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Table 1
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Manuscript Type	Word Limit	Abstract Word Limit	Reference Limit	Table Limit	Figure Limit
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Invited Review	5000	350	100	6	10
Case Report	1500	200	15	2	5
Image corner	500	No abstract	5	-	3
Letter to the Editor	1000	No abstract	5	1	1

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Journal Article:

1. Ang KK, Price RE, Stephens LC, et al. The tolerance of primate spinal cord to re-irradiation. *Int J Radiat Oncol Biol Phys*. 1993;25:459–464.

Journal Article published in non-English Languages:

2. Altuntaş N, Çelebi DT, Koçak M, Andıran N. Yenidoğan bebeklerde direkt coombs testi taraması ve pozitifliğinin morbidite üzerine, etkisi; tek merkezd eneyimi. *Pam Tıp Derg* 2015;8:39-44. (in Turkish)

Book Chapter:

3. Dimery IW. Chemotherapy in head and neck cancer. In: Myerhoff WI, Rice DH, eds. Otolaryngology: head and neck surgery, 2nd ed. Philadelphia: WB Saunders, 1992:1027–1045.

Entire Book:

4. Virchow R. Cellular Pathology. Philadelphia: JB Lippincott, 1863.

Software:

5. Epi Info [computer program]. Version 6. Atlanta, GA: Centers for Disease Control and Prevention; 1994.

Online Journals:

6. Friedman SA. Preeclampsia: a review of the role of prostaglandins. Obstet Gynecol [serial online]. January 1988;71:22–37. Available from: BRS Information Technologies, McLean, VA. Accessed December 15, 1990.

Database:

7. CANCERNET-PDQ [database online]. Bethesda, MD: National Cancer Institute; 1996. Updated March 29, 1996.

World Wide Web:

8. Gostin LO. Drug use and HIV/AIDS [JAMA HIV/AIDS Web site]. June 1, 1996. Available at: http://www.ama-assn.org/special/hiv/ethics. Accessed June 26, 1997.

URL (Uniform Resource Locator)

9. (J. M. Kramer, K. Kramer [jmkramer@umich.edu], e-mail, March 6, 1996).

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Invited Review

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Hereditary Angioedema: Diagnosis, Management, Current State of Art and Advances

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Abstract

Hereditary angioedema (HAE) is a rare, mostly inherited disorder however 25% of patients have de novo mutations. Although it's rarity, it can be life threatening due to laryngeal involvement. Along with understanding the basis of swelling, several new treatment options aside from C1-inhibitory protein (C1-INH) replacement have been developed and are avaible on the markets. However the avaibility of approved drugs for attacks of HAE varies world wide. Treatment management requires angioedema attacks treatment, pre-procedural treatment and long term prophylaxis (LTP). C1-INH which was firstly developed and approved for on-demand treatment, pre-procedural treatment and LTP by iv route, nowadays for LTP, other developed and approved options are used by orally and sc route. Despite the new developing medications, permanent treatment such as gene therapy is needed.

Keywords: Hereditary angioedema, C1-inhibitory protein, C1-INH replacement, bradykinin, plasma kallikrein, lanadelumab,

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History of hereditary angioedema

Angioedema is known as recurrent, localized and self-limiting swelling at subcutaneous or submucosal tissue that can be resolve spontaneously in 1-3 days. Firstly in 1586, Marcello Donati[1] was termed angioedema in a patient with egg allergy. After for a long time, in 1876 John Laws Milton[2] is renamed angioedema as "giant urticaria" in a woman with swelling of her both eyes. Then in 1882, Quincke delinated angioedema in a group of patients but the term of "angioneurotic edema" was first used by Paul Strübing[3], in 1885. The term of angioneurotic affirms that edema is associated with neurotic affects. In 1888, the denomination of hereditary form "hereditary angioneurotic edema" was renamed by Osler.[4] Osler highligted the hereditary inclination of the disease based on a patient and her affected family members expansion back to five generation. In 1961, Lepow and et al.[5] found out an enzyme that inhibits Complement 1 and named it as C1-esterase inhibitor (C1-INH). In 1963, Donaldson and Evans[6,7] explained the basis of the disease with absence of C1-INH in hereditary angioedema (HAE) patients. In 1965, Rosen et al.[8] reported impaired C1-INH function with normal C1-INH level in a family and HAE was then defined as Type I and Type II. HAE type I has low both C1-INH antigen and function and Type II has normal C1-INH level but low C1-INH function. Crowder[9] was defined inheritance pattern of the disease as autosomal dominant heritage in 1917. Genetic sequences with DNA revealed that C1-INH is a member of serin protease inhibitor (SERPING1) family and located on 11q12-q13.1 position. The mutation in the SERPING1 gene was found in the mid 1980s. C1-INH protein regulates several proteases inclusive of the complement, contact-system, coagulation, and fibrinolytic pathways. Although all of these developments, until 1998, Nussburger et al.[10] stated that bradykinin was the main molecule that leads to vascular permeability and angioedema as a result of contact system activation.

