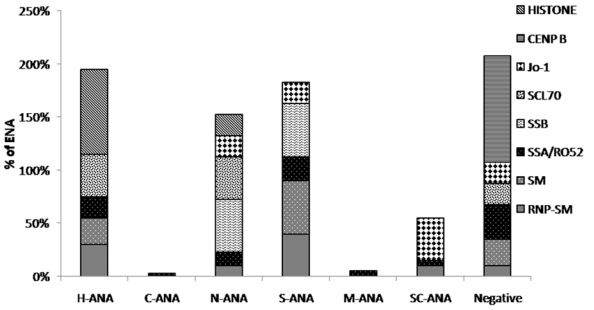

Figure 3. Frequency of extractable nuclear antigens (ENA) in different patterns of antinuclear antibodies (ANA) among males. The different ANA patterns are abbreviated as follows: Homogenous (H-ANA), Speckled (S-ANA), Nucleolar (N-ANA), Cytoplasmic (C-ANA), Centromere (Cen-ANA), Mitochondrial (M-ANA), and some mixed patterns such as Speckled + Cytoplasmic (SC-ANA), Homogenous + Mitochondrial (HM-ANA), Homogenous + Nucleolar (HN-ANA).

Homogenous + Nuclear (HN-ANA). The frequency of different patterns in males and females was compared by Wilcoxon signed rank test (a non-parametric test) that resulted in insignificant differences in the frequencies of ANA patterns in both genders.

Furthermore, to study the most prevalent ENA among the different ANA patterns, we carried out a study of ENA in these patterns, specifically RNP-Sm, Sm, SSA, SSB, Scl-70, Jo-1, Cenp B, and histones. ENA was detectable at different percentages in different patterns. The highest percentage of ENA was observed in the S-ANA pattern. The percentage of ENA in different ANA pattern in males is shown in Figure 3. RNP-Sm, Sm, SSA/Ro52, and SSB were the most frequent ENA identified (about half of the ANA patterns). Scl-70, Jo-1, Cenp B, and histones exhibited altered results. SCl-70 was predominant in H-ANA and N-ANA, Jo-1 was predominant in SC-ANA, histones in H-ANA, and Cenp B in a negative pattern. C-ANA and M –ANA were positive only to SSA. In contrast, SSA was detectable in all the ANA patterns. Of the total 55 sera, 16 sera with ANA negative (-) tested positive for ENA. The ENA studied in females yielded similar results. Figure 4 displays the percentage of ENA in different ANA patterns in females. The highest percentage of ENA was observed in S-ANA followed by H-ANA. Of the 398 sera studied in females, 66 sera were ANA negative with ENA positive. SSA positivity was recorded in all ANA patterns, including M-ANA exclusive of SSA. The most prevalent ENA observed was SSA and RNP-Sm compared to all the studied ENA.

To evaluate the inter-relationship between ENA and the correlation between the ENA and the ANA pattern, Spearman's rank correlation was performed. No

significant correlation was observed for both genders by the Kruskal test. However, there was a significant difference in the occurrence of ENA in all ANA patterns at $p < 0.05$ in males and $p < 0.001$ in females. Table 2 shows the interrelationship in the frequency of the ENA in all ANA patterns in both genders. Though different ENA were comparative in the ANA patterns, it suffices to observe that for the males, SSA was considerably correlated to the RNP-Sm and Sm at $p < 0.05$ and $p < 0.01$, respectively. There was significant correlation between Scl-70 and histones ($p < 0.01$) for the females. Similar observations on SSA were observed for Sm and Scl-70 at $p < 0.05$. Scl-70 was strongly correlated with SSA and SSB at $p < 0.05$. A considerable and significant correlation was also seen between Jo-1 /Sm, Jo-1/cenp B, RNP-Sm/Sm, and Jo-1/histone ($p < 0.05$). Essentially, there existed no correlation between ENA within and between the patterns. For a majority of these, it was noted that the ENA was SSA and the RNP-Sm within and between the patterns. Mainly, these were observed to be among the speckled patterns. For the males, ENA frequency in M-ANA and C-ANA was significantly correlated at $p < 0.001$. Conversely, among the females, there was a notable correlation in the frequency with S-ANA and H-ANA, and S-ANA and N-ANA at $p < 0.01$ and $p < 0.05$, respectively. ENA in C-ANA, also, was significantly related to the ENA in M-ANA at $p < 0.01$. Furthermore, comparison of the ENA within the specific patterns showed significant correlations between RNP-Sm with Sm and SSA with Jo-1 at $p < 0.05$.

It is thus crucial to note that the most frequent ANA patterns identified were homogenous (H) and the speckled (S) patterns. Between the genders, it was observed that S>H for the females and, for the males, H > S. In the present findings, the most frequent ENA identified was the RNP-Sm and SSA. Following these were the histones observed to be associated with the H-ANA and the S-ANA patterns. There was a statistical correlation in the frequency of these ENA: SSA with RNP-Sm and Sm; RNP-Sm with Sm in males. In females, the SSA correlated with Sm. The correlation between the most frequent ENAs, SSA and RNP-Sm, was non-significant. SSA was significantly correlated in different ANA patterns at $p < 0.001$ and non-significant in a specific pattern in males. Females showed no significant association between the SSA and ANA patterns. However, RNP-Sm exhibited a significant correlation with all ANA patterns including homologous and speckled ANA. It is thus crucial to observe the analysis of correlation between all ANA patterns.

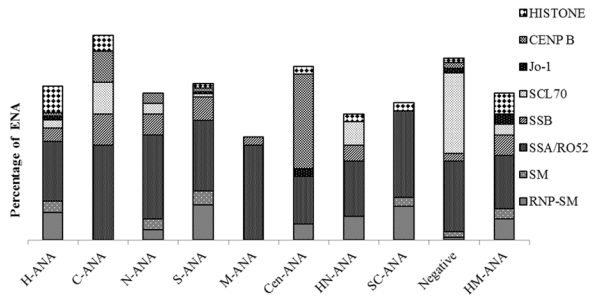

Figure 4. Frequency of extractable nuclear antigens (ENA) in different patterns of antinuclear antibodies (ANA) among females. Homogenous (H-ANA), Speckled (S-ANA), Nucleolar (N-ANA), Cytoplasmic (C-ANA), Centromere (Cen-ANA), Mitochondrial (M-ANA), and some mixed patterns such as Speckled + Cytoplasmic (SC-ANA), Homogenous + Mitochondrial (HM-ANA), Homogenous + Nucleolar (HN-ANA).

4. Discussion

In this study, we found that S-ANA and H-ANA were the most frequent ANA patterns in patients with AID associated with the antigens SSA, RNP-Sm, histones, and SSB. The frequencies of ENA in all ANA patterns were found significant at $p < 0.05$ in males and $p < 0.001$ in females.

Table 2. Correlation between extractable nuclear antigens (ENA) among different ANA pattern in males and females

ENA	SSA	RNP-Sm	Sm	Histone	Scl-70
Males					
RNP-Sm	0.7 (0.05)*	-	0.8 (0.01)*	0.37 (NS)	-
SSA	-	-	0.8 (0.01)*	0.18 (0.66)	-
SSB	0.31 (0.43)	0.49 (0.21)	0.26 (0.54)		-
Scl-70	0.46 (0.25)	0.32 (0.43)	0.16 (0.66)	0.87 (0.05)*	-
Jo-1	0.29 (0.4)	0.30 (0.38)	0.06 (0.8)	0.22 (0.60)	-
Cenp B	0.61 (0.12)	0.0 (0.9)	0.34 (0.38)	-	-
Histone	0.18 (0.66)	0.37 (0.38)	0.07 (0.84)	-	-
Females					
RNP-Sm	0.5 (0.07)	-	0.73 (0.01)*	0.42 (0.21)	0.52 (0.10)
SSA	-	-	0.78 (0.05)*	0.59 (0.05)*	-
SSB	0.71 (0.015)	0.5 (0.10)	0.80 (0.002)**	0.58 (0.06)	0.91 (0.0001)***
Scl-70	0.62 (0.04)*	0.52 (0.10)	0.66 (0.033)*	0.55 (0.089)	-
Jo-1	0.46 (0.10)	0.44 (0.1)	0.62 (0.021)*	0.81 (0.001)**	0.62 (0.04)*
Cenp B	0.42 (0.21)	0.15 (0.65)	0.42 (0.2)	0.3 (0.38)	0.38 (0.25)
Histone	0.54 (0.09)	0.42 (0.21)	0.59 (0.05)*	-	0.5 (0.08)

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The existence of ANA in blood constitutes a significant criterion for the diagnosis of connective tissue diseases (CTD). Identification of ANA subtypes plays a key role in diagnosis of specific CTD. The present study verified the profile of patient samples tested for ENA antibodies and correlated the ENA results with ANA patterns. The most common pattern was speckled S-ANA (32.4%) in females and homologous H-ANA pattern (25.4%) in males. Indeed, females were found to exhibit some mixed patterns apart from the defined ANA pattern compared to males.

Despite ANA specificity to nuclear antigens, cytoplasmic patterns correlated with autoantibodies against cytoplasmic antigens were also observed in some patients. Additionally, some mixed patterns observed could be due to similarity in the epitopes/antigenic determinants of the cellular antigens (*8*). Among the ENA, SSA/Ro52, RNP-Sm, and histones were found in higher frequency in different ANA patterns. Within the study, SSA was the most prevalent ENA identified, which was found associated with a speckled ANA pattern, around 81.8% in males and 75% in females. The second most was RNP-Sm, with the highest frequency in speckled and homologous patterns. Spearman's rank correlation of ENA within and among the ANA patterns was non-significant. Nevertheless, the frequency of ENA in all ANA patterns was found significant at $p < 0.05$ in males and $p < 0.001$ in females.

A comprehensive understanding on the significance of various patterns assists clinicians in confirming the diagnosis of AID. Notably, the H-ANA pattern has been observed in association with SLE (*9*). Mainly, the patterns result from the reaction of the antibodies against dsDNA, histones, and DNA/histone complex on HEp-2 cells (*10*). The S-ANA is released by the antibodies while targeting the extractable nuclear antigens (*11*). As such, it suffices to observe that the nucleolar pattern results from the antibodies such as RNA polymerase and Scl-70 (topoisomerase-1) (*10,12*). In a Swedish study by Frodlund *et al*., H-ANA was reported as the most dominant IF-ANA pattern in patients with SLE (*10*). The most frequent pattern identified in this study was speckled pattern followed by homologous pattern. The results are in line with a previous study by Peene *et al*. (*13*).

However, studies of ANA pattern with relevant ENA testing with diagnostic importance are scarce, particularly in the Saudi population. The ENA commonly observed in this study was inclusive of the RNP-Sm and SSA found in association with the speckled pattern. This was in line with the observations by Mutasim *et al*. (*14*). Similarly, the highest frequency of the anti-SSA/Ro autoantibody in anti-ENA-positive patients was also reported by Lora *et al*. and evidenced by Banhuk *et al*. (*15,16*). Homologous to the finding of Li *et al*. (*17*), higher ANA positivity (83.4%) in females compared to males (71%) was observed. Few of the mixed patterns were found in females compared to males. It is possible that Scl-70, a type of ENA found attached to DNA and extrachromosomally in the nucleoplasm, draws from a mixed IF staining pattern. Similar to Scl-70, the La/SSB antigen may partially localize in nucleoli. Hormonal profiles, and fetal microchimerism, are regarded as potential discriminating factors for such patterns.

As observed, the determination of ENA or anti-ENA profiling contributes to an improved differentiation among the different types of autoimmune rheumatic diseases (ARD). The presence of RNP autoantibodies constitutes an efficient marker in the diagnosis of mixed connective tissue disease (MCTD). Similarly, ANA positivity with dsDNA or Sm positivity forms the criteria for diagnosis of SLE (*18*). In addition, the anti-SSA/Ro and anti-SSB/La antibodies contribute as a significant immune marker in the detection of Sjogren syndrome, subacute cutaneous SLE, and neonatal lupus syndrome (*19*). Jo-1; histidyls RNAsynthetase is observed to be capable immunomarker linked with polydermatomyositis. Likewise, the CENP-B and Scl-70 positivity or the appearance of anti-centromere antibodies (CENP-B) or topoisomerase 1 (Scl-70) facilitates the diagnosis of systemic sclerosis (*20,21*).

Of the many constraints conspicuously displayed with ANA testing, is the ENA positivity displayed in ANA negative samples among patients with AID. Unpredictably, ENA-positivity with ANA-negative patients in 16 AID males (29%) and 66 AID females (16.5%) was noted in the current investigation. These findings are common as these could be cases of patients undergoing immunosuppressive therapy (*22*). Additionally, immunoassay was observed to be more sensitive for the detection of the SSA/Ro52 relative to ANA-IF for even the Hep-2cells. Scl-70 and Jo-1 that is associated with systemic sclerosis and polymyositis, and may not be detected in preliminary IF –ANA screening. Mainly, this follows the antibodies' cytoplasmic positivity rather than nucleic staining patterns on IF. Therefore, in such cases, ANA could have been reported, as negative. Negative ANA samples also require further assessment with anti-ENA profiles to facilitate the identification of the relevant ENA associated with the disease in case there is a strong suspicion of AID. Regardless of this, positive results of ANA testing should always be interpreted within the confines of a clinical context.

Additionally, Spearman's rank correlation between different ENA within and between the patterns yielded unsatisfactory results. As such, there was no significant correlation between ENA and ANA. Paramount in the present findings was the significant correlation between different ENA studied. SSA was significantly correlated with RNP-Sm, Sm, and Scl-70. Based on the above associations, it can be postulated that the study comprised a mixed population with different AID such as SLE and Sjogren syndrome. From these perspectives, it can be concluded that IF serves well as a standard ANA testing

method showing high specificity and sensitivity, along with specific ENA detection that assists in diagnosis of autoimmune diseases more accurately, while minimizing cost and time required for conventional immunological evaluation for diagnosis of these diseases. The study, however, had some limitations. Though it confirmed the prevalence of ANA and ENA patterns among patients with autoimmune diseases, the evaluation of IF ANA patterns from specific autoimmune diseases as a comparative study would have added additional information to the outcome of the present investigation.

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Brief Report

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Identification of novel compound heterozygous mutations of the *DYNC2H1* gene in a fetus with short-rib thoracic dysplasia 3 with or without polydactyly

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SUMMARY A prenatal sonograph revealed a 26-week-old fetus with short limbs and a narrow chest in a 23-year-old woman with a history of fetal skeletal dysplasia. A single nucleotide polymorphism-based chromosomal microarray (CMA) indicated a normal karyotype, and no chromosomal segments with abnormal copy numbers were noted in the fetus. Whole exome sequencing identified compound heterozygous mutations in the *DYNC2H1* gene responsible for a lethal type of bone growth disorder, short-rib thoracic dysplasia 3 with or without polydactyly (SRTD3), and revealed a missense mutation c.515C>A (p. Pro172Gln) of paternal origin and a missense mutation c.5983G>A (p. Ala1995Thr) of maternal origin. These variants were further confirmed by Sanger sequencing. To the extent known, the c.515C>A (p. Pro172Gln) mutation is novel for SRTD3, and the site is conserved across species. This study found a novel mutation of the *DYNC2H1* gene for SRTD3 and it has increased the number of reported cases and expanded the spectrum of mutations causing this rare disease.

Keywords short-rib thoracic dysplasia 3 with or without polydactyly, *DYNC2H1*, compound heterozygous mutations

1. Introduction

Short-rib thoracic dysplasia 3 with or without polydactyly (SRTD3) covers a range of autosomal recessive or digenic recessive skeletal dysplasia characterized by shortened limbs, a narrow trunk, and associated visceral abnormalities with or without polydactyly (*1*).

Currently, short-rib polydactyly syndromes (SRPSs) have been classified into short-rib thoracic dysplasias with or without polydactyly types 1-17 (SRTD1-17). Short-rib thoracic dysplasia 3 with or without polydactyly (SRTD3; 613091) is caused by homozygous or compound heterozygous mutations in the dynein heavy chain, isotype 1B (*DYNC2H1*) gene, which encodes a protein involved in ciliary intraflagellar transport (*2,3*). Currently, more than 140 mutations in the *DYNC2H1* gene have been identified in SRTD3.

Reported here is a case of a Chinese woman with three consecutive pregnancies that were diagnosed with SRTD3 without polydactyly according to clinical and ultrasound results revealing novel compound heterozygous mutations in *DYNC2H1*.