Hereditary angioedema with normal C1-INH level and function (nlC1-INH-HAE) was defined in 2000s.[11,12] In 2006, Dewald and Bork[13] found out FXII gene mutations in patients with nlC1-INH-HAE, in exon 9, that encodes coagulation factor (Hageman factor). Nevertheless FXII mutations in HAE accounts for only up to 25% of European patients with nlC1-INH-HAE. Afterwards widening next-generation sequencing technologies utilization, 4 novel target genes (ANGPT1, PLG, KNG and MYOF) discovered in patients with nC1-INH-HAE.[14]

Highlights

- Hereditary angioedema is a rare autosomal dominant trait disease.
- Mutation on SERPING1 leads to C1-inhibitor protein deficiency.
- Hereditary angioedema Type I and II have low C4, and low C1-inhibitor protein level and/or function.
- Other forms of hereditary angioedema have normal C4 and C1-inhibitor protein level /function with different mutations.
- Some identified mutations causing hereditary angioedema with normal C1-inhibitor are factor XII, angiopoietin-1, plasminogen, kininogen-1, myoferlin and heparan sulfate glucosamine 3-sulfotransferase 6.
- Treatment options are replacement of C1-INH protein intravenously or subcutaneously, and targeting inhibition of bradykinin– kallikrein pathways by orally, and subcutaneously.

Estimated prevelance of HAE is 1-9/100.000.[15] Well known and firstly defined form is HAE with C1-INH deficiency, that has hypocomplementemia and caused by SERPING1 mutation. Afterwards nonhypocomplementemic HAE with normal C1-INH due to mutations other then SERPING1 was discovered. The known mutations to cause nlC1-INH HAE are FXII (HAE-FXII), PLG (HAE-PLG), ANGPT1 (HAE-ANGPT1), high molecular KNG1(HAE-KNG1), and myoferlin (HAE-MYOF) and lastly heparan sulfate glucosamine 3-O-sulfotransferase 6 (3-OST-6), HS3ST6. Although the majority of HAE patients with nlC1-INH may have unknown mutations, namely as HAE-U.[16]

Pathophysiology of HAE

Unlike urticaria-angioedema, underlying mechanism of edema in HAE patients' is activation of contact system or kallikrein-kinin system which leads to exaggerated bradykinin generation or signaling. Bradykinin, a potent vasodilatory mediator, enhances vascular permeability and acts as a main mediator of swelling in C1-INH-HAE and probably in nlC1-INH-HAE. C1-INH is an inhibitory protein and act as a check point protein on classical, lectin complement pathways, as well as contact system, fibrinolytic and kinin-generating pathways. Increased bradykinin engages bradykinin B2 receptors, that is mainly expressed on endothelial cells, and leads to disintegration of the adherens junction, with resultant increases pore size of endothelial cells and vascular leakage and angioedema.[17] Swelling occurrs without wheals and pathologic basis is non-histaminergic. The feature of the angioedema is chronic, non pruritic and recurrent episodes of swelling in any part of the body. Fortunately edema resolves spontaneously within 2-5 days, however larynx, tongue and uvula involvement can cause asphyxia and death. To provide to control of the plasma contact system crucial functional threshold of C1-INH is approximately 40%.[18] In C1-INH-HAE, functional C1-INH level is commonly 5-30% of the normal level. In nlC1-INH-HAE, underlying mechanism of angioedema is not fully understood but is considered that increased bradykinin plays an important role.

Diagnosis of Hereditary Angioedema

To make a diagnosis of hereditary angioedema, firstly a highly suspicion is needed. Episodic cutaneous or subcutaneous angioedema without urticaria and accompanying abdominal semptoms includes reccurrent pain, swelling of bowel walls, and vomiting in patients

with a positive family history are warning signs of HAE. Swelling may occurs at any part of the body other than abdomen and skin. During lifetime, a laryngeal attack rate is more than 50% and even if only one laryngeal attack should be accepted as a warning sign for HAE.[19,20] In cases with unresponsive to antihistamines or glucocorticoids therapy should also be watched out for HAE. In HAE with C1-INH deficiency, angioedema attacks usually proceed in childhood, and worsen during puberty with the difference of in nlC1-INH patients angioedema attacks usually begins at adulthood. Classifications of angioedema without urticaria is illustrated in **Figure 1**.

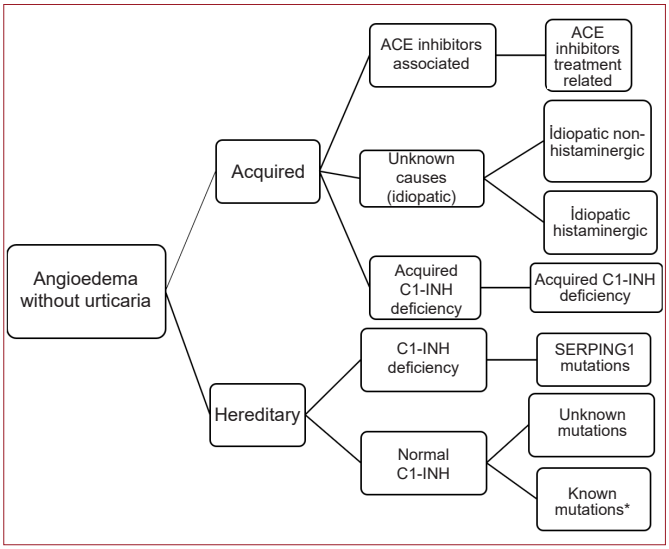

Figure 1. Classification of angioedema without wheals.

ACE inhibitors: angiotensin-converting enzyme inhibitors; C1INH deficiency: C1 inhibitor deficiency. * Mutations in the genes encoding factor XII, angiopoietin-1, plasminogen, and kininogen-1, myoferlin and heparan sulfate glucosamine 3-O-sulfotransferase 6 are associated with hereditary angioedema.

Laboratory diagnosis

Currently, HAE is basically classifed based on C1-INH levels and Complement 4; HAE with normal C1-INH or HAE with C1-INH deficiency. HAE with C1-INH deficiency is also classifed as Type I that accounts for 85% of cases and Type II accounts for 15%. In C1-INH-HAE, C4 levels are low in both suptypes. HAE type I has low C4 and low level of C1-INH antigen and functions. HAE type II has normal C1-INH antigen level but C1-INH protein function is low. Both subtypes can not be distinguished clinically. C1-INH levels could be measured by enzyme-linked immunosorbent assay or the chromogenic assay but if avaible the chromogenic assay should be preferred because of more sensitive.[21] Complement tests should be assessed twicely, at least one month interval. In at least 10% percent of patients with C1-INH-HAE has normal C4 level and C4 level should be performed during an angioedema episode. **Table 1** shows C4 and C1-INH assesment and comparison of HAE and other forms of angioedema. Differential diagnosis should be done with allergic reactions and anaphylaxis, idiopathic angioedema, drug induced angioedema, allergic contact dermatitis, autoimmune conditions, thyroid disorders, chelitis granulomatosa and Merkersson-Rosenthal Syndrome and trichinosis. In HAE with normal C1-INH, making diagnosis needs genetic evalaution on suspected cases because of lack of spesific diagnostic tests.

Genetic testing

To make a diagnosis of HAE with C1-INH deficiency, genetic analysis is not required but in some cases HAE with normal C1-INH, detecting a genetic mutation allows for diagnosis. Although HAE has an inheritance trait patterns, approximately 25% of mutations are de novo. By this time, a total of 748 diverse SERPING1 variants associated with C1-INH-HAE have been revealed. To make a diagnosis of HAE with normal C1-INH is difficult because of absence of commercially avaible diagnostic tests and biomarkers except for known genetic mutations.[22,25] Well known genetic mutations in nlC1-INH-HAE are FXII, PLG, ANGPT1, KNG1, and MYOF. Although widen utilization of genetic sequencing, a group of non-histaminergic/bradykininergic patients can not be diagnosed. The diagnosis of nlC1-INH-HAE should be based on consensus guidelines.[26]

HAE-FXII

Gain of function mutation in prolin-rich domain of FXII gene is the most frequently detected in patients with nlC1-INH-HAE.[27,28] FXII gene mutation is inherited by autosomal dominant trait with a female predominance. FXII converts prekallikren to kallirein which is later turned into bradykinin via high molecular weight kininogen (HMWK). The clinical phenotype is similiar with C1-INH-HAE and more dependent to estrogen. Estrogen increases the quantity of FXII in the plasma.