2. Patients and Methods

2.1. Fetus with SRTD3 and samples

A woman in her third pregnancy at 26 weeks of gestation was seen at Zibo Maternal and Child Health Hospital in 2015. A fetus with abnormal dysplasia had developed in her first and second pregnancies. An ultrasound of the third pregnancy revealed a maximal depth of amniotic fluid of 3.6 cm, a biparietal diameter of 6.4 cm, a femur length of 2.8 cm, and a humerus length of 1.6 cm. The fetal chest is bilaterally symmetrical but narrow. In addition, a prenatal ultrasound also indicated that the long bones of the lower extremities were clearly short and the epiphyses were irregular. Abnormalities in the brain, liver, or kidneys were not noted. The parents of the fetus with SRTD3 are healthy and have no family history of genetic diseases or dysplasia.

The pregnancy was terminated after diagnosis, and amniotic fluid cells of the fetus and whole blood

samples of the parents were collected after obtaining informed consent from the parents. This study was approved by the ethics committee of the Shandong Medical Biotechnological Center.

2.2. DNA extraction

Genomic DNA was extracted from fetal amniotic fluid cells and whole blood cells using a blood DNA extraction kit (Omega Bio-tek, Norcross, GA, USA). DNA purity was measured with the NanoDrop® 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the concentration of extracted DNA was measured using the Qubit 3.0 Fluorometer (Thermo Scientific, Waltham, MA, USA).

2.3. Genome-wide copy number analysis

Genomic alterations of fetal amniotic fluid cells were used to analyze single nucleotide polymorphism by chromosomal microarray following the provided standard protocol (Affymetrix, CA, USA). Purified DNA was first fragmented and biotin-labeled and subsequently hybridized and scanned. Data were analyzed using the software Chromosome Analysis Suite (Affymetrix, CA, USA).

2.4. Whole exome sequencing (WES)

A DNA libraries was constructed using the Illumina TruSeqDNA sample preparation kit (Illumina Inc. San Diego, CA, USA). Exome capture was performed using the Agilent Custom sureselect Enrichment Kit (Agilent, Santa Clara, CA, USA). Massive parallel sequencing was performed with the Illumina Genome Analyzer IIx instrument (Illumina Inc. San Diego, CA, USA). Raw data were assessed to generate clean reads, and the reference human genome (GRCh37/hg19) was mapped using the Burrows-Wheeler Aligner (BWA). The genome analysis toolkit (GATK), samtools, and Picard tools were used to remove duplicates and false mutations introduced by library construction and to re-calibrate map quality scores. Single nucleotide variants (SNVs) and small insertion-deletions (InDels) were detected with GATK and Varscan. Numerous databases were used to annotate the identified SNP and InDel variants. The pathogenicity of the detected SNP variants was assessed using the tools PolyPhen-2, SIFT, and PROVEAN.

2.5. Sanger sequencing

Probable mutations identified by WES in the fetus and parents were verified using Sanger sequencing. The PCR primers used to amplify *DYNC2H1* exons 4 and 38 are shown in Table 1. Two hundred unrelated healthy controls were subjected to Sanger sequencing to verify the identified variants.

Table 1. Primer sequences used to amplify *DYNC2H1* exons 4 and 38

Primer name	Sequence (5′-3′)
DYNC2H1-4F	TCAAATATTTTGCTGCTCTGC
DYNC2H1-4R	CATTTTAAAACAGAAGAGAGGCTGT
DYNC2H1-38F	GGCAATACCTTCCACTGAAGAAA
DYNC2H1-38R	CCCCCTCCAAAGTAAAATATCAAAT

3. Results and Discussion

SRTD3 represents a type of severe autosomal recessive or digenic recessive fetal skeletal dysplasia characterized by shortened limbs, a narrow thorax, and with or without polydactyly. Signs of polydactyly were not noted in the current case. Moreover, this fetus with SRTD3 reflected an obvious family history, and similar symptoms were noted during two previous pregnancies.

Chromosomal microarray (CMA) analysis indicated that the karyotype of the fetus was normal (46, XX), and no chromosomal segments with abnormal copy numbers were noted in the whole genome. WES of the fetus revealed a mean target coverage of 76.5%, a total sequencing depth of 100×, and a target region sequencing depth of 35×-50×. In addition, coverage of the targeted bases for > 10 reads was 92.19%, that for > 20 reads was 86.22%, and that for > 30 reads was 78.81%. Two compound heterozygous variants c.515C>A and c.5983G>A in the *DYNC2H1* (NM_001377) gene were screened out by filtering out known variants in the relevant database using an autosomal recessive inheritance model. Sanger sequencing confirmed the finding of *DYNC2H1* mutations in the fetal sample from WES; the father carried the c.515C>A mutation while the mother carried the c.5983G>A mutation, both of which were heterozygous mutations (Figures 1 and 2).

The c.515C>A mutation in the *DYNC2H1* gene was located in exon 4, and the c.5983G>A mutation was located in exon 38; both were missense mutations. The c.515C>A mutation will give rise to a substitution of a glutamine for a proline at amino acid 172 (Pro172Gln), and the c.5983G>A mutation causes a substitution of a threonine for an alanine at amino acid 1995 (Ala1995Thr). The c.515C>A mutation affects the highly conserved proline residues in the N-terminal region 1 (DHC_N1) (Figure 3). The pathogenicity of this mutation was predicted using a series of online pathogenesis prediction programs; it was "Probably Damaging" according to polyphen-2, "Affect Protein Function" according to SIFT, and "Deleterious" according to PROVEAN. Moreover, the c.515C>A (Pro172Gln) missense mutation was not present in the Human Gene Mutation Database (HGMD), the Exome Aggregation Consortium (ExAc), ClinVar, or

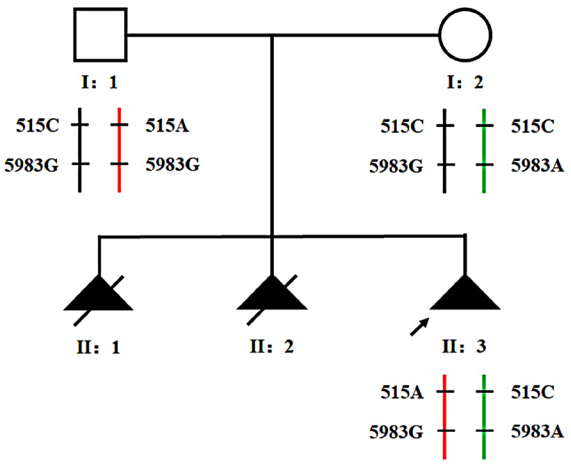

Figure 1. Pedigrees of the family members with STRD3 and mutation analysis of the *DYNC2H1* gene. The proband (II:3) is indicated with a black arrow.

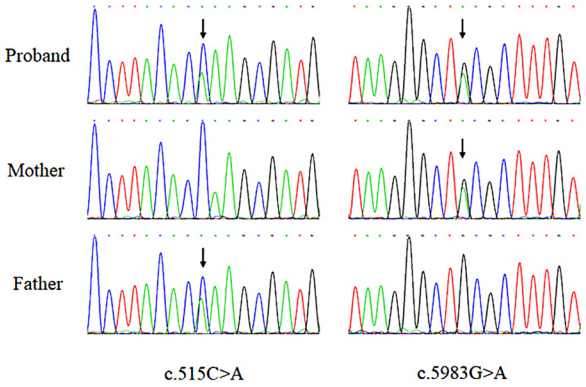

Figure 2. A DNA sequence chromatogram of the family members indicating that compound heterozygous mutations (c.515C>A and c.5983G>A) of the *DYNC2H1* gene in the proband (II:3) were respectively inherited paternally and maternally.

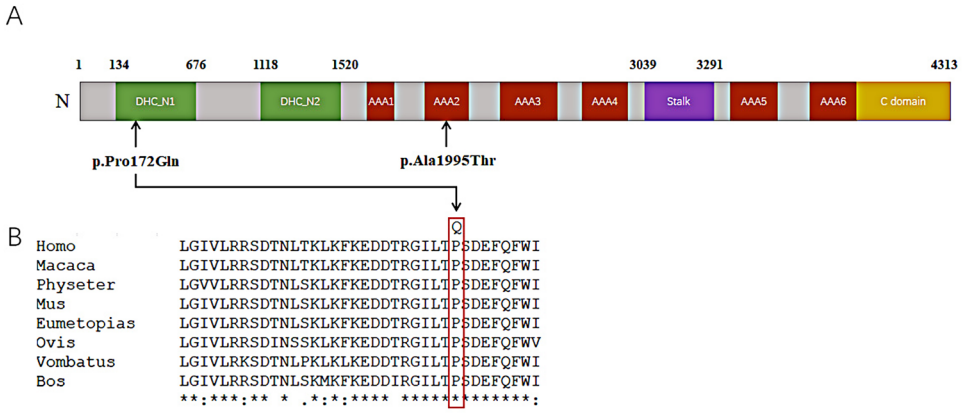

Figure 3. (A) Schematic diagram of the *DYNC2H1* protein and the locations of the mutations detected in the proband. **(B)** Conservation analysis of *DYNC2H1* indicated that the proline residue at position 172 is highly conserved in various species.

1000 genomic databases. No such mutation was found in 200 unrelated healthy controls according to Sanger sequencing. Thus, c.515C> A in the *DYNC2H1* gene may be a novel pathogenic mutation.

DYNC2H1, the gene responsible for SRTD3, is located at chromosome 11q22.3 and encodes a large cytoplasmic dynein protein involved in the structure and function of cilia (*4,5*). It consists of an N-terminal tail (DHC_N1), a linker domain (DHC_N2), six identifiable AAA-ATPase domains, a stalk between AAA domains 4 and 5 in the microtubule binding domain (stalk MTBD), and a C-terminal tail (C domain) (*6*). The c.515C>A (p. Pro172Gln) variant is located in the DHC_N1, which along with DHC_N2 is considered to be the tail domain of a dynein heavy chain. This domain is considered to be the motile element of the dynein heavy chain, directing its movement along the dynein microtubules (*7*). A recent study reported that disruption of the dynein-2 tail domain can eliminate the ciliary localization of dynein-2, suggesting its important role in the ciliary entry of dynein-2 (*8*). The c.5983G>A(Ala1995Thr) variant is located in the AAA2 domain. This variant was first reported in Chinese SRTD3 cases, and three cases of this variant have been reported overseas, one of which is homozygous mutation and two of which are heterozygous mutations ([c.195G>T] + [c.5983G>A] and [c.5983G>A] + [c.10594C>T]) (*9*).

Currently, a total of 93 SRTD3 cases caused by *DYNC2H1* mutations have been reported (*3,9-13*). Shortened limbs and a narrow chest are common in these cases, but polydactyly, irregular epiphyses, and visceral abnormalities are not found in all cases. A total of 147 pathogenic mutants of the *DYNC2H1* gene have been identified for SRTD3, including 92 missense mutations, 21 nonsense mutations, 22 insertion/deletion mutations, and 12 splicing mutations. When these mutations were mapped on the domain of dynein protein, 16 mutations were located in the DHC_N1, 9 were located in the DHC_N2, 62 were located in the six AAA+ domains, 6 were located in the stalk MTBD, and 11 were located in the dynein heavy chain. In all cases, most of those mutations were compound heterozygous mutations, and only four were homozygous mutations.

In summary, the current study involving WES identified two missense mutations of *DYNC2H1* in a fetus with SRTD3, including a novel c.515C>A (p. Pro172Gln) mutation with probable pathological significance. This finding expands the spectrum of *DYNC2H1* mutations found in patients with SRTD3 and it also provides helpful information to better understand the molecular function of the *DYNC2H1* gene.

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Brief Report

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Increased CD27 expression in the skins and sera of patients with systemic sclerosis

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SUMMARY Systemic sclerosis (SSc) is a kind of collagen disease and has an acquired autoimmune activation as represented by the production of autoantibodies. CD27 is a type I glycoprotein and a member of the tumor necrosis factor receptor family. It binds to the CD70 ligand, CD27-CD70 signaling is implicated in the development of various autoimmune diseases, but its role in the regulation of extracellular matrix expression and its contribution to the phenotype of SSc both remain to be elucidated. This study aimed to investigate the associations between CD27 and SSc in the skins and sera. Immunohistochemistry were performed to determine the expression of CD27 in the skin. Enzyme-linked immunosorbent assays were done to the sera of the 54 patients with SSc and 23 normal healthy controls. CD27 expression was significantly increased in the affected regions of the skin and the sera of patients of SSc. Thereafter, we evaluated the correlation between the serum soluble CD27 (sCD27) levels and the clinical symptoms. The study subjects with increased sCD27 levels had a significantly higher ratio of dcSSc and to showed higher modified Rodnan's total skin thickness scores (mRSS) than those with normal sCD27 levels. These results suggest that sCD27 levels might be useful for diagnosis of SSc and its severity.

Keywords CD27, tumor necrosis factor (TNF) receptor family, systemic sclerosis, fibrosis

1. Introduction

Systemic sclerosis (SSc) is an acquired autoimmune disorder, characterized by microvascular damage and excessive fibrosis of the skin and various internal organs. Based on the extent of skin fibrosis and the pattern of internal organ involvement, patients with SSc are commonly classified into either the diffuse cutaneous SSc (dcSSc) subset or the limited cutaneous SSc (lcSSc) subset (*1*). Although the pathogenesis of the disease is unclear, the clinical and pathological manifestations of SSc have been characterized as abnormalities in the innate and adaptive immune system that lead to the production of autoantibodies and cell-leading to the accumulation of excessive collagen and other extracellular matrices in the skin, blood vessels, and internal organs (*2*). Fibrosis is a major contributor to the high level of morbidity and mortality in SSc patients, and it is believed that the expression levels of pro-inflammatory chemokines, cytokines, and connective tissue growth factors are elevated, which leads to the activation of fibroblasts and the abnormal accumulation of the extracellular matrix. This results in progressive endothelial damage, edema, and sclerosis of the skin (*3*).

CD27 is a type I glycoprotein that is expressed on the majority of T cells and some B cells, and it is a member of the tumor necrosis factor (TNF) receptor family with unique cysteine-rich motifs (*4*). In subsequent studies, it was revealed that CD27 is expressed on CD4 and CD8 T lymphocytes, NK cells, and hematopoietic stem cells (*5,6*). The activation of T cells can also lead to the shedding of CD27 from the cell surface, resulting in the secretion of soluble CD27 (sCD27) (*7*). On the contrary, CD27 is not expressed by naïve B lymphocytes, but is upregulated in activated and antigen-experienced B lymphocytes (*8*). CD27 is also uniformly found on memory B cells (*9*). It binds to the ligand CD70 and transduces signals that lead to the activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase 8 (MAPK) / c-jun N-terminal kinase(JNK). Adaptor proteins, such as TNF receptor-associated factor (TRAF) 2 and TRAF5, which have been shown to mediate the signaling process of this receptor. The CD27-binding protein (SIVA), a pro-apoptotic protein, can bind to this receptor, and then regulates many cellular processes, including cell proliferation, differentiation, and survival (*10,11*). Thus, a key biological role for the interactions of CD27-CD70

in T cell priming and in the subsequent promotion of their survival, which results in the formation of effector and memory T cells, has been well documented (12,13). In the B cell compartment, CD27-CD70 interactions are important for T cell-dependent antibody production by promoting B cell activation, germinal center formation, expansion of B cells, and differentiation into plasma cells, and by enhancing immunoglobulin production (*14-16*). However, the involvement of CD27 in the pathogenesis of SSc remains unknown.

In this study, we examined the expression of CD27 in the skin of SSc patients by immunohistochemistry. The amount of CD27 was measured in dcSSc, lcSSc patients and healthy controls. Furthermore, we examined the serum levels of sCD27 in SSc patients by enzyme-linked immunosorbent assay (ELISA). We evaluated the correlation between the serum sCD27 levels and clinical symptoms in patients with SSc.

2. Materials and Methods

2.1. Patients

The study was approved by the Ethical Committee of the Kumamoto University School of Medicine and was carried out according to the guidelines set by the Institutional Review Board. We obtained tissue samples from 8 patients with SSc and serum samples from 54 patients with SSc. All patients with SSc fulfilled the new criteria proposed by the American College of Rheumatology/European League against Rheumatism (*17*), and were grouped according to the classification system proposed by LeRoy *et al.* (*18*). Of the 8 patients, 5 patients had diffuse cutaneous SSc (dcSSc), and 3 patients had limited cutaneous SSc (lcSSc), as described above. Skin biopsy specimens for the SSc patients were obtained from the involved skin. Control skin samples were obtained from the routinely discarded skin of healthy human subjects undergoing skin grafts. The samples were fixed in formalin immediately after removal and were then embedded in paraffin. On the contrary, the serum samples were obtained from 23 patients with dcSSc and 31 patients with lcSSc. Control serum samples were also collected from 18 healthy age- and sex-matched volunteers. All serum samples were stored at −80°C prior to use.