Table 1
Assesment and comparison of C4, C1-INH on HAE and other forms of angioedema

Angioedema types	C4	C1-INH level	C1-INH function	C1q	Genetic tests
C1-INH-HAE Type I,	Low	Low	Low	Normal	SERPING1 mutation/not needed
C1-INH-HAE, Type II,	Low	Normal/elevated	Low	Normal	SERPING1 mutation/not needed
Acquired angioedema with C1-INH deficiency	Low	Normal/low	Low	Normal/low	Genetic test is not needed. Anti-C1-INH antibodies positivity
Idiopathic acquired angioedema (histaminergic or nonhistaminergic)	Normal	Normal	Normal	Normal	Not identified
ACE inhibitor-associated angioedema	Normal	Normal	Normal	Normal	Not identified
HAE with normal C1-INH	Normal	Normal	Normal	Normal	Factor XII, angiopoietin-1, plasminogen, kininogen-1, myoferlin, heparan sulfate glucosamine 3-O-sulfotransferase 6, unknown mutations

HAE: hereditary angioedema, C1-INH:C1 inhibitor protein, C4: Complement 4, ACE: Angiotensin-converting enzyme

HAE-PLG

Bork et al.[24] defined a genetic mutation in the PLG gene in patients nlC1-INH-HAE, in 2018. Plasminogen converts to plasmine that activates FXII which has a role to turn prekallikrein into kallikrein. The diversity between C1-INH-HAE and HAE-PLG is age of onset of attacks. In PLG mutations, symptoms occurs usually in adulthood with a tendency of head and neck swelling.

HAE-ANGPT1

In 2018, Baffuna et al.[23] revealed a mutation in the gene encoding Angiopoietin 1 (ANGPT1) in a family diagnosed as HAE-U. ANGPT1 does not play a role in kinin or complement pathways. ANGPT1 plays a role in diminishing vascular permeability via tunica interna endothelial cell kinase 2 signaling cascade. Deficiency of ANGPT1 or decrease ratio of ANGPT1/ANGPT2 was reported to leads to enhaced vascular permiability.[29]

HAE-KNG1

A variant in the Kininogen 1 (KNG1) gene is founded in patients with nlC1-INH-HAE. KNG1 mutation is present in both high and low molecular weight kininogen isoproteins. Autosomal dominant inheritance pattern is seen in cases and the mutation is located in a functional domain, the cleavage region for kinins including bradykinin.

HAE-MYOF

In 2020, a variant in the myoferlin (MYOF) gene is reported in an Italian family diagnosed with HAE-U. The gene encodes myoferlin, an integral membrane protein, and regulates vascular endothelial growth factor signaling.

HAE-HS3ST6

In 2021, a mutation encoding heparan sulfate glucosamine 3-O-sulfotransferase 6 (3-OST-6), HS3ST6, was detected in all 3 members of one family diagnosed with nlC1-INH-HAE. This mutation likely causes to defective synthesis of heparan sulfate, a glycosaminoglycan on endothelial surfaces.[30]

New biomarkers for diagnosis and prognosis of HAE were detected during the last years. These are ready to enter market, the cleavage of high molecular weight kininogen in plasma, the spontaneous amidase activity[31], the threshold-stimulated kallikrein assay[32], the fragmentation patterns of serum glycoprotein 120.[33]

Therapeutic management of Hereditary angioedema

The main causative mediator, bradikinin, is considered to lead increased vascular permiability and swelling. Edema formation occurs slower as over hours or a few days and is generally self- limited.

Some drugs such as ACEIs, hormone replacement therapy and estrogen containing pills should be avoided in all group of HAE patients.[34,35] Pills consisting of only progestin is considered safe in these patients.[36-38] Apart from some drugs, other known angioedema triggers are trauma, infectious diseases and psychoemotional stress. Surgery, endotracheal entubation, tooth extraction and endoscopic interventions are also considered mechanical trauma and should done with cautions and under short-term prophylactic therapy. Also lactation, breastfeeding, pregnancy or menstruations may cause attacks of angioedema.

All vaccinations are considered safe in HAE patients and especially, hepatitis A and B vaccines should be advised to patients in whom receive regular plasma derived C1-INH.

Approved therapies for HAE attacks by FDA has been avaible since 2009 except for fresh-frozen plasma (FFP). FFP is helpful in some cases with a risk of increasing the severity of an attack and transmission of some infectious diseases.[19] FDA and EMA approved dosing for HAE acute attack, long term prophylaxis (LTP) and short term prophylaxis (STP) treatment is summarized in **Table 2**.