Institutional review board approval and written informed consent were obtained before the patients and healthy volunteers entered in this study, according to the Declaration of Helsinki.

2.2. Immunohistochemical (IHC) staining and IHC evaluation

Immunohistochemical analysis was performed on 4μm sections of formalin-fixed, paraffin-embedded tissue. The sections were deparaffinized with Clear Plus® (Falma, Tokyo, Japan) and were subsequently rehydrated with ethanol. Antigen retrieval was performed in a microwave oven with a citric acid (pH 6.0) buffer. Endogenous peroxidase activity was blocked by incubation with 1.0% hydrogen peroxidase. After the addition of normal goat serum (FUJIFILM Wako Pure Chemical, Osaka, Japan), a monoclonal rabbit anti-CD27 antibody (Abcam, Cambridge, UK) (1:250 dilution) was used as the primary antibody, and tissue samples were incubated with this antibody overnight at 4°C. These sections were further incubated with Histofine® Simple Stain MAX-PO(R) (Nichirei Biosciences, Tokyo, Japan) for 30 min. Finally, the chromogen was 3,3′-diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin to facilitate the recognition of structures.

2.3. Soluble CD27 measurement

Serum levels of sCD27 were measured with a specific ELISA kit (Human CD27/, Abnova, Taipei, Taiwan). Briefly, human CD27 capture antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by the peroxidase-conjugated antibodies for CD27. The color was developed with hydrogen peroxidase and tetramethylbenzidine peroxidase, and the absorbance at 450 nm was measured. Wavelength correction was performed based on the absorbance of a blank well at 450 nm. The concentration of sCD27 in each sample was determined by interpolation from a standard curve.

2.4. Statistical analysis

Statistical analysis was carried out with the Mann-Whitney test for the comparison of the median, and Fisher's exact probability test was used for the analysis of the frequency. A p value of less than 0.05 was considered significant.

3. Results and Discussion

3.1. Abundant expression of CD27-positive lymphocytes in the skin of SSc patients

First, we attempted to evaluate the expression pattern of CD27 in the involved skin of SSc patients. Skin tissue samples were obtained from 5 patients with dcSSc and 3 with lcSSc. The immunohistochemistry of CD27 was examined in the skin tissues from patients with dcSSc and lcSSc. CD27 was found on the surface of lymphocytes around the capillaries in the reticular dermis. Both lymphocytes and CD27-positive lymphocytes were more abundant in the skin of patients with dcSSc (Figure 1A and 1B). Then, the ratio of CD27-positive lymphocytes to overall lymphocytes was calculated for each of five high-power fields,

respectively. The ratios of CD27-positive cells were significantly higher in the skin of the dcSSc patients than in the lcSSc patients (32.2% *vs.* 19.7%, $p < 0.05$), as shown in Figure 2. Jacobi AM *et al.* reported that CD27-positive plasma cells were elevated in the PBMC of patients with SLE (*19*). However, whether CD27 is involved in the pathogenesis of skin sclerosis in SSc patients remains to be elucidated. Our results suggest that CD27 is activated in the skins of dcSSc patients and may be useful for the diagnosis.

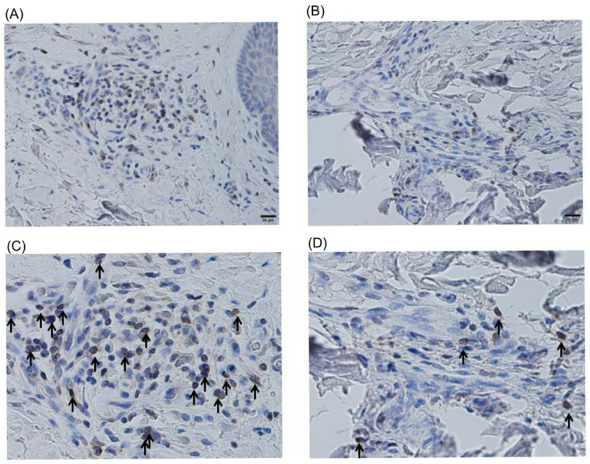

Figure 1. Abundant expression of CD27-positive lymphocytes in the skin of SSc patients. Paraffin sections were subjected to the immunohistochemical analyses. Both the lymphocytes and CD27-positive lymphocytes were abundant in the skin of patients with dcSSc **(A,C)** compared to lcSSc **(B,D)**. Black arrow indicates CD27-positive cells in high-power fields. It shows at Low-power field (× 100) **(A,C)** and high-power field (×200) **(B, D).**

3.2. Elevated soluble CD27 levels in the sera of SSc patients

Next, we examined the expression of sCD27 in serum by sandwich ELISA, as described in the methods section. Serum samples were obtained from 54 patients with SSc (23 dcSSc and 31 lcSSc). Samples were also obtained from 18 healthy control subjects. The serum levels of sCD27 in patients with dcSSc and lcSSc and in the healthy control subjects are shown in Figure 3. Patients with dcSSc had significantly higher sCD27 levels than those with lcSSc (4,319 ± 1,701 pg/mL *vs.* 3,305 ± 1,189 pg/mL, $p < 0.05$). Additionally, the lcSSc patients had significantly higher sCD27 levels than the healthy controls did (3,305 ± 1,189 pg/mL *vs.* 1,781 ± 593 pg/mL, $p < 0.05$). These results suggest that CD27 is also activated in the sera of SSc patients, particularly in those of dcSSc.

3.3. Systemic activation of CD27 in SSc patients

According to our results as described above, CD27 was activated not only in the skin tissues of patients with SSc, but also in the sera of patients with SSc. These results suggest that the systemic activation of CD27 is associated with the pathogenesis of SSc. In SLE patients, Font J *et al.* reported that the serum levels of sCD27 were elevated and correlated with disease activity measures (*20*). However, the efficacy of CD27 as a biomarker for the diagnosis or prognosis prediction of SSc is still unknown.

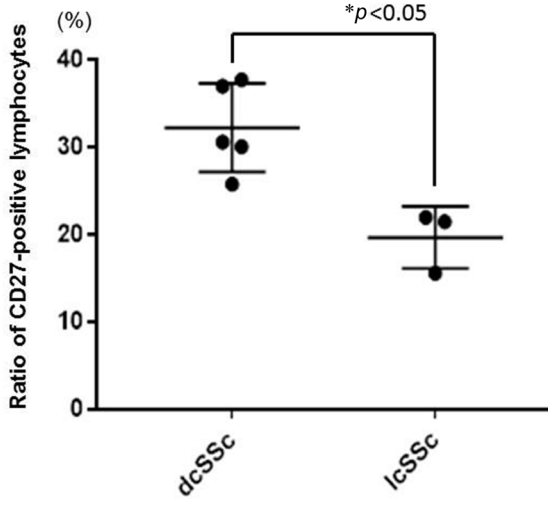

Figure 2. High ratios of CD27-positive lymphocytes in dcSSc patients. The samples were prepared from skin tissues obtained from patients with dcSSc ($n = 5$) and those with lcSSc ($n = 3$). The ratio of CD27-positive cells to overall lymphocytes was calculated for each of the five high-power fields from each sample, respectively. The ratios of CD27-positive cells in dcSSc patients were significantly higher than those in lcSSc patients (32.2% *vs.* 19.7%). Bars indicate means (SD). *p* values less than 0.05 are interpreted as significant.

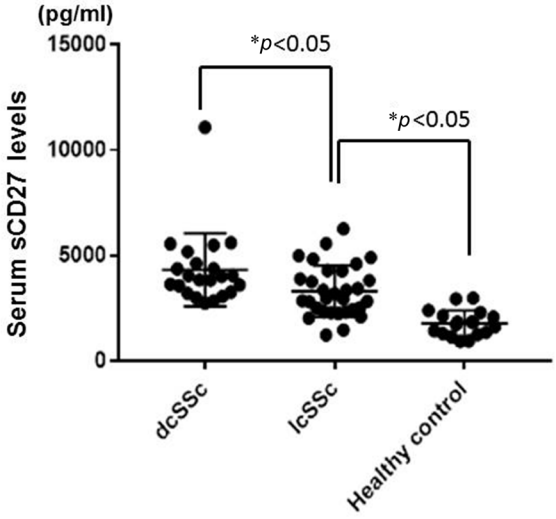

Figure 3. Elevated soluble CD27 levels in the sera of SSc patients. Serum CD27 levels were measured with ELISA kits. Patients with dcSSc had significantly higher sCD27 levels than the patients with lcSSc (4,464 pg/mL *vs.* 3,305 pg/mL, $p < 0.05$). Additionally, the lcSSc patients had significantly higher sCD27 levels than the healthy controls did (3,305 pg/mL *vs.* 1,781 pg/mL, $p < 0.05$). Bars show means (SD). *p* values less than 0.05 are interpreted as significant.

Then, we evaluated the correlation between sCD27 and the clinical symptoms. We divided the SSc patients into two groups. The increased sCD27 group was defined as having much higher sCD27 levels than the mean + 2SD of the healthy controls, while the normal sCD27 group was defined as having less than the mean + 2SD. The ratio of the dcSSc patients and modified Rodnan's total skin thickness scores (mRSS) were statistically significantly higher in the increased sCD27 group, whereas the number of patients with calcinosis was larger in normal sCD27 group. There were no statistically significant differences in the sex, age of onset, disease duration, other clinical symptoms, or organ involvement (Table 1). These results were consistent with those of the immunohistochemical analysis of the skin tissues and suggested that CD27 could play a key role in skin fibrosis.

CD27-CD70 is co-stimulatory molecule, the signaling of which leads to the activation of T cells and B cells and induces the proliferation of plasma cells and the production of immunoglobulin G (*21*). Furthermore, CD27-CD70 signaling is considered to trigger the pathophysiology of autoimmune diseases. In SSc patients, Jiang H, *et al.* reported that the proportion of CD4+ T cells expressing CD70 was significantly increased compared to the controls (*22*). CD70 was significantly more expressed in CD4 T cells of patients with RA than in those of age-matched healthy controls (*23*). Oflazoglu E, *et al.* reported the efficacy of treatment of collagen-induced arthritis (CIA) with an anti-CD70 antibody in a murine model. Treatment with an anti-CD70 antibody, both before onset and after established disease in a CIA model resulted in significant improvements in the disease activity and reduction in the production of autoantibodies (*24*). However, it remains to be seen whether a therapeutic blockade of CD70 might be a useful approach for the treatment of RA or other inflammatory arthritides, or whether CD27-CD70 signaling is involved in the pathogenesis of SSc. Clinical trials of an anti-CD70 antibody for the treatment of malignant tumours have been ongoing. Similarly, inhibition of CD27-70 signaling might be a target for the treatment of SSc.

Table 1. Correlation of serum sCD27 with clinical symptoms

sCD27	Increased (n = 36)	Normal (n = 19)
Male:Female	8:28	0:19
Age (average)	67.2 (31-84)	60.6 (33-84)
Disease duration (year)	6.51	5.53
Diffuse:limited	21:15*	3:16
topo1:ACA:RNP	12:13:4	5:8:1
mRSS	14 (1-41)*	8.5 (0-34)
Clinical symptoms (%)		
Raynaud's phenomenon	82.4	93.3
Pitting scar	38.7	42.9
Didital ulcer	38.2	18.8
Nailfold bleeding	50.0	61.5
Telangiectasia	36.0	40.0
Contracture of phalanges	87.1	84.6
Caltinosis	4.55*	25.0
Sicca sumptoms	64.3	57.1
Organ involvement (%)		
ILD	52.8	41.2
Heart	51.4	40.0
Esophagus	35.3	28.6
Liver	14.3	12.5
Kidney	8.57	0.00
Joint	24.0	50.0
Thyroiditis	12.1	6.25

ACA, anti-centromere antibody; ILD, intestinal lung disease; mRSS, modified Rodnan total skin thickness score; RNP, anti-U1 RNP antibody; topo1, anti-topoisomerase 1 antibody. *$p < 0.05$.

In conclusion, the CD27 expression levels in skin tissues and sCD27 levels in serum samples are useful as diagnostic markers for SSc. Furthermore, they have the possibility of being markers for the disease activity or for the prediction of the response to treatment for SSc. In this study, only a small number of patients were evaluated, due to the rarity of SSc; large-scale studies on CD27 and CD70 in patients with SSc should be conducted in the future.

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Brief Report

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Reporting one very rare pathogenic variation c.1106G>A in *POMT2* gene

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SUMMARY Dystroglycan (DG) is a major cell membrane glycoprotein, which is encoded by the *DAG1* gene. α-DG is one of DG subunits, belongs to O-mannosylated protein of mammals and was identified in brain, peripheral nerves and muscle. Dystroglycanopathies are a group of heterogeneous congenital muscular dystrophies, which can result from defective α-DG mannosylation. First line of α-DG glycosylation is catalyzed by protein O-mannosyltransferase family (PMT). In this study, the mutation was identified in the *POMT2* gene, which encodes O-mannosyltransferase 2 protein and its mutations can be contributed to dystroglycanopathies. A very rare missense mutation in the *POMT2* gene (NM_013382: exon9: c. 1106G>A) was identified by next generation sequencing (NGS) and was subsequently confirmed using Sanger sequencing in both affected siblings. There was no report of this mutation in the literature, therefore, the significance was uncertain. Our findings confirmed the pathogenicity of mutation and expanded the mutation spectrum of *POMT2*, which will be helpful in further molecular evaluations of muscular diseases.

Keywords dystroglycanopathy, alpha-dystroglycan, rare mutation, *POMT2*

1. Introduction

Protein glycosylation is a complex process which takes place in the ER and Golgi apparatus. This post translational process maintains main protein features such as stability, conformation and function. Glycans attach to protein in two distinctive sites: N-glycans are attached to asparagine residues and O-glycans to threonine and serine residues of the protein. These glycosylated proteins can be distinguished by the sugar subunits, such as mannose, N-acetylgalactosamine and fucose (*1*).

Dystroglycan (DG) is a major cell membrane glycoprotein that links cytoskeleton of muscles and nerve cells to the extracellular matrix (ECM) and plays a major role in dystrophin associated protein complex. *DAG1* gene encodes DG which is cleaved into two subunits, α-DG and β-DG, as a post translational proteolytic process. α-DG is a kind of O-mannosylated cell surface protein in mammals, which was identified in brain, peripheral nerves and muscle. Protein O-mannosylation is a vital complex process and conserved from bacteria to humans but not found in plants or nematodes. It is an essential protein modification for central nervous system (CNS) development and function. Defective α-DG mannosylation results in congenital muscular dystrophy (CMD) with CNS malfunction, generally named dystroglycanopathies (*2,3*).

Dystroglycanopathies are a group of heterogeneous CMD, often characterized by variable neurological and ocular involvement and are inherited as an autosomal recessive pattern. The relevant genes are *POMT1, POMT2, POMGNT1, LARGE, GTDC2, B4GAT1, B3GALNT2, DPM(1,2,3), DOLK, POMK, GMPPB, FKTN, FKRP, ISPD, TMEM5* and *DAG1* that all encode a series of enzymes involved in glycosylation of α-DG (*4,5*).

Hypoglycosylation of α-DG can lead to dystroglycanopathies including progressive muscular dystrophy and eye involvement. First line of α-DG glycosylation is catalyzed by protein O-mannosyltransferase family (PMT), O-mannosyltransferase 2 protein and its homolog O-mannosyltransferase 1. PMTs were originally discovered in yeast and tend to be evolutionarily conserved. Co-expression of *POMT1* and *POMT2* enzymes is essential for sufficient enzymatic activity.

O-mannosyltransferase 2 protein, an integral ER membrane protein, is encoded by *POMT2* gene on chromosome 14 (14q24.3). This gene expands to 46 kb of the entire genome and has 21 exons (*2,6,7*).

POMT2 mutations can result in three distinct forms of muscular dystrophy-dystroglycanopathies, including severe congenital muscular dystrophy with brain and eye involvement (type A2, formerly named Walker-Warburg Syndrome (WWS) or muscle-eye-brain disease, MIM number# 613150), a less severe congenital muscular dystrophy with mental retardation (type B2, MIM number# 613156) and mild adult onset limb-girdle muscular dystrophy (type C2, MIM number# 613158) (*8*).

2. Materials and Methods

The patient was 10-year-old presented with poor growth, microcephaly, mental retardation, muscle weakness, contractures and movement and speech impairment. She had an older sister (11-year and 6-month- old) presenting with similar symptoms. Both children were the product of full term C/S delivery with normal birth weight, head circumference and normal APGAR scores.

Brain computerized tomography (CT) findings included periventricular calcification foci, ventricular dilation, cerebral atrophy and microcephaly in patient I (Figure1A, 1B).

Other physical examination indicated low head circumference in both cases (42 cm, < 2.5 percentile in younger and 43 cm, < 5 percentile in older sister). Face muscle weakness with severe drooling was obvious. They showed stiff neck, scoliosis and chest deformity (Figure 1C) and additionally, the younger sister had a sign of hip dislocation. Ophthalmologic studies showed astigmatism in both siblings, while the younger one had strabismus eyes with normal vision. The viral markers of both cases were negative. Elevated serum creatine kinase and seizures were reported in both cases.

Whole exome sequencing (WES) was performed on younger affected sib in order to capture, enrich and sequence all exons of protein coding genes in addition to other critical genomic segments. Next generation sequencing was carried out using illumine Hiseq 2000 machine to sequence 100 million reads approximately and standard illumine protocol for paired-end 99 nucleotide sequencing. The test platform assessed > 95% of the target regions with sensitivity of above 99%.

The next generation sequencing (NGS) data was analyzed using various online Bioinformatics tools. WES reads was aligned against human genome using BWA aligner and genome variants were identified by GATK, which were annotated with the use of ANNOVAR software. In order to confirm pathogenic variants, standard bioinformatics software such as CADD-Phred, SIFT, Polyphen, Phastcons, Mutation Assessor, I-Mutant 2.0, and Mutation taster were used.

The novel pathogenic variant should be confirmed by Sanger sequencing in the next step of investigation. Whole blood samples of all family members were collected in EDTA tubes to extract DNA using QIAamp DNA blood Mini kit (Germany) according to protocol. The genomic DNA concentration was assayed by NanoDrop One (Thermoscientific, USA) and stored at -20°C until use.

Polymerase chain reaction (PCR) and Sanger sequencing were then performed on patients, non-affected sister, parents and one of their cousins, in

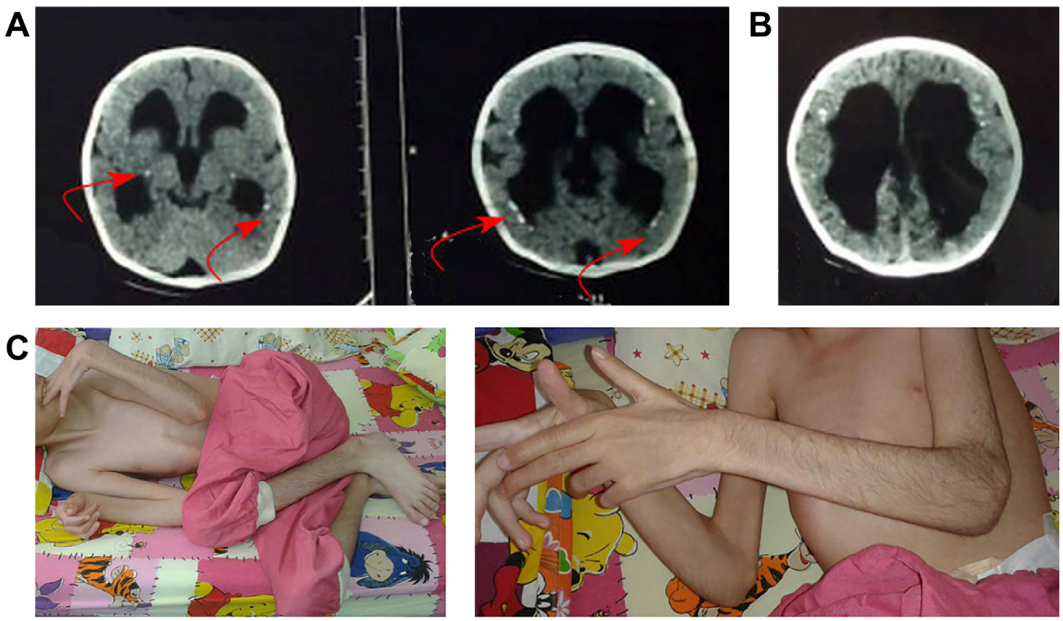

Figure 1. Brain CT scan. (A) Numerous periventricular calcified nodules (red arrows). **(B)** Periventricular Dilation. **(C)** Clinical presentation: The proband was 10 years old with poor growth, contractures, movement impairment, severe drooling, scoliosis and chest deformity.

both directions on the resulting PCR products using ABI BigDye terminator cycle sequencing kit (Applied Biosystems®, USA).

3. Results and Discussion

NGS data analysis of younger patient revealed one extremely rare deleterious homozygous missense mutation in *POMT2* gene (NM_013382: exon9: c.1106G>A: p.R369H).

Mutations of *POMT2* gene are shown to cause dystroglycanopathies with various organ involvements and severity. The mutation was confirmed using Sanger sequencing. Sanger confirmation revealed two mutant homozygous alleles in two affected siblings and two wild type alleles in healthy sib. Their parents and cousin were heterozygous carriers of mentioned mutation. Therefore, the inheritance pattern must be autosomal recessive and the mutation is segregated in this family (Figure 2).

According to mutation taster results, this variation is disease causing leading to a single base exchange. 1106 G>A nucleotide alteration affects amino acids sequence (The replacement of Arg by His) (*9*). Conservation analysis of this amino acid was evaluated using comparative multiple amino acid sequence alignment of POMT2 protein across various animal kingdoms and revealed a high level of Arginine conservation during evolution (Figure 3A). Mutation taster uses Phastcons values to determine the grade of nucleotide conservation. Phastcons is a program for evaluating conserved elements by multiple alignment of genome sequence of 46 different species. The scores closer to 1 reflect a higher conservation of the nucleotide. In this case Phastcon score was 0.998, reflecting strong nucleotide conservation.

I-Mutant 2.0 is a tool to predict protein stability changes upon various point mutations. As figure 3b shows protein stability will be decreased upon replacement of Arginine 369 with Histidine. The result also shows the effect of other amino acid substitutions in the 369 residue (Figure 3B).

STRING online software v11.0 shows *POMT2* gene in close co-operation with various partners, which have critical functions in glycosylation of α-DG (Figure 4).

O-Man glycosylation pathway was originally discovered in yeast. Mammalian homologs were later recognized as POMT1/POMT2. Co-expression of these two multi-pass transmembrane enzymes is essential for the catalysis of the first step in O-Man glycosylation of α-DG. Dolichol phosphate-β-D-mannose (Dol-P-Man) is used as a donor substrate to catalyze the attachment of α-linked mannose to serine and threonine residues in ER lumen and further elongation in the Golgi apparatus. This process is critical for accurate structure and function of α-DG (*2*).

Yoshida *et al*, identified muscle-eye-brain disease with defective O-mannosyl glycan in 2001 and introduced a novel pathological pathway for muscular dystrophy and neuronal migration disorders (*10*). Investigations have shown that mammalian POMT2 is an ER integral membrane protein and is expressed in all tissues but predominantly in testis (*11*).

POMT2 mutations were first identified in severe

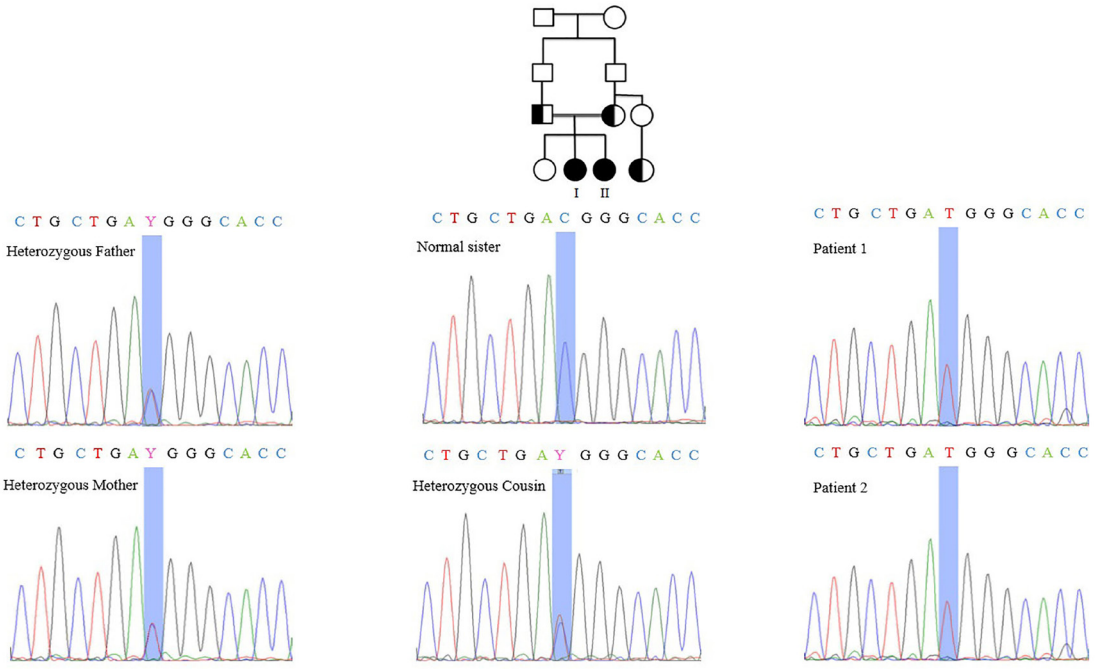

Figure 2. Family pedigree and Sanger sequencing chromatogram. Both Parents and cousin are heterozygous for c.1106G>A. Non-affected sister and two affected sisters are homozygous for wild type and mutant alleles, respectively. The autosomal recessive pattern of inheritance seems obvious in the pedigree.

Figure 3. (A) Comparative amino acid sequence alignment of POMT2 protein across various animal kingdoms shows the conservation of arginine amino acid during evolution. The conserved residue is marked in the rectangle. (B) I-Mutant V2.0 analysis of protein stability revealed the reduction of POMT2 protein stability upon the substitution of Arg 369 with His. This program also predicted the effect of Arg 369 substitution with all other amino acids on protein stability. As is clear in the picture, most of the alterations can reduce protein stability.

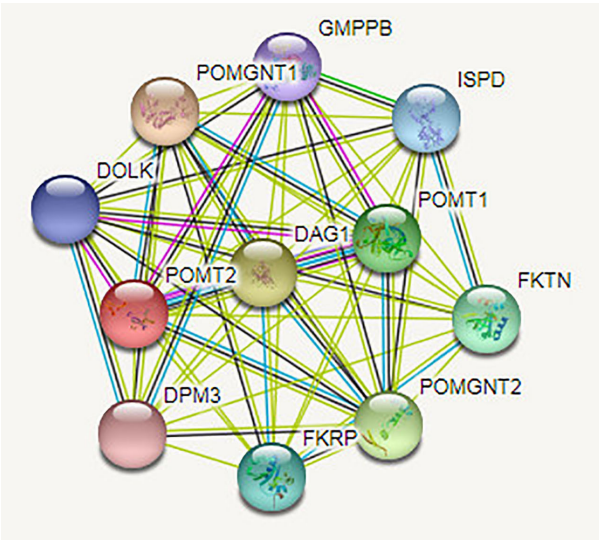

Figure 4. STRING online software v11.0 shows the close interactions of various proteins in the process of α-DG glycosylation. All mentioned genes play a role in coding the series of enzymes involved in glycosylation of α-DG.

WWS (currently labelled as severe congenital muscular dystrophy with brain and eye involvement). However, various clinical severities of dystroglycanopathies from severe congenital muscular dystrophy to mild adult onset limb-girdle muscular dystrophy have been reported so far.

Dystroglycanopathies are an autosomal recessive group of muscular dystrophy, in which functional glycosylations of α-DG decline or are completely absent. α-DG, a critical extracellular component of dystrophin-glycoprotein, is glycosylated by a series of proteins and disruption of each step will interfere with protein function and the attachment to ECM ligands such as laminin, neuexin and agrin (*12,13*).

Here, a novel rare mutation of *POMT2* was identified in a family with two affected siblings (NM_013382: exon9: c. 1106G>A: p.R369H). Sanger sequencing was performed on all family members to confirm NGS data results. The mentioned mutation was confirmed in the family as an autosomal recessive pattern of inheritance. The homozygous A mutant allele in two patients, the homozygous G wild-type allele in normal sib, and the heterozygous G/A alleles in their parents and cousin were confirmed.

c.1106G>A single nucleotide variant was reported in dbSNP with reference SNP: rs398124260 and T allele global minor allele frequency is 0.0000 on average. It is classified as an unknown significant variant and no patients have been identified for this variant so far.

Using STRING software V11.0 to identify *POMT2* functional partner, revealed its interaction with all O-mannosylation biosynthesis genes. Defective function of each enzyme due to gene mutation can lead to distinct forms of dystroglycanopathies with a broad spectrum of clinical phenotypes that range from severe congenital muscular dystrophy with brain and eye

involvement to a benign limb girdle form. In the current study, Muscular and CNS abnormalities in addition to ocular involvement were apparent in two patients, early in life. These characteristics help us to classify mentioned patients in type A2 of muscular dystrophy-dystroglycanopathy (severe congenital muscular dystrophy with brain and eye involvement).

Homozygous c.1106G>A mutation in two affected sibs leads to substitution of the evolutionary highly conserved arginine 369 with a histidine and various bioinformatics analysis provide evidence of pathogenicity for the mentioned mutation. This amino acid substitution (Arg369His) is located at the highly conserved domains mannosyl-IP3R-RyR (MIR), which encompasses position 334-514 in the protein. This domain was first identified in three proteins, mannosyltransferase, Inositol 1,4,5-trisphosphate receptor (IP3R) and Ryanodine receptor (RyR), and so named MIR. It had been reported in fungi that a hydrophilic region of PMT facing the ER lumen played an important role in protein function and it encompassed the triplet of the MIR domain. However, the exact functions of MIR domain have not been completely identified (*14,15*). Two pathogenic missense mutations (p.V373F and p.G353S) were reported in the vicinity of the current mentioned mutation (R369H), which are located in MIR domain (*8,16*). On the basis of this evidence it can be concluded that this mutation is pathogenic in the mentioned family.

In conclusion, this study revealed the pathogenicity of a single nucleotide variant in *POMT2* gene (rs398124260), which expands the knowledge of pathogenic mutations of *POMT2* to identify the underlying cause of muscle problems and such studies can be useful in performing genetic counseling and prenatal diagnosis accurately.

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Brief Report

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A novel frame shift mutation in *STIM1* gene causing primary immunodeficiency

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SUMMARY Immunodeficiency 10 is an autosomal recessive disorder presenting with iris hypoplasia, muscular hypotonia and nonprogressive myopathy, recurrent bacterial infections, autoimmune hemolytic anemia, hypohidrosis and nail dysplasia caused by the mutation of stromal interaction molecule 1 gene (*STIM1*). Herein, we present a new case of *STIM1* mediated immunodeficiency, carrying a novel frameshift mutation. Our patient presented with nephrotic syndrome, hypotonia, myopathy, recurrent bacterial infections, thrombocytopenia and autoimmune hemolytic anemia. She is now 23 months old and is on steroid, cyclosporine and monthly IVIG. She has had no recent significant infections and is receiving rehabilitation therapy to improve her motor skills. Rare genetic syndromes should be suspected in patients of consanguineous parents, who present with a set of different manifestations. Gathering all the patient's manifestations together and looking them as one disease should be encouraged.

Keywords genetics, immunology, recurrent infections, pediatrics

1. Introduction

Immunodeficiency 10 is an autosomal recessive disorder presenting with iris hypoplasia, muscular hypotonia and nonprogressive myopathy, recurrent bacterial infections, autoimmune hemolytic anemia, hypohidrosis and nail dysplasia (*1*). The genetic base of this syndrome is due to the mutation of stromal interaction molecule 1 gene (*STIM1*) located on chromosome 11p15.4. The *STIM1* gene provides instructions for encoding STIM1 protein, which is involved in controlling the entry of positively charged calcium ions into the cells through calcium-release activated calcium (CRAC) channels when levels of the ions are low (*2*). These proteins have been showed to be localized to the endoplasmic reticulum and the plasma membrane, serving as Ca sensors and, also, mediators for Ca influx (*3*).