On-Demand Treatment For Acute Attacks

Nowadays several treatment modalities including replacement treatment with human and recombinant C1-INH and targeted therapies which targets bradykinin receptors and plasma kallikrein are under use.

C1-INH replacement

As C1-INH deficiency was defined for the cause of HAE in 1963, development of a rescue treatment for angioedema attacks took ten years time, up to1974. Therefore the first plasma derived C1-INH concentrate (pdC1-INH) was produced by Central Laboratory of Netherlands Red Cross Blood Transfusion Service in 1974 but it was approved in 2008, in the United States. Additionally recombinant human C1-INH (rhC1-INH) was approved for marketing in Europe and USA in 2010 and 2014, respectively. It is produced from transgenic New Zealand White rabbits' milk with a distinction in the glycosylation pattern from human C1-INH protein. Probably because of this difference, plasma half life is about 2-3 hours and shorter then pdC1-INH which is 33±19 hours aproximately.[39]

The recommended dose for attack treatment and short term prophylaxis in pdC1-INH is 20U/kg intravenous and if needed a second dose can be given after 60 minutes. For rhC1-INH, the approved dose is 50 U/kg (<84 kg), or 4200U (>84 kg) and if needed a second dose can be given within 4 hours. Although rhC1-INH has short plasma-life, studies showed no more frequent infusion needs compared to pdC1-INH.[40,41] pdC1-INH can be used as intravenous route both at the hospital by a health care professional or at anywhere if adequate training is completed. Self-administering of pdC1-INH is accepted safe, well-tolerated and applicable.

Recombinant human C1-INH should not be used in patients with rabbit dander allergy because of a severe allergic reactions developed in a patients with rabbit allergy after 3 minutes of administration.[42]

Table 2
Approved dosing of medications for LTP, STP, and acute attack treatment

Medications	Plasma derived-C1 inhibitor concentrate Berinert (iv, sc), Haegarda (sc) Cinryze (iv)	Recombinant C1-inhibitor Conestat alfa Ruconest, Rhucin (iv)	Bradykinin B2-receptor antagonist Icatibant (sc) (Firazyr)	Kallikrein inhibitor: Ecallantide (sc) (Kalbitor)	Monoclonal Kallikrein inhibitor antibody Lanadelumab (sc) Takhzyro	Kallikrein inhibitor Berotralstat (orally) Orladeyo
Approval	FDA and EMA approved Cinryze for LTP ≥6 years. EMA approved Cinryze for STP and acute attack treatment in ≥2 years. FDA approved Haegarda and Berinert for LTP in ≥6 years	EMA and FDA approved for acute attack treatment in adolescents and adults	EMA approved for acute attack treatment in ≥2 years, FDA approved for acute attack treatment in ≥18 years.	FDA approved for acute attack treatment in patients ≥12 years. EMA not approved	EMA and FDA approved for LTP in patients ≥12 years	FDA and EMA approved for LTP in ≥12 years.
Dosing for acute attacks	**Adults:** Berinert :20U/kg,iv Cinryze: 1000 U **Children:** Berinert :20U/kg, Cinryze: 500 units for body weight <25 kg, 1000 units ≥25 kg	**Adults**: 50U/kg body weight for patients <84 kg. 4200 U (2 vials) ≥84 kg.	**Adults**: 30 mg sc **Children:** 10 mg if weight is 12-25kg,(1mL) 15 mg if weight is 26-40 kg (1.5 mL) 20 mg if weight is 41-50kg (2mL) 25 mg if weight is 51-65 kg (2.5mL) 30 mg if weight is >65 kg (3 mL)	**Adults:** 30 mg (3 doses of 10 mg each) given at three separate sites subcutaneously **Children:** ≥12 years, same as adults		
Dosing for STP	**Adults**: Berinert:1000U, 1-6 hours before, iv Cinryze:1000 U, 1-24 hours earlier, iv. **Children:** Berinert:15-30 U/kg, 1-6 hours before, iv					
Dosing for LTP	**Adults**: Cinryze:1000U, every 3-4 days, iv Haegarda,Berinert : 60 IU/kg, twice weekly, sc **Children:** Cinryze: 500U twice weekly (6-11 years) Haegarda,Berinert: 60 IU/kg, twice weekly, sc				300 mg, subcutaneously every 2 weeks	150 mg/day, orally

iv:intravenously, sc: subcutaneously, LTP: Long Term Prophylaxis, STP: Short Term Prophylaxis, FDA:US Food and Drug Administration, EMA: European Medicines Agency

Icatibant

Icatibant targets bradykinin B2 receptors as competitively and selectively and is used with subcutaneous manner. The mean plasma half-life is 1.4±0.3 hours for a single 30 mg/3 mL injection. In a day maximum 3 injections is recommended with 6 hours intervals. In clinical studies with icatibant were carried out maximum 8 injections in a month.[43] Icatibant is yet approved for only attacks treatment. It can be administered in anywhere with a good safety profile with only local injection site events (erythema, swelling and pain).