Here we report a case of immunodeficiency 10 presented with nonprogressive myopathy and hypotonia, and nephrotic syndrome.

2. Patients and Methods

Our patient is a 2-year-old girl of consanguineous parents. During early infancy, her parents noticed sucking and swallowing discoordination of their daughter. In her monthly pediatrician visits, mild degrees of delay in motor developmental milestones was observed. At 7 months old, she developed sudden onset fever accompanied with lethargy, poor feeding and generalized edema; work-ups showed nephrotic range proteinuria along with hyperlipidemia and hypoalbuminemia with normal renal function and normal liver enzymes. Kidney biopsy pathology was suggestive of classic features of Focal Segmental glomerulosclerosis, Not Otherwise Specified (NOS) variant. Prednisolone and Enalapril was started for her and, fortunately, due to her good response to the prescribed medicine, proteinuria abated after few months; therefore, steroid dosage was tapered off. One year later when she was 17 months old, generalized petechial and ecchymotic rashes were appeared on her body. Lab data revealed thrombocytopenia, normal hemoglobin (Hb) level and normal white blood cell (WBC) count. Erythrocyte sedimentation rate (ESR) was 47 mm/hr and C-reactive protein (CRP) was 7 mg/dL. Due to previous history of kidney involvement and nephrotic syndrome, lupus serology and complements were checked which were all normal.

She underwent bone marrow aspiration and biopsy which revealed normocellular marrow and Flow cytometric results for Bernard-Soulier syndrome and Glanzmann's thrombasthenia were both negative. Therefore, she was diagnosed as Idiopathic Thrombocytopenic Purpura (ITP) and methylprednisolone was administrated for

her, but no satisfactory clinical response was detected. Few days later, she developed sudden onset hemolytic anemia with positive indirect coombs test ,normal G6PD level, reticulocyte count: 10% and erythropoietin level > 750 IU/L. Considering ITP and hemolytic anemia, possibility of Evans' syndrome was deliberated and due to ineffectiveness of steroid pulse administration, Rituximab was initiated 375mg/m^2 weekly for four consecutive weeks. Fortunately, our patient had good clinical response after the fourth dose of Rituximab, with normal platelet and Hb level; moreover, she did not have proteinuria any more. In Parallel to these manifestations and considering the patient's history and evidences of repeated infections including otitis media and urinary tract infection, consultation with an expert pediatric immunologist was measured and cluster of differentiation (CD) marker flow cytometry, Dihydrorhodamine (DHR) test and immunoglobulin levels were requested. CD flow cytometry demonstrated low CD19, CD20 and CD8 and high CD3 and CD4. However, as the results could be attributed to rituximab, repeating the work-ups later was recommended. DHR was negative and immunoglobulin levels were in normal range. Therefore, steroid dose was tapered gradually.

At 19 months old, she was hospitalized due to coughing, respiratory distress and episodes of lip cyanosis. Chest x-ray revealed bilateral ground glass appearance but no evidence of infectious process was found in her lab data (normal CRP and procalcitonin). Spiral Chest CT scan demonstrated bilateral patchy infiltrations, diffuse ground glass appearance and mosaic pattern in middle lobe and lingula more likely associated with vasculitis. Her desired clinical response to methylprednisolone pulse was also corroborative of vasculitis rather than infections. After 1 week of respiratory care and mechanical ventilation, she was extubated (however, the patient was still oxygen dependent). Bronchoscopy was also done which documented swallowing discoordination by fiberoptic endoscopic evaluation of swallowing (FEES) and malacia of larynx, trachea, and left main stem and revealed mucosal plugging in right main stem and right lower lobe. Microscopic examination of the bronchoalveolar lavage was negative for bacterial and fungal infections. During PICU admission, she was noticed to have mid-dilated pupils, which were both non-reactive to light stimulation. Hence, brain MRI, MR Angiography and MR venography were requested which revealed diffuse microbleeding in different part of both cerebral hemispheres, suggestive of vasculopathy. Later, with ophthalmologist consult, it was specified that the patient has iris hypoplasia (partial aniridia) and non-reactive pupils. As stated earlier, our patient had mild motor skill developmental delay and hypotonia; therefore, electromyography was performed for her, reported mild myopathic process, and found no evidence of peripheral neuropathy.

3. Results and Discussion

Considering her delayed motor skill development, renal involvement, history of thrombocytopenia, recurrent infections and autoimmune disorders including autoimmune hemolytic anemia and vasculitis, something syndromic were suspected; hence, whole exome sequencing (WES) was ordered which revealed a novel homozygous mutation in *STIM1* gene (with 11p15.4 cytogenetic location) and based on our patient clinical picture, "immunodeficiency 10 syndrome" was suggested which was compatible with her clinical manifestations.

Immunodeficiency 10 is a rare genetic disorder presenting with variable features and severity, most commonly recurrent episodes of infections, nonprogressive myopathy and muscular hypotonia (*4,5*). The defective known gene associated with this kind of primary immunodeficiency is *STIM1* which is located on Chromosome 11 and at least three loss-of-function mutations are detected to have a pivotal role in pathogenesis of immunodeficiency 10 (*6,7*). These mutations result in impaired calcium ions influx into cells which lead to defects in gene expression, cell growth and division, and immune dysfunction particularly NK-cell and T-cell inactivation (*8,9*). Immunodeficiency 10 syndrome is of disorders with autosomal recessive heritance with rare allele distribution throughout population (*10*); so, this pattern suggests a common ancestor to be present between parents. All reported cases with this immunodeficiency (Table 1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=66*) had positive parental consanguinity, as our patient had.

Based on the determining role of *STIM1* gene in biologic intracellular and extracellular cascades, its manifestations usually emerge early, in neonacy or infancy (*1*). The clinical manifestations and the extent of immune system dysfunction mainly depends on the type of the mutation that affected the *STIM1* gene. The first three reported patients (based on Table 1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=66*) had complete alteration of *STIM1* gene functions due to homozygous nonsense mutations in the *STIM1* gene (E136X mutation) and therefore, significantly reduced STIM1 protein production, which resulted in severe immunologic features including immunodeficiency, autoimmune disease, and myopathy (*5*). The first two siblings died early in age, one at the age of 18 months from encephalitis and the other at the age of nine years from complications of hematopoietic stem-cell transplantation (HSCT); however, fortunately, the other sibling survived after HSCT and there are no immunological or infectious complications. He does suffer from moderate muscular weakness but he is completely ambulatory and does not need a wheelchair or other support. The fourth reported patient, died at the age of 2 years and four months from severe pulmonary

infection, was shown to have a homozygous loss-of-function point mutation in STIM1 alleles, determined by studying EBV-transformed B cells (EBV-B cells) from the patient (the only material remained from the deceased patient). This point mutation leads to a complete impaired production of STIM1 protein and therefore an impaired store-operated Ca^{2+} entry (SOCE) (*11*). The next two siblings, both, with homozygous R429C point mutation however had residual *STIM1* gene expression, the SOCE was completely depleted leading to pronounced defect in T-cell activation and NK cell function with partial response to viral infections (*12*). One sibling died in 21 months due to suspected sepsis but the other one is alive now at the age of 14-year-old after two times of HSCT (rejected the first graft).

Patients 8 and 9 were reported to have a hypomorphic homozygous missense *STIM1* mutation (mutation c.494C>A in exon 4 resulting in p.165P>Q) which get rise to reduced but residual STIM1 protein production and SOCE which is sufficient for some levels of T-cell proliferation and activation (*10*); therefore, this could explain different immunologic features of these patients comparing to the aforementioned reported cases that these two siblings had no immune cytopenia and no episodes of lymphoproliferation. These two patients would have such a prolonged survival without HSCT. They are both alive now- the boy (patient 8) suffers from severe colitis and the girl (patient 9) have recurrent episodes of skin infection. Likewise, the patient 10 and 11 (cousins) with missense homozygous STIM1 mutation (p.L74P- c.221T>C) was reported to have no permanent immune cytopenia and overt clinical immunodeficiency despite severe SOCE impairment (*13*). Another extraordinary feature seen in these two patients is that in contrast to all aforementioned reported patients, they were not affected with any level of myopathy. In our patient, a novel homozygous frameshift deletion mutation in exon 7 (p. T273fs- c.818_831del) was reported.

Recurrent severe infections are of characteristics of *STIM1*-associated immunodeficiency. Despite bacterial infections such as sepsis and upper respiratory tract infections, these patients due to obvious defective T- and NK-cell function have a pronounced susceptibility to intracellular infections and particularly to viruses (*14*). As demonstrated in Table 1 (*http://www.irdrjournal.com/action/getSupplementalData.php?ID=66*), viral infections especially Herpesviridae infections (Herpes Simplex Virus, Varicella Zoster Virus, Cytomegalovirus, Epstein Barr virus, and Human Herpes Virus 8) are of pathogens of concern. In 2009, Picard *et al*. reported three patients from one kindred with immunodeficiency 10 syndrome. The older child had caught CMV infection at 1 month of age and two episodes of chickenpox (VZV infection) before 4 year old. The middle child, also, had been reported to contract EBV infection and enteroviral encephalitis (*5*). In another case-report by Minji Byun *et al*., their patient had been found to have classic Kaposi sarcoma caused by HHV8 infection (*11*). In 2012, Feske *et al*. reported two sisters with *STIM1*-mediated immunodeficiency, which both of whom had suffered recurrent viral pneumonias, recurrent HSV stomatitis, and chronic EBV and CMV viremia (*12*).

Whereas nephrotic syndrome has not been directly included in immunodeficiency 10 entity (*1*), nephrotic syndrome was present in our patient and some of other patients with *STIM1*-mediated immunodeficiency. Nephrotic syndrome was detected in our case after presenting with lymphadenopathy, poor feeding, proteinuria, and edema at 7 month old. In a case-report by Picard *et al*., one of patients with immunodeficiency 10 syndrome had severe nephrotic syndrome (*5*) and in another one by Stefan Feske *et al*., one of patients with *ORA11* and *STIM1*-mediated immunodeficiency had suffered from nephrotic syndrome (*6*). Maybe in future with advance of scientific methods and research, the role of *STIM1* gene in underlying pathways involved in nephrotic syndrome pathogenesis become uncovered. Of another manifestations in primary immunodeficiencies is vasculitis, either in internal organs such as brain (*15*), and lung (*16*) or in external organ, the skin (17). The most common specific vasculitis found in patients with primary immunodeficiency has been reported to be CNS vasculitis (*18*). CNS vasculitis was observed in our patient based on some evidences of diffuse microbleeding in both cerebral hemispheres. In the same way, both lungs especially middle lobe and lingula had been involved by vasculitis based on evidences of patchy infiltrations, diffuse ground glass appearance and mosaic pattern reported in CT scan.

To sum up, it would be of great importance to remind that however, this genetic disorder has its complications, but with several measures, their life quality and life expectancy would be improved. Using steroids would be protective against nephrotic syndrome and autoimmune processes including autoimmune hemolytic anemia, immune thrombocytopenia and vasculitis and since steroids may have long-term adverse sequelae such as neurocognitive impairment, stunting, obesity, cardiac dysfunction and infertility (*19*), steroid sparing agents could be administrated, permitting partial withdrawal of glucocorticoids and reducing glucocorticoids' adverse effects (*20*). Use of HSCT for those with moderately to severely suffered immune system, would be helpful in immune system improvement, preventing fatal infections (*21*). Immunization is also another effective choice that can be measured in patients with primary immunodeficiencies (*22*). Fortunately, their myopathy is nonprogressive (*1*); so, with occupational therapy and physical therapy, their ambulation would improve (*23*). Our patients is now 23 months old and is on steroid and cyclosporine as an steroid sparing agent as she has developed signs of steroid toxicity in appearance. She has had no recent significant infection and is receiving

rehabilitation therapy to improve her motor skills. Reviewing the previous articles on this rare genetic disorder, we initiated treatment with monthly intravenous immunoglobulin (IVIG) for her.

4. Conclusion

Rare genetic syndromes should be suspected in patients of consanguineous parents, who present with a set of different manifestations. Gathering all the patient's manifestations together and looking them as one disease should be encouraged. Such approaches are the key to earlier identification of congenital genetic disorders and will lead to awareness of the underlying problem of the patient instead of symptomatic therapy, and by reviewing the past reported articles in this regard; stronger decisions will be made about the course of the disease, recommended medications and, the patient's future, and prognosis.

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Case Report

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Fragile X associated neuropsychiatric disorders in a male without FXTAS

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SUMMARY Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability and autism spectrum disorder. In most cases, it is due to an expansion of the CGG triplet to more than 200 repeats within the promoter region of the *FMR1* gene. In the premutation (PM) the trinucleotide is expanded to 55-200 repeats. PM carriers can present with disorders associated with the PM including fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated ovarian insufficiency (FXPOI). Recently fragile X-associated neuropsychiatric disorders (FXAND) was proposed as an umbrella term to include the neuropsychiatric disorders that are more prevalent in PM carriers compared to the general population such as anxiety, depression, chronic fatigue, alcohol abuse, and psychosis, among others. The patient in our study was evaluated by a team of clinicians from the University del Valle in Cali who traveled to Ricaurte, a Colombian town known for being a genetic geographic cluster of FXS. A detailed medical history was collected and complete physical, neurological and psychiatric evaluations were performed in addition to molecular and neuroradiological studies. We report the case of a 78-year-old man, PM carrier, without FXTAS whose main clinical presentation consists of behavioral changes and psychosis. Brain imaging revealed white matter lesions in the periventricular region and mild cerebral atrophy. Although anxiety and depression are the most common neuropsychiatric manifestations in PM carriers, it is important to perform a complete psychiatric evaluation since some patients may present with behavioral changes and psychosis.

Keywords fragile X mental retardation 1 gene, fragile X-associated neuropsychiatric disorders, premutation, FXAND, psychosis

1. Introduction

Fragile X Syndrome (FXS) is the most common inherited cause of intellectual disability and autism spectrum disorder (ASD) and is caused in the majority of cases by an expansion of the CGG repeat to > 200 repeats, in the 5' untranslated region of the fragile X mental retardation (*FMR1*) gene on the X chromosome (Xq27.3). This expansion, called the full mutation, leads to methylation of the promoter and low or absent levels of the *FMR1* protein (FMRP) (*1*). Individuals with FXS present with intellectual disability of varying degrees and, approximately 50-60% present with ASD. Common phenotypical features include an elongated face, prominent ears, and macroorchidism in males (*2*). The normal number of CGG repeats is < 40 repeats, 41-54 CGG repeats are considered the gray zone, and 55-200 is considered the premutation (PM) (*3*). PM carriers are known to develop *FMR1* associated disorders such as fragile X-associated tremor/ataxia syndrome (FXTAS) which is a late-onset neurodegenerative disease with varying clinical manifestations including intention tremor, cerebellar ataxia, neuropathic pain, parkinsonism, memory, and executive function deficits among others (*4*). Fragile X-associated premature ovarian insufficiency (FXPOI) is another premutation disorder in approximately 20% of women with the premutation and is characterized by amenorrhea at an age earlier than 40 years (*5*).

Over time, neuropsychiatric manifestations in PM carriers with and without FXTAS have been reported including depression, anxiety, attention-deficit/hyperactivity disorder (ADHD), and insomnia (*6-9*). A new clinical term has been proposed in order to raise

awareness of the neuropsychiatric manifestations in PM carriers, fragile X-associated neuropsychiatric disorders (FXAND) (*6*). Although some studies have not found an increased risk of neuropsychiatric disorders in carriers (*10,11*), the majority of controlled studies have found significantly higher rates of neuropsychiatric disorders in carriers including depression, anxiety, ADHD, social phobia, chronic fatigue and personality traits such as those present in obsessive-compulsive disorder (OCD) (*12-20*). A recent blinded study from a large population associated with a health care program, Marshfield, demonstrated several psychiatric problems at a much higher rate in premutation carriers compared to controls (*21*). An increase in alcohol consumption and psychoactive substance abuse among PM carriers has been reported which is detrimental and has been shown to accelerate neurodegeneration (*22-25*). Since the proposal of FXAND as an umbrella term for neuropsychiatric manifestations in PM carriers, there have been two case reports published reporting patients who presented mainly with behavioral and psychiatric manifestations exemplifying FXAND as a condition that is separate from the other well-known premutation disorders (*26,27*).

Here, we report the case of a male PM carrier without FXTAS whose primary presentation consists of neuropsychiatric manifestations and therefore contributes to cases of PM carriers who could be categorized under FXAND. To date, only two cases that exemplify FXAND have been published. This case adds to the varied neuropsychiatric manifestations patients present with, including psychosis.

2. Case Report

This patient was evaluated in one of the medical brigades done in Ricaurte, a Colombian town known for being a genetic geographic cluster of FXS (Saldarriaga 2018). A detailed medical history was collected and physical, psychiatric, and neurological examinations were completed. The Scale for the Assessment and Rating of Ataxia (SARA) (*28*) and the Fahn-Tolosa Marín Clinical Rating Scale for Tremor (FTM) (*29*) were applied for the evaluation of clinical signs of FXTAS. Genetic testing results were obtained from previous research work done by Saldarriaga and colleagues in 2018 (*30*). The patient signed an informed consent to have his case history and neurological imaging published. Here, we describe the patient's medical history, neuropsychiatric symptoms, and findings on medical and imaging evaluations.

This is the case of a 78-year-old man, diagnosed as a PM carrier with 61 CGG repeats, who presented with a longstanding history of alcohol abuse, irritability and in the past 3 years delusional jealousy, depression and aggressive behavior. His birth history was unremarkable and he didn't present any medical condition during childhood. He did not report any learning difficulty at school nor problems with attention or ADHD symptoms.

At the age of 30, he started working as a boatman and began drinking alcohol almost daily for the subsequent 38 years. He reported he regularly drank 2-5 bottles (375 mL) of brandy or 20 beers throughout the day. He would on some occasions spend all his salary on drinking during payday. He experienced anxiety only on the days when he didn't drink alcohol. He didn't feel guilty about his alcohol consumption but was asked by doctors and family members to reduce consumption. He stopped drinking at the age of 68 when he was diagnosed with hypertension and was advised to quit his drinking habits. He denied the consumption of any nicotine product and denied the consumption of any psychoactive substance.

Three years ago he started having delusional jealousy with fixed ideas about his wife, a 71-year-old woman, being unfaithful, and having secret codes with her lovers at night. He would awaken at 1 am due to sounds he related to lovers coming for his wife, such as whistles, scratching at the door, and motorcycles. He then started sleeping with a machete (knife), hammers or a club in his nightstand to be prepared in case of an eventual confrontation. He would stand outside the house if he heard a motorcycle at night with his machete believing it was a lover approaching his wife. His family reported that since last year he locks all doors with chains and big locks and blocks the main door with sofas and chairs. He admitted to previously having thoughts about killing his wife due to her alleged unfaithfulness but claimed he no longer has these thoughts. His wife reported that he has never been jealous before nor aggressive. Six months ago they had to call the police because he was aggressive towards his wife and daughters but responded to verbal containment. The family members reported that when he becomes suspicious he threatens his wife. His wife also reported that although it has been years since they stopped having sexual encounters, on occasions he has made extravagant sexual proposals mentioning sexual positions she finds to be strange.

While experiencing the described personality changes, he has also become more irritable and the family stated that he at times becomes angry with otherwise innocent triggers, for example when lights are turned on or when his grandsons take his fruits. They claimed that when he gets upset, he presents with rigidity and clenches his teeth. Although he denies sleep problems, he remains vigilant throughout the night. He usually goes to bed at 8 pm but may wake up at 4 am to monitor for any sounds or movements in the surroundings. He reported recent feelings of hopelessness associated to his current worries. He met diagnostic criteria in DSM5 for depression.

During the interview, he denied any movement-related concerns, cognitive issues, or pain. He reported the onset

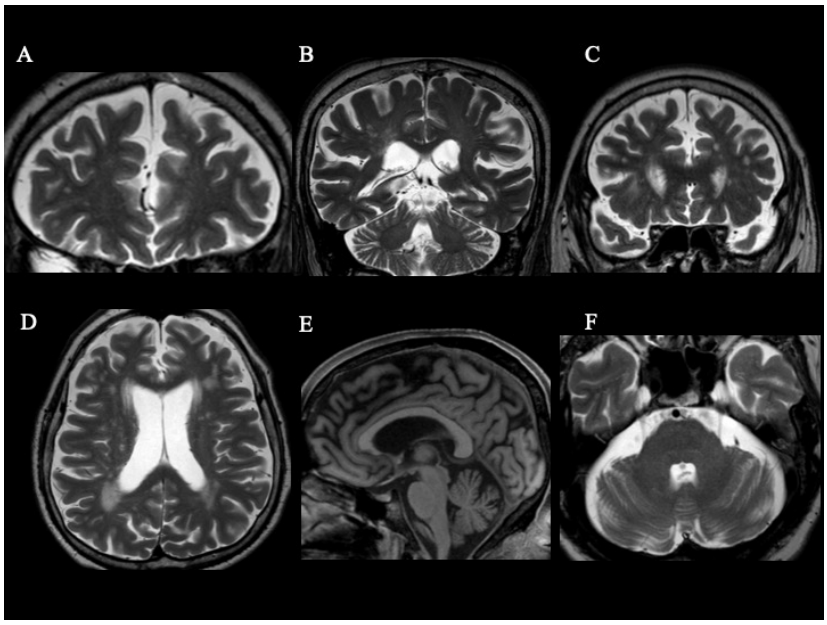

Figure 1. Brain Magnetic Resonance Imaging: T2-weighted images (A, B, C, D, F) and T1-weighted image (E). In this brain MRI, scattered white matter lesions affecting mainly frontal and parietal cortex are observed **(A) (B) (C)**. Periventricular white matter lesions are also observed **(B) (C) (D)**. There are white matter lesions affecting anterior and posterior horns bilaterally **(D)**. Mild-moderate cerebral atrophy is evident in the frontal cortex **(E)**. No white matter changes in the corpus callosum were observed, splenium sign was not present **(E)**. No white matter changes in the middle cerebellar peduncles were observed **(F)**.

of progressive hearing loss 5 years ago that has not been documented clinically nor treated with aides.

Current medications include monthly cobalamin, gemfibrozil, metformin for diabetes type 2 diagnosed in 2001, enalapril, and nifedipine for hypertension. He was prescribed quetiapine 25 mg/night 5 months ago but only took it once, suspending it due to somnolence. Five months ago his wife started giving him non-prescribed amitriptyline 50 mg at night powdered and hidden with his night time coffee. His daughters and wife reported that since he has been taking amitriptyline, he has been less aggressive, sleeps better and has stopped sleeping with a weapon next to him, although he has continued to lock the doors at night.

On neurological exam, there was no tremor with finger to nose touching, no rest tremor was evident and he was able to perform tandem gait without difficulty. He had normal evaluation when applying both SARA, score 1/40, and FTM scales. Vibration and pinprick evaluation were normal in all extremities. He did not have any primitive reflexes and his strength was normal in all extremities. There was bilateral hearing loss which was more prominent in his right ear. He scored 27/30 in the Mini-Mental State Examination (MMSE). During the examination, there was a pause for coffee when the examiners could observe the patient holding a cup and handling utensils without difficulty and without tremor.

This patient's cerebral MRI showed mild frontal atrophy, mild ventriculomegaly and white matter disease in the periventricular regions (Figure 1). There was no evidence of symmetrical white matter hyperintensities in the middle cerebellar peduncles - (MCP) sign - nor white matter lesions in the corpus callosum. No other abnormalities were observed.

The patient was seen in a medical team evaluation in 2019. He was prescribed quetiapine 25 mg/night and sertraline 50 mg in AM. Due to current traveling restrictions, a telemedicine follow-up was performed. The family reports issues with compliance to medications but improvement regarding his delusional thinking and sleep pattern. This patient does not meet diagnostic criteria for FXTAS and most of his symptoms involve neuropsychiatric problems: a history of alcohol abuse, delusional jealousy, irritability, depression, disinhibition and hearing loss. The hearing loss is associated with the premutation but his other symptoms are all related to FXAND exacerbated by long term alcohol abuse.

3. Discussion

Here, we present a case of an adult male PM carrier with a long history of alcohol abuse, irritability and recent development of delusions and behavioral changes. He does not meet the FXTAS diagnostic criteria in carriers of a *FMR1* premutation, only presenting one minor radiologic criteria (MRI white matter lesions in cerebral white matter) (*4*). Although hearing loss is associated with aging in premutation carriers his main clinical problem list is most consistent with the recently described FXAND umbrella of neuropsychiatric symptoms.

FXAND is a term that encompasses various neuropsychiatric conditions associated with fragile X PM carriers. In this patient his neuropsychiatric

symptoms are also associated with white matter disease on MRI but he does not meet criteria for FXTAS currently, such as intention tremor, cerebellar ataxia, and parkinsonian features (*4*).

Neuroradiological abnormalities have been widely described for PM carriers with FXTAS, consisting of white matter disease in the cerebrum and/or cerebellum, white matter hyperintensities in the middle cerebellar peduncles, known as the MCP sign, and in the splenium of the corpus callosum and brain atrophy (*31*). The MCP sign is present in approximately 60% of male patients with FXTAS and is part of the major diagnostic criteria for this condition (*32,33*). White matter abnormalities in PM carriers without FXTAS, such as in our patient, have also been described and it has therefore been proposed that white matter pathology may be the initial point in the pathophysiology of neurological symptoms of carriers and perhaps puts him at high risk to develop FXTAS in the future (*34-36*).

This patient presented a long-standing history, thirty-eight years, of alcohol abuse. Excessive alcohol consumption has been reported to be a common condition in PM carriers compared to controls (*25*), although some studies have shown alcohol consumption rates to be similar to family control groups (*37*). In 1994 Dorn *et al.* evaluated psychiatric and behavioral problems among fathers of women with the PM and found that the prevalence of alcohol abuse/dependence for male PM carriers was significantly higher compared to controls, exceeding as well the prevalence rate of alcohol abuse/dependence reported for first-degree relatives of known alcoholics in a previous study (*25*). It has been proposed that alcohol abuse and illicit drug use may be common among carriers due to co-occurring anxiety, depression and ADHD (*6,38*). This patient reported anxiety symptoms when not consuming alcohol which may be explained by alcohol withdrawal but may also indicate that his long-standing history of alcohol abuse may have masked an anxiety disorder. It is important to address substance abuse in PM carriers since it has deleterious effects in the brain. Excessive alcohol consumption has a negative effect in white matter integrity, myelination, and promotes a neuroinflammatory state, which aggravates the already present neuronal oxidative stress state present in aging carriers (*6,39*). PM carriers must be advised about the increased rate of neurodegeneration due to excessive alcohol consumption and an increased risk of developing FXTAS (*22,40*).

The main neuropsychiatric symptom reported by the family of this patient was his delusional disorder-jealous type. Psychotic symptoms have been reported at increased rates in PM carriers in some studies, although they have been reported less frequently compared to other disorders such as depression and anxiety. Hessl *et al.* reported a prevalence of 26% of psychoticism/psychosis in males with the PM. They found that elevated levels of *FMR1* mRNA in PM carriers was significantly associated with increases in psychological symptoms, mainly obsessive-compulsive symptoms and psychoticism/psychosis in PM males with and without FXTAS (*41,42*). Our patient additionally presented with disinhibition, irritability, and behavioral changes which have been reported in PM carriers, especially in those with FXTAS (*10,43*). It is important to note that some studies have not found an elevated risk of significant psychiatric symptoms in asymptomatic males with the PM, but this conflicted evidence may be due to different methodological approaches used in the different studies (*25,37*).

Other neuropsychiatric disorders reported in PM carriers without FXTAS include anxiety, which is the most common neuropsychiatric problem encountered in PM carriers, and depression, occurring in approximately 40% of PM carriers (*6*).

Physicians need to be aware of neuropsychiatric symptoms in PM carriers. More studies and case reports are needed in order to delineate and further characterized FXAND. FXAND constitutes a separate entity and it is important for clinicians evaluating a carrier of the premutation to evaluate for psychiatric and cognitive issues, even in the absence of symptoms of the other well-known premutation disorders. FXAND was proposed as an umbrella term in order to raise awareness into the problems that fall under it. To date, there are only two published case reports of patients that exemplify this condition. In the case report by Santos and colleagues, they present a 26-year-old male with the premutation with depression, anxiety, and ASD (*26*). In the case by G Tan and colleagues, they present a female with the premutation and a complex neuropsychiatric history including anxiety, depression, substance abuse, and chronic pain (*27*). This case differs in that the main psychiatric symptom is psychosis manifested as delusional disorder-jealous type, and behavioral changes in an older patient with no signs and symptoms of FXTAS. The patient had been evaluated by psychiatrists and primary care providers who didn't associate his clinical picture to the *FMR1* premutation. It exemplifies a case that can fall under the diagnosis of FXAND as a different condition associated to the premutation that should be considered during the evaluation of a patient with the premutation.

Although the main neuropsychiatric manifestations in PM carriers are anxiety and depression it is important to perform a complete neurologic and psychiatric evaluation since there have been reports, such as in our patient, in which the primary manifestations are psychosis and behavioral changes.

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Case Report

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Efficacy of trazodone for treating paroxysmal sympathetic hyperactivity presenting after left temporal subcortical hemorrhage

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SUMMARY Paroxysmal sympathetic hyperactivity (PSH) is a clinical condition characterized by abnormal paroxysmal surges in sympathetic nervous system activity. PSH is known to occur after severe head injury and hypoxic encephalopathy. Cases of PSH that develop after stroke have been reported worldwide; however, PSH is not commonly reported in the field of stroke research in Japan. Some studies have suggested that gabapentin may improve the symptoms of PSH. To our knowledge, this is the first case report demonstrating the efficacy of trazodone for the treatment of PSH that developed after temporal subcortical hemorrhage. A 49-year-old woman presented to our clinic with mild confusion and sensory aphasia after experiencing left temporal subcortical hemorrhage; a conservative treatment was initiated at our hospital. Immediately upon hospitalization, she developed prolonged consciousness disorder, high fever, tachycardia, malignant hypertension, tachypnea, constipation, and overactive bladder. The patient's symptoms improved after the administration of trazodone. She was diagnosed with PSH after intracranial hemorrhage and was subsequently transferred to a recovery and rehabilitation hospital unit where the oral administration of trazodone continued. Prolonged PSH contributes significantly to the impairment of daily activities in patients with stroke; therefore, early diagnosis and treatment are critical. Here, we report on the efficacy of trazodone as an effective treatment option for improving clinical outcomes and reducing the stay in the stroke care unit.

Keywords paroxysmal sympathetic hyperactivity, temporal subcortical hemorrhage, trazodone

1. Introduction

Paroxysmal sympathetic hyperactivity (PSH) is a clinical condition characterized by abnormal paroxysmal surges in sympathetic nervous system activity. Although the symptoms of PSH have been identified for longer than 60 years, it has had over 31 different names, including dysautonomia, paroxysmal autonomic instability with dystonia, paroxysmal sympathetic storm, sympathetic storm, autonomic storm, diencephalic seizure, and autonomic dysfunction syndrome, to name a few, which makes it very difficult to identify (*1-3*). PSH often occurs after severe head injury and hypoxic encephalopathy, although it is also known to develop after stroke. However, in Japan, limited evidence regarding a connection between PSH and stroke exists (*3-5*). Therapeutic drugs, including morphine, benzodiazepines, beta-blockers, baclofen, gabapentin, and clonidine, are commonly used to suppress PSH. The inadequate therapeutic effect of these drugs necessitates the inclusion of bromocriptine (a dopamine agonist) to the treatment regimen (*3,6-9*). However, evidence suggests that such treatment regimens are therapeutically ineffective (*8*). Moreover, antiepileptic drugs are generally ineffective for treating PSH. Alternatively, multiple papers have reported on the efficacy of gabapentin for treating PSH (*1,2,4,6, 9,10-14*), which is considered to improve the symptoms by controlling the suppressive nerve stimulation (*6*). However, there is few report on the therapeutic effect of sympathetic blockers, *i.e.*, α blockers (*3*). To our knowledge, the efficacy of trazodone for treating PSH that developed after temporal subcortical hemorrhage has not been reported. Here, we describe the case of a patient who developed PSH after left temporal subcortical hemorrhage, which was successfully treated with trazodone.