Ecallantide

Ecallantide inhibits plasma kallikrein as reversibly and selectively. It is recommended for only attack treatment at 30 mg (3x10 mg/ml) in subcutaneous manner and a second dose could be administered after 24 hours. By reason of reported cases with anaphylaxis after ecallantide administrations, it is strongly recommended that drug should be used at settings that can manage anaphylactic reactions. Beside from hypersensitivity reactions, other commonly reported adverse events associated with ecallantide are upper respiratory tract infection, fatigue, headache, nause, vomiting, upper abdominal pain, diarrhea, injection site reactions, pruritus and pyrexia.

Management of HAE attacks in special populations

Children

Data are limited about HAE attacks treatment in children and a few randomized controlled trials showed similar effects in decreasing the time to symptom resolution to adults. Approved medications are considered well tolerated and safe in children.[44,45] The recommended dose for attacks treatment in children is 20 U/kg, intravenous for Berinert (pdC1-INH). For Cinryze (pdC1-INH), the recommended dose for children aged ≥12 years old and >25 kg is 1000U, and 500U intravenous for children 2-11 years and <25 kg. For Rocunest (rhC1-INH), the approved dose is 50U/kg (<84 kg) and up to 4200U/kg (≥84) for children aged ≥2 years in Europe and aged ≥12 years in U.S.

Icatibant is approved for children aged ≥2 years as a single injection dosed per kg who weigh <65 kg with subcutaneous manner. Data about repeated icatibant dosing in children has not been evaluated yet.

Ecallantide is approved for children ≥12 years with a subcutaneous manner and as the same in adults, should be administered in settings that staff has experience to treat severe hypersensitivity reactions including anaphylaxis.[46,47]

Pregnancy and breastfeeding

According to case reports series and observational studies, HAE attacks in pregnant could be managed with pdC1-INH replacement safely.[36,48] Recent data shows rhC1-INH replacement can also be used during pregnancy with well-tolerance.[49,50] Data about icatibant usage in pregnant is limited to only case reports.[51,52] There is no data about the use of ecallantide during pregnancy.

During lactation and breastfeeding, phC1-INH replacement is accepted as safe. For rhC1-INH, case series have been reported during breastfeeding. But there is no information for the use of ecallantide and icatibant during lactation.

Treatment of attacks in patients with nlC1-INH

For patients with FXII gene mutations, there is no approved medications for attacks treatment. Although, pdC1-INH and rhC1-INH replacement were found out significant reduction of resolution time of swelling compared to nontreated attacks.[22,53] Additionally icatibant has also showed good improvement of swelling in abdominal attacks of patients of FXII mutated HAE.

According to a recently published retrospective study, 23 patients with nlC1-INH-HAE were evaluated and lanadelumab, rhC1-INH, icatibant showed favorable effect on prophylaxis and acute attacks. The frequency and severity of angioedema attacks reduced with good disease control.[54]

For patients with HAE-PLG, according to a review of 111 patients, icatibant was demonstrated more effective in reduction the duration of attacks than C1-INH replacement.[55]

For patients with HAE-ANGPT1 gene, tranexamic acid was found effective as 2 patients for prophylaxis with significant reduction of severity and frequency of attacks.[23]

For patients with KNG1 gene mutations, only 1 patient with facial swelling twicely was improved with 1000U intravenous pdC1-INH infusion.

For patients with MYOF gene mutation, there is no information about effective attacks treatment of these patients.

HAE with a pathogenic mutation of the HS30ST6 gene, there is no evidence for effective attacks treatment of these patients.[30]

Prophylactic therapy

To preclude HAE attacks in certain settings, patients should be advised to receive STP or LTP. STP should be used before encountering of triggers of attacks such as a surgery or medical interventions cause to mechanical trauma and aims to decrease the risk of angioedema attacks.[49] Angioedema attacks can develop up to 72 hours after an intervention and patients should be advised to be aware of this possible risk. Even though there is no randomized controlled trials (RCT) about pre-procedural prophylaxis, a few retrospective reviews and studies reported reduction of the rate of angioedema attacks in adults and children who received prophylactic treatment.[56-59] Thus, STP before medical interventions (surgery, endoscopy, dental procedures, and intubation…) is recommended by recent guidelines and also medicine should be avaible during and after any interventions.