2. Case Report

A 49-year-old woman presented to our clinic with mild confusion and sensory aphasia after a left temporal subcortical hemorrhage. She had a medical history

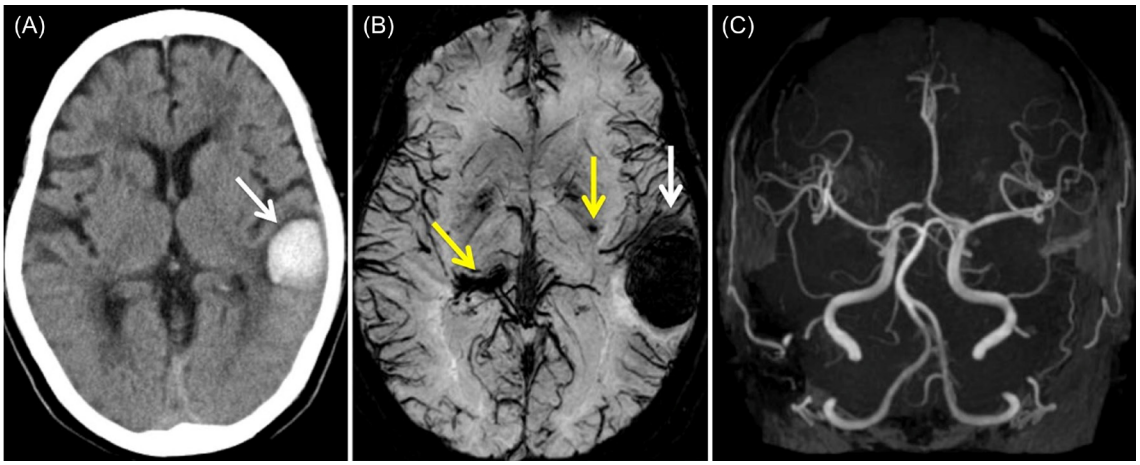

Figure 1. Clinical imaging for stroke signs upon initial presentation. (A) Head plane computed tomography image reveals a left temporal subcortical hemorrhage (white arrowhead). **(B)** Susceptibility-weighted magnetic resonance image shows cerebral microbleeds (yellow arrowhead) in the bilateral basal ganglia but no blood vessel malformations (white arrowhead). **(C)** Magnetic resonance angiography image reveals no aneurysms or blood vessel malformations.

of untreated diabetes only. Neurological assessments revealed no paralysis and sensory disturbances, but mild confusion and sensory aphasia. She scored 5/42 on the National Institutes of Health Stroke Scale; her modified Rankin scale score at admission was 2, and her blood pressure at hospitalization was 201/104 mmHg, indicative of a hypertensive emergency. Electrocardiography revealed sinus tachycardia and blood analyses, including blood cell counts, biochemistry, and coagulation parameters, revealed no aggressive abnormalities requiring treatment. A plain head computed tomography showed left temporal subcortical hemorrhage (Figure 1A), and susceptibility-weighted magnetic resonance imaging showed cerebral microbleeds in the bilateral basal ganglia without any blood vessel malformations (Figure 1B). Magnetic resonance angiography revealed no aneurysms or blood vessel malformations (Figure 1C)).

The patient was administered nicardipine as a conservative treatment for management of her blood pressure; the targeted systolic blood pressure was ≤ 140 mmHg. However, the controls were poor. Immediately upon hospitalization, the patient developed a sudden high fever, accompanied by mass sweating, prolonged consciousness disorder, tachycardia, a significant increase in blood pressure, tachypnea, constipation, overactive bladder. Head computed tomography images showed no enlargement of the hematoma on day 1 of hospitalization, and she was prescribed azilsartan (40 mg/day), amlodipine (10 mg/day), to manage the hypertension.

Electroencephalogram showed no obvious abnormal wave. Based on the series of systemic symptoms experienced, including the autonomic symptoms that were indicative of an intracranial hemorrhage associated with PSH, she was administered trazodone (100 mg/day), beginning on day 15 of hospitalization. As we had experienced a case in which trazodone was effective as a sympathetic blocker for Barré-Lièou syndrome (BLS), based on the post-traumatic sympathetic hyperactivity theory, we hoped that it would also be effective for PSH, which is a similar pathological condition (*3,15*).

Under this treatment regimen, the patient's tachycardia and malignant hypertension improved promptly, and prolonged consciousness disorder, constipation, overactive bladder, and fever gradually improved. However, aspiration pneumonia was complicated at the time of prolonged consciousness disorder, and antibiotic treatment was required temporarily, Because the patient's symptoms improved significantly after the administration of trazodone, she was diagnosed with PSH after intracranial hemorrhage.

By day 21 of hospitalization, her general condition had stabilized, and she was moved from the stroke care unit to the general ward. She was transferred to a separate recovery rehabilitation hospital 50 days after admission, where the trazodone treatment (50 mg/day) continued. Trazodone was gradually reduced and eventually stopped 3 month after initial admission. No relapse of PSH was observed until 6 months after admission, and the patient had a modified Rankin scale score of 2 at the outpatient follow-up examination 10 months after admission. Written informed consent was obtained from the patient for publication of this case report and the accompanying images, and the study design was approved by the appropriate ethics review board.

3. Discussion

To our knowledge, the present report is the first to demonstrate the efficacy of trazodone for the treatment of PSH that developed after temporal subcortical hemorrhage, while there is the reportsof thalamic hemorrhage (*3*). PSH has only recently been defined (*1*), and is characterized by excessive autonomic

symptoms, including high fever, high blood pressure, tachycardia, tachypnea, perspiration, and muscle tone abnormality. PSH occurs after severe brain injury, usually during a state of paroxysmal sympathetic excitement (*11*). Following paroxysmal excitement, the autonomic symptoms typically occur approximately five times a day, each episode lasting approximately 30 min. PSH causes hyperthermia, dehydration, muscle mass reduction, and muscle contracture and has a serious effect on reversion, such as symptom recurrence or prolonged requirement of intensive care unit management, or causes serious secondary sequelae (*6,12-14*). Although these complications can be avoided by early diagnosis and treatment (*7-9,16,17*), the detection of PSH is impossible without any knowledge of the underlying pathophysiology. Previously, the lack of a clear definition and diagnostic criteria resulted in poor understanding of the condition, and moreover, the variations in the symptoms complicated the diagnosis of PSH. In our case, we did not observe an epileptic wave on electroencephalography and non-convulsive status epilepticus was negative. Since the series of her general symptoms resembled post-traumatic sympathetic hyperactivity to the prevailing BLS (*15,18*), we suspected PSH, and the diagnosis was confirmed once the symptoms met the known diagnostic criteria (*1*). We previously reported on the efficacy of trazodone for BLS (unpublished observations), and as PSH, similar to BLS, is a sympathetic condition, we assumed that trazodone use would be effective in this case (*3,15*).

Currently, there are two theories (*12,17*) that explain the pathophysiology of PSH. Specifically, it is theorized that the decoupling of the sympathetic excitement center of the hypothalamus and brainstem from the control of higher functioning brain regions, such as the cerebral cortex, results in a state of sympathetic excitement. The second theory suggests that when the midbrain or brainstem, regions that control afferent stimulation in the spinal cord, is injured, it becomes impossible to suppress the stimulation, which leads to hyperexcitability in the afferent pathway of the spinal cord. Currently, the latter theory has greater support (*12,17*).

Research has shown that PSH most commonly occurs in younger individuals; indeed, Hughes *et al.* (*17*) reported that the mean age of patients with PSH was 33.6 years, which is consistent with the age of our patient. Few reports of stroke-associated PSH in Japan have been published (*1,3,18*). Although the reason for this is unknown, awareness and understanding of the pathological condition are poor; therefore, it is possible that the occurrence of this condition has not been accurately reported.

The patient was diagnosed with PSH associated with intracranial hemorrhage. Trazodone, a well-known antidepressant drug widely used worldwide, works as a 5-hydroxytryptamine (5-HT2) and α1-adrenergic receptor antagonist and a serotonin reuptake inhibitor (*19*). Symptoms improved after trazodone administration to patients with BLS and PSH, which are considered similar pathological conditions involving sympathetic hyperactivity, and we assume that trazodone's alpha blocking action was responsible for this effect. It was considered that this action could suppress sympathetic hyperactivity (*3,15*). There have been few reports of trazodone side effects, such as 270 cases of drowsiness (3.64%), 215 cases of dry mouth (2.90%), and 134 cases of constipation (1.81%) (*20*) and it is relatively safer for use in the elderly. In our case, trazodone, which is often used for treating depression because of its mechanism related to alpha-adrenoceptor inhibition (*19*), was effective for treating PSH.

Recognition of PSH is crucial for the rapid recovery of patients with traumatic brain injury or stroke, even when they are still in intensive care units. It is also important to reduce complication rates and the length of hospitalization (*7*). PSH is a common syndrome, and failure to recognize this condition is associated with increased morbidity and mortality, higher health costs, longer hospitalization, and poorer outcomes (*4*). In the present case, we believe that the early diagnosis and treatment of PSH, considering the symptoms of paroxysmal sympathetic hyperactivity that occurred after intracerebral hemorrhage, contributed to the overall short duration of hospitalization (in the stroke care unit and general hospital ward), while the diagnosis was delayed than the previous report (*3*). In the future, it is desirable to accumulate more cases to conclusively comment on the efficacy of trazodone for PSH.

In conclusion, trazodone was effective in treating PSH in our case, and its use may reduce the overall duration of hospitalization and improve clinical outcomes in affected individuals. Trazodone can be an effective drug for PSH treatment, although further evidence accumulation from a larger number of cases is needed.

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Case Report

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Successful early diagnosis and treatment of non-convulsive status epilepticus-induced Takotsubo syndrome

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SUMMARY Takotsubo syndrome (TTS) is often preceded by emotional or physical stress. Epileptic seizures have been described in more than 100 TTS cases. Due to the lack of typical symptoms, seizure-induced TTS can be overlooked. Here, we describe a rare case where TTS induced by non-convulsive status epilepticus (NCSE) was diagnosed early and successfully treated. An 82-year-old man presented to our hospital with confusion, anorexia, aphagia, and abnormal behavior beginning a few days earlier. Head computed tomography and magnetic resonance imaging did not show any structural abnormalities. Upon hospitalization, blood sampling revealed elevated levels of myocardial escape enzymes; however, cardiac ultrasonography showed apical asystole, and emergency coronary angiography did not show any significant stenosis or occlusion. The patient's symptoms improved after the administration of antiepileptic drugs consisting of diazepam, fosphenytoin, and levetiracetam. On day 2 of hospitalization, an electroencephalogram showed high amplitude slow waves in the left cerebral hemisphere and NCSE-induced TTS was diagnosed. The patient was discharged after 2 weeks with a modified Rankin Scale score of 0 and continuing oral administration of levetiracetam. Delay in the diagnosis of NCSE-induced TTS can lead to a poor prognosis. Early diagnosis and treatment for NCSE and NCSE-induced TTS may result in favorable outcomes for the patient.

Keywords non-convulsive status epilepticus, Takotsubo syndrome, trazodone

1. Introduction

Takotsubo syndrome (TTS) is often preceded by emotional or physical stress. Epileptic seizures have been described in more than 100 TTS cases.. Due to the lack of typical symptoms, seizure-induced TTS can be overlooked (*1*). Specifically, there are few reports of non-convulsive status epilepticus (NCSE)-induced TTS (*2*). NCSE (*3*) is common in the elderly. It most often involves prolonged focal seizures with impaired contact, known as complex partial status epilepticus. A form of *de novo* absence status epilepticus can also occur, which is much rarer. The identified risk factors for the onset of NCSE are precession by a generalized tonic-clonic seizure, a known history of epilepsy, female gender, and known brain injury (especially a stroke sequela). The presence of one of these risk factors combined with a confusional clinical symptom of unknown origin should lead us to consider a diagnosis of NCSE. As the clinical presentation is often quite general, for example involving stupor, confusion, and even coma, the diagnosis is based on electroencephalography (EEG) with validated criteria known as the Salzburg EEG criteria. The treatment first involves injection of benzodiazepines and then, intravenous, oral, or gastric tube administration of antiepileptic drugs. Intubation/ventilation are not generally recommended, except when absolutely necessary for example under respiratory distress and multi-organ failure (*1*). The prognosis is generally poor with approximately 30% mortality (*1*).

Here, we describe a rare case where TTS induced by non-convulsive status epilepticus (NCSE) was diagnosed early and successfully treated.

2. Case Report

An 82-year-old man presented to our hospital with confusion, anorexia, aphagia, vomiting, and abnormal behavior beginning a few days earlier. He had history of hypertension but did not use any antihypertensive medication. Neurological assessments revealed a Japan Coma Scale grade 3, aphagia, and no limb paralysis.

Upon hospitalization, his heart rate was 100 bpm and his blood pressure was 169/102 mmHg. Blood sampling revealed elevated levels of myocardial escape enzymes (troponin T 1.34 ng/mL, creatine kinase MB 59.9 U/L), but no other abnormalities were noted. An electrocardiogram showed an ST-T wave rise at V2, 3. However, a cardiac ultrasound displayed apical asystole and an emergency coronary angiography did not show any significant stenosis or occlusion (Figure 1A). Plain head computed tomography (Figure 1B) and magnetic resonance imaging (Figure 1C) showed no abnormalities. The patient's symptoms improved after the administration of the antiepileptic drugs diazepam, fosphenytoin, and levetiracetam.

On day 2 of hospitalization, EEG revealed high amplitude slow waves in the left cerebral hemisphere (Figure 2). The patient was diagnosed with NCSE-induced TTS and was discharged 2 weeks later with a modified Rankin Scale score of 0; he was advised to continue the oral administration of levetiracetam 1,000 mg/day.

Written informed consent was obtained from the patient for publication of this case report and the accompanying images, and the study design was approved by the appropriate ethics review board.

3. Discussion

To the best of our knowledge, reports of successful early diagnosis and treatment for NCSE-induced TTS, such

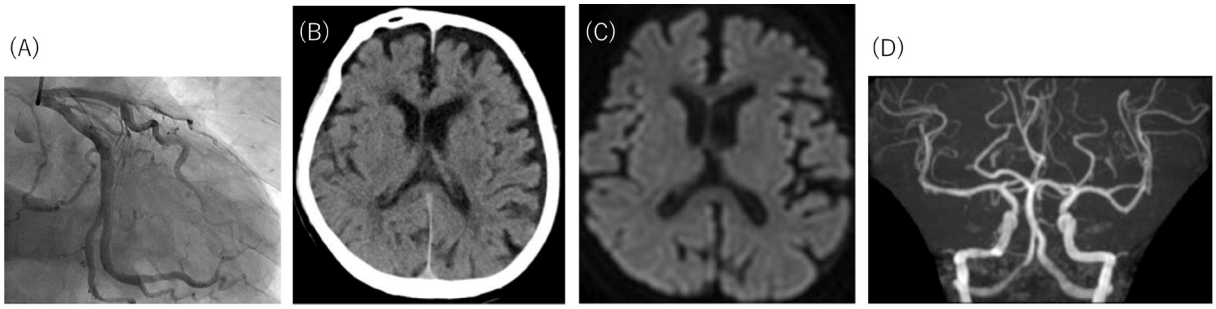

Figure 1. Clinical imaging at hospitalization. (A) Coronary angiography images acquired on day 1 of hospitalization do not show any significant coronary artery stenosis or occlusion. **(B)** Head plane computed tomography reveals no abnormalities. **(C)** Diffusion-weighted imaging shows no abnormalities. **(D)** Magnetic resonance angiography reveals no aneurysms or blood vessel malformations.