The recommended dose with pdC1-INH for STP is 20U/kg by intravenous route. Cinryze should be administered within 24 hours of the intervention as late as possible and for Berinert within 6 hours or if possible before the procedure.

For rhC1-INH, STP dosage is 50U/kg intravenously and administration should be done as soon as possible before procedure.

Anabolic androgens (AAs), danazol, could be used for SPT as a second-line choice and at 2,5-10 mg/kg/day dose (maximum 600 mg/day) and should be started before 5 days of the intervention and continued 2-3 days after the procedure.[58]

Fresh frosen plasma might also be used for SPT, on the other hand FFP should be preferred as a third-line therapy if C1-INH concentrates and AAs are not avaible. The recommended dose of FFP for SPT, based on published data, is 2U in adults and 10mL/kg in children before 1-2 hours of the procedure.[60,61]

According to recent data, other therapeutic medicine used for HAE treatments are not recommended for STP.

STP for children with regard to European pediatric guidelines, the recommended dose for Berinert is 15-30 U/kg within 6 hours, for Cinryze 500 U in children weight 10-25 kg within 24 hours of scheluded procedure. As same as adults anabolic androgens could be used as STP for children at a 2,5-10 mg/kg/day, mean 5mg/kg/day (maximum 600 mg/day) dose, however it is recommended for a second line choice when C1-INH concentrate is not avaible.

For pregnant, C1-INH concentrate is only accepted with well-safety profile as the same dose in adults. Vaginal delivery should be preferred, because of however in case of ceserian section, STP and epidural anesthesia are strongly recommended. Intubation always has to done under STP. In vaginal delivery setting, STP is not routinely recommended but C1-INH concentrate should be present for on demand use if needed. But in certain cases such as frequent attacks during third trimester or a history of serious, life-threatening angioedema attacks or a history of vaginal edema caused by mechanical trauma, STP should be preferred.[62] Anabolic androgens are contraindicated during pregnancy and breastfeeding, because of crossing placenta and milk. If C1-INH is not avaible during breastfeeding period, AAs could be used for STP after discontinuation of breastfeeding.

There is not any randomized controlled studies about STP in case of HAE with nlC1-INH, therefore any strong recommendations can not be made. However based on little experience regarding SPT, the same management with C1-INH-HAE is accepted for cases with nlC1-INH-HAE.

Long term prophylaxis

The aim of LTP is reducing the frequency, duration and severity of episode of HAE. There is no certain rules for whom and when will to start LTP but LTP management should to be individualized based on case's needs. Although regular LTP does not make sure the risk of angioedema attacks completely, patients should be warned about the risk and also obtained on-demand therapy. Many factors such as attacks severity and frequency and a history of life threatening attacks, access to emergency treatment and patient's decision should be taken into account to commence LTP.

C1-INH-HAE

Options for LTP in C1-INH-HAE are C1-INH concentrate replacement by intravenous/subcutaneous route, lanadelumab, a selective inhibitor of plasma kallikrein with subcutaneous route and, orally AAs, transexamic acid and plasma kallikrein inhibitor.

C1-INH concentrate replacement is considered as a first choice for LTP. The recommended dose of Cinryze for LTP is 1000 U every 3-4 days by intravenous route for adults.[63] Recently Cinryze and Berinerts have approval for LTP by intravenous and subcutaneous manner, respectively. Rocunest has not approval for LTP but when it was administered one or twice weekly for prophylaxis, it was reported a good safety profile and a decrease in the frequency of angioedema attacks.[41] Studies showed routine administration of C1-INH by intravenous route could cause several thrombotic events, therefore physician should be careful about the risk factors and symptoms.[64] Long term intravenous prohylaxis has some difficulties such as displeasure of repetitive intravenous access, long infusion time and maintaining long term venous access.