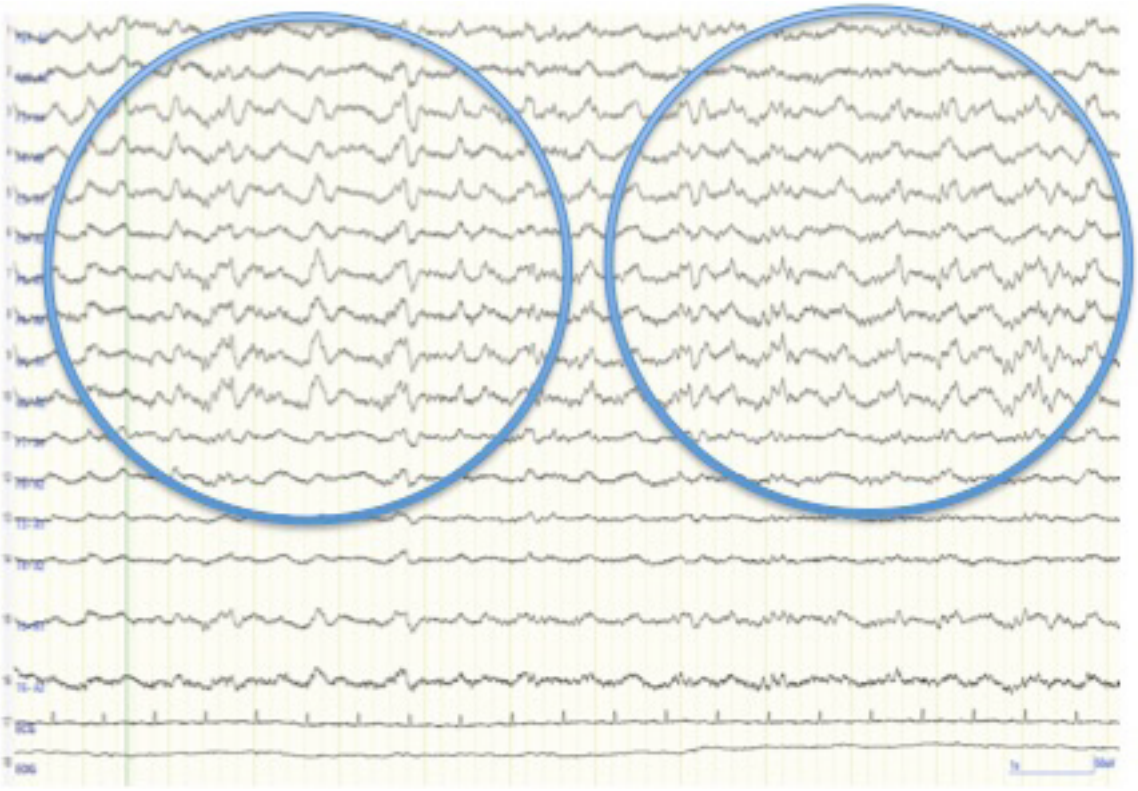

Figure 2. Examinations performed on day 2 of hospitalization. lectroencephalogram showing high amplitude slow waves in the left cerebral hemisphere.

as the present one, are rare. Enhanced catecholamine production may cause TTS (*2,4*). Catecholamines in the blood are elevated in diseases with high severity such as those involving convulsions and are implicated in the onset (*2*). Our case suggests that partial seizures may also be complicated. Uemura *et al*. (2) showed increased blood flow in the left temporal lobe and basal ganglia at the time of the seizures by single-photon emission computed tomography, and a high signal area in the left thalamus by head magnetic resonance diffusion weighted imaging. Based on these findings, they speculated that hyperactivation of neurocircuits may be associated with the onset of TTS (*2*).

Acute complications of takotsubo cardiomyopathy include pump ataxia, left ventricular apical thrombus formation, left ventricular outflow tract obstruction, and arrhythmia (torsade de pointes), each of which should be managed. Sudden death has been reported in cardiomyopathy cases (*5*), and takotsubo cardiomyopathy has been indicated as a cause of sudden death associated with epileptic seizures (*6*) and it has been reported that NCSE-induced takotsubo cardiomyopathy can result in sudden death (*6*). In takotsubo cardiomyopathy, chest pain is usually the main complaint, but in our case, there was no symptom of chest pain during the course. There is a possibility that consciousness disorder may influence the symptoms of NCSE-induced TTS. In our case, early diagnosis and subsequent treatment of NCSE-induced TTS enabled successful discharge of the patient within 14 days of admission.

In conclusion, delay in the diagnosis of NCSE-induced TTS can lead to a poor prognosis. Early diagnosis of NCSE and NCSE-induced TTS by clinical symptoms, elevated levels of myocardial escape enzymes (troponin T and creatine kinase MB), electrocardiography, and EEG followed by a prompt and appropriate treatment may contribute to favorable clinical outcomes for the patient.

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Communication

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Food intolerance/malabsorption may occur in rare diseases

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SUMMARY Sugars including lactose and fructose, or proteins (gluten), or biogenic amines (histamine), and combinations thereof may cause food intolerance/malabsorption. However, in usually asymptomatic patients with rare diseases, who present with functional, non-specific, non-allergic gastrointestinal (GI) complaints the etiologic factors of food intolerance/malabsorption need to be evaluated. We summarize patients with rare diseases, such as primary epiploic appendagitis, beta-thalassemias minor, Gullo syndrome and anomaly of the inferior vena cava, who presented functional, non-specific, non-allergic GI complaints. As conclusion, these GI symptoms in patients with otherwise asymptomatic, rare diseases were due to fructose malabsorption, histamine-, lactose intolerance and *Helicobacter pylori* (*H.p.*) infection. A registered and experienced dietician was employed to design an individually-tailored diet which ensured effective treatments and *H.p.* infection was accordingly eradicated.

Keywords primary epiploic appendagitis; Gullo syndrome; beta-thalassemia minor; inferior vena cava anomaly; lactose; fructose, histamine

1. Introduction

Functional, non-specific, non-allergic gastrointestinal (GI) symptoms, including various abdominal complaints, are widespread and troublesome. Carbohydrates, including lactose and fructose, or proteins *e.g.* gluten, or biogenic amines, including histamine, and many of their combinations may cause food intolerance/malabsorption (*1,2*). However, in usually asymptomatic patients with rare diseases, who presented with functional, non-specific, non-allergic GI complaints all etiologic factors of food intolerance/malabsorption were evaluated and published. This included testing for fructose malabsorption, celiac disease, histamine-, and lactose intolerance, and *Helicobacter pylori* (*H.p.*) infection in each patient (*3*). Here, we review these patients with rare diseases, such as primary epiploic appendagitis, beta-thalassemias minor, Gullo syndrome and anomaly of the inferior vena cava, whose symptoms were caused by food intolerance/malabsorption and *H.p.* infection. As therapy a registered and experienced dietician was necessary to design an individually-tailored diet to ensure successful treatments and *H.p.* infection was subsequently eradicated.

2. Food intolerance/malabsorption

Mainly abdominal pain, bloating, postprandial fullness, diarrhea and/or obstipation, are functional, non-specific, non-allergic gastrointestinal (GI) symptoms and represent widespread, troublesome complaints. Adverse reactions to ingested foods are reported as food intolerance/malabsorption and affect up to 20% of populations in westernized countries (*1*). Pathophysiology is still not well understood (*4*) but, food ingredients are described to cause various functional, non-specific, non-allergic GI symptoms and extra-intestinal complaints. Carbohydrates, mainly sugars including lactose and fructose, or proteins (gluten), biogenic amines (*e.g.* histamine), may cause food intolerance/malabsorption complaints (*3*). These widely used food components are not digested well and/or absorbed properly during GI passage and then influence the microbiome. Therefore, they may result in symptoms due to bacterial metabolism and fermentation (*5,6*).

Lactose intolerance is related to a deficiency of an enzyme, namely lactase, and causes gastrointestinal complaints with the consumption of dairy products. It was described with an estimated high global prevalence of 70 percent in the world's population (*7*). Depending on the ingested quantity of lactose and on the activity of lactase, patients with lactose intolerance may be asymptomatic or experience GI symptoms. If functional,

non-specific, non-allergic GI complaints are present, then, besides other GI evaluations, a clinical diagnosis of lactose intolerance may usually be performed with a hydrogen breath test (*8*).

The increasing commercial use of high-fructose corn syrup, results in a growing occurrence of complaints caused by fructose malabsorption. GLUT-5 protein is the major fructose transporter in enterocytes of the small intestine and its limited absorption capacity causes malabsorption of fructose (*9*). This absorption capacity varies widely within populations, but it has been estimated that up to 50% of the U.S. population cannot absorb 25g of pure fructose. Then it was demonstrated, that up to 80% of healthy persons were unable to absorb 50g of fructose (*10*). Clinical diagnosis of fructose malabsorption is also performed with a hydrogen breath test (*8*).

Histamine intolerance (HIT) occurs with an unbalanced metabolism and through disproportionate amounts of histamine in the body. This is caused by a reduced ability of enzymes to degrade histamine and/or the consumption of histamine-containing foods. Within digestion, the enzyme diamine oxidase (DAO) is thought to be responsible for degradation of histamine (*11*). Due to the high variability of symptoms observed in many organs, the diagnosis of HIT is challenging. Standardized *in vitro* diagnostic tests for evaluation of HIT are not existing. Although, serum DAO values have not been established to reflect gastrointestinal DAO activity, the diagnosis of HIT may be supported with the measurement of DAO in serum. Patients with low serum DAO values (< 10 U/mL) (*12*), two or more GI symptoms described for HIT, and a reduction of symptoms due to a histamine-reduced diet may be diagnosed with HIT (*13*). Its exact prevalence is not known, but numbers of up to 3 percent in populations were estimated (*14*).

The absence of celiac disease (CD) or gluten malabsorption (*15*), and absence of a *H.p.* infection need to be confirmed also in all rare-disease patients with functional, non-specific, non-allergic GI symptoms. Although, the involvement of *H.p.* in patients with functional, nonspecific, non-allergic GI symptoms is controversial (*16*), an *H.p.* infection needs to be considered and, if *H.p.* is present then eradication therapy is required (*17*). Generally, in patients aged > 50 years GI endoscopy and ultrasound of the abdomen are valuable extra approaches for evaluation of functional, nonspecific, non-allergic GI symptoms (*18*).

3. Rare diseases and food intolerance/malabsorption

Appendices epiploicae are up to 100 subserosal fat pouches attached to the colon wall with a vascular stalk. Clinically, a primary epiploic appendagitis (PEA) is accompanied by localized pain, mainly in the lower left or right abdominal quadrant. PEA with its characteristic and diagnostic appearance has computed tomography (CT) as the diagnostic modality of choice (Figure 1) (*19*). However, clinicians are frequently unfamiliar with PEA (*20*). Currently, approximately 270 publications on appendagitis epiploica can be found in the U.S. National Library of Medicine PubMed. Because of our recent publications with descriptions of PEA, and due to the presence in the world-wide-web, our treatment centre was contacted by several patients with PEA during the last years (*21*). Usually, after a radiological PEA diagnosis these patients were asking for additional clinical treatment advice. In some of them anamnesis revealed, that they had had a CT-confirmed epiploic appendagitis months prior to presentation at our institution (*22,23*). These patients presented with various functional, non-specific, non-allergic GI symptoms, and were concerned about the possibility of continuing complaints due to PEA. Generally, PEA resolves within days to a few weeks without any medical or surgical treatment. However, months thereafter with evaluations of food malabsorption/intolerance the cause for their ongoing symptoms was found (Table 1).

Beta-thalassemias minor, characterized by a reduced β-hemoglobin chain synthesis, are genetically heterogeneous autosomal recessive anemias (*24*). Due to mobility and migration, beta-thalassemias minor

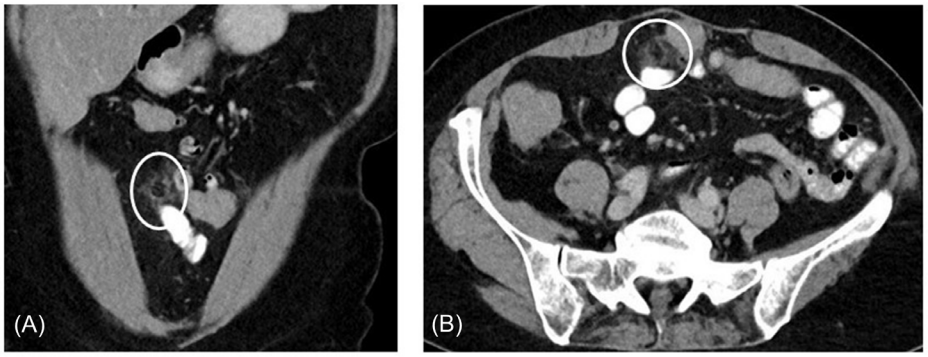

Figure 1. (A), Example of contrast enhanced longitudinal abdominal CT demonstrating a primary epiploic appendagitis adjacent to the transversal colon in a 68-year-old white female patient. The lesion, with a size of size 2.3 x 1.2cm, shows fat attenuation and surrounding inflammation. **(B)**, In the same patient an axial abdominal CT with contrast enhancement showing primary epiploic appendagitis adjacent to the transversal colon in. The lesion demonstrates fat attenuation and surrounding inflammation.

Table 1. Patients with rare diseases and food intolerance/malabsorptions

Rare disease	Number of patients (*Reference*)	Food intolerance/malabsorption
Primary epiploic appendagitis	1 (*21*)	LI, FM, *H.p.*
	1 (*22*)	FM, HI
	1 (*23*)	FM
Beta-thalassemia minor	1 (*26*)	FM, HI
	1 (*27*)	LI
Gullo syndrome	1 (*28*)	LI, HI
Inferior vena cava anomaly	1 (*31*)	LI

Legend: FM, fructose malabsorption; HI, histamine intolerance; LI, lactose intolerance; *H.p.*, *Helicobacter pylori*.

have spread from endemic areas like the Mediterranean, Africa and Asia, to northern Europe. Diagnosis of haemoglobinopathies is well defined, but there are poor data on the precise prevalence and trends of these diseases. Therefore, haemoglobinopathies are considered rare diseases in Central Europe (*25*). Usually, beta-thalassemias minor are asymptomatic in carriers of disease, although they may cause mild anaemia. We described food malabsorption/intolerance in two patients with beta-thalassaemias, which caused recurring functional, nonspecific, non-allergic GI symptoms (*26*).

Gullo syndrome is a rare, benign pancreatic hyperenzymemia. It is was described with a more than three-fold, above normal range, increase of serum pancreatic enzymes. This elevation of lipase and amylase occurs in the absence of a pancreatic disease. Diagnosis of Gullo syndrome is made when all evaluations, including radiologic examinations, for pancreatic diseases are without abnormality during the time period of at least one year. The additional diagnostic criterion is, that significant elevations and undulations of both pancreatic enzymes, lipase and amylase, occur on a day-to-day basis for five consecutive days (*27*). Only single patients are reported and a prevalence for Gullo syndrome is not known. Generally, patients with Gullo syndrome are asymptomatic. Although, we reported on a Gullo syndrome patient with recurring functional, nonspecific, non-allergic GI symptoms and these were found to be due to food malabsorption/intolerance (*28*).

Congenital anomalies of the inferior vena cava (IVC) are detected in young patients with unprovoked both-sided deep vein thrombosis in lower limbs. Occasionally, the discovery of a malformation of IVC is incidental during abdominal radiologic evaluations. Literature suggests that there exists a pro-thrombogenic effect in patients with these various congenital IVC anomalies. Generally, without thrombosis or without other associated additional, congenital defects, malformations of IVC are asymptomatic (*29*). The course and number of collateral veins in congenital anomaly or absence of the IVC are highly variable and if it is detected during abdominal surgery, then it may be detrimental for the patient. Although, anomalies of the IVC are well-recognized anatomic abnormalities they are considered rare diseases (*30*). Food malabsorption/intolerance may cause functional, nonspecific, non-allergic GI symptoms in otherwise asymptomatic congenital anomalies of the IVC (*31*).

4. Conclusion

However, in usually asymptomatic patients with rare diseases, who present with functional, non-specific, non-allergic GI complaints etiologic factors of food intolerance/malabsorption need be evaluated. This includes testing for fructose malabsorption, celiac disease, histamine-, and lactose intolerance, and *H.p.* infection in each patient. Studies have shown various combinations of these to occur in patients with functional, non-specific, non-allergic GI complaints (*2,3*).

The worldwide high prevalence of food intolerance/malabsorption stresses the likelihood of co-occurrence in patients with rare diseases, too. In conclusion, functional, non-specific, non-allergic GI symptoms in these described patients with rare diseases, including primary epiploic appendagitis, beta-thalassemias minor, Gullo syndrome and anomaly of the inferior vena cava, were due to fructose malabsorption, histamine-, lactose intolerance and *H.p.* infection. A registered and experienced dietician was employed to design an individually-tailored diet which ensured effective treatments and *H.p.* infection was accordingly eradicated. Each patient's tolerance level was considered when recommending the dietary restrictions for long-term symptom reduction. Dietary advice ought to include nutritional variety, ensure alimentary adequacy and cause negligible impact on the gastrointestinal microbiome.

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Manuscripts are suggested to be prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at http://www.ICMJE.org.

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

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

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

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

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

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

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

Examples are given below:

Example 1 (Sample journal reference):

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

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

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

Example 3 (Sample book reference):

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

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever.*http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

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

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained. Any individually labeled figure parts or panels (A, B,*etc.*) should be specifically described by part name within the legend.

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Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g.* mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide www.bipm.org/en/si/ for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and Intractable & Rare Diseases Research accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g.*, Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to Intractable & Rare Diseases

Research for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to Intractable & Rare Diseases Research online at https://www.irdrjournal.com. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at office@irdrjournal.com

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author *via* e-mail. Corrections must be returned to the editor (office@irdrjournal.com) within 3 working days.

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