Haegarda (in USA) is the first subcutaneous C1-INH concentrate, was approved at the dose of 60 IU/kg twice weekly by the FDA in 2017 for LTP in patients 6 years age and older. Berinert (subcutaneous form in Europe) showed a consequential decrease (95% reduction)[18] in the rate of attacks compared to plasebo. C1-INH administration by subcutaneous route is well tolerated with a good safety profile.[65] Nasopharyngitis, hypersensitivity, dizzines, and localized injection site reactions are the most common reported adverse events of subcutaneous C1-INH administraiton.[66]

Lanadelumab is the first human monoclonal antibody that inhibits plasma kallikrein reversibly for approximately 2 weeks. It is approved for LTP at a dose 300 mg, subcutaneously, every 2 weeks and if cases are stable for 6 months, injections interval could be change as 300 mg every 4 weeks.[67-69] Lanadelumab is well-tolerated with a good safety profile. According to HELP study, injection site pain (42.%), viral upper respiratory tract infection (23.8%), headache (20.2%), injection site eritema and bruising (9.5% and 7.1%, respectively) and dizzines (6%) are the most common reported advers event related to the drug.[70] HELP study showed a rapid onset of effect and continuous effectiveness in decreasing frequency of HAE attacks.[70] In 2% percent of active drug group had developed increased aspartate and alanine transaminase levels while zero on placebo group. These were asymptomatic and transient and did not require drug discontinuation. Lanadelumab can increase activated partial thromboplastin time but has not been associated with abnormal bleeding.

Berotralstat is the first orally used, plasma kallikrein inhibitor that is approved for LTP as once daily in adults and ≥12 years old by FDA in 2020 and EMA in 2021. The approved dose 150 mg once daily showed a good safety profile and effectiveness. Berotralstat should be taken with foods. The most common reported adverse events are abdominal pain, vomitting, diarrhea and back pain in 10% percentage of cases and alleviated with continued use. A phase 3, placebo controlled study called APeX-2, carried out on 121 adults and adolescents for two different doses, 110 and 150 mg once daily. The study demonstrated a significant decrease in the rate of attacks for both doses compared placebo.[71]

Anabolic androgens and transexamic acid should be used as a second-line choice for LTP unlike other options are not avaible. Danazol and stanozolol are the mostly used worldwide. The exact mechanism of AAs is not clear, however it is considered they enhance the level of C1-INH.[72] AAs are used orally and in several studies they were provide a reduction in HAE attacks.[73-76] The most important side affects related to AAs are hepatotoxicity, hepatocelular adenoma and carsinoma and may develop dose dependent.[77,78] Other common side affects are acne, hirsutism, menstrual disorders, weight gain and depression.[79] AAs are contraindicated in pregnants and in cases with hepatitis and androgen dependent malignancies.[80,81] Danazol should be started at 400-600 mg daily, then dose should be tapered to the lowest effective dose that maintain to prevent or reduce HAE attacks, usually at 200 mg daily or every other day. Patients under AAs treatment should be checked for liver enzymes, lipid profile, urine examination, alpha-feto-protein, and complete blood cell count every 6 months and abdominal ultrasound yearly.

Antifibrinolytic agents (tranexamic acid and epsilon aminocaproic acid) is the last option in cases with HAE for LTP in circumtance that other medications are not avaible and AAs are contraindicated. Tranexamic acid (TXA) is the most preferred drug because of less side effects compared to epsilon aminocaproic acid. The recommended dose for TXA is 1-3 gr/day up to 6 gr daily. Abdominal discomfort, diarrhea, headache, nause are the most reported side effects related to tranexamic acid.

LTP in special populations

pdC1-INH, Cinryze is approved for LTP in children ≥6 years old, at a dose of 500 U for 6-11 years old and 1000 U for 12-17 years old every 3-4 days. Berinert is approved for LTP for children ≥6 years old via subcutaneous manner. Berinert is provided a good safety profile and effective treatment for LTP in patients aged <17 years old in OLE and COMPACT studies.[82-84] AAs should not used for LTP in children because of adverse events.

In pregnant for LTP pdC1-INH should be preferred as the first line therapy according to current guidelines. Any newborn abnormatilies related to exposure of pdC1-INH during pregnancy has not been showed up to date in researches.[85-88] AAs must not used for LTP in pregnant and also have to be stopped before a planned pregnancy at least 1 month. TXA is the last option for LTP in pregnant otherwise pdC1-INH is not avaible. In lactation and breastfeeding period, pdC1-INH should be preferred firstly.[36]

nlC1-INH-HAE:

There is not enough data based on randomize controlled trials about LTP for nlC1-INH- HAE and approved medicine to these cases. According to experimental data, TXA showed a significant reduction of attacks in FXII- HAE, PLG-HAE and ANGT1-HAE for LTP.[23,55] Also AAs and progestin provided a significant decrease of angioedema attacks in FXII-HAE patients. Data are not avaible in patients with nlC1-INH-HAE, although medicine that could inhibits bradykinin may be an effective treatment options in these cases. Also studies about progestins therapies were reported a benifical effect for prophylaxis in HAE with normal C1-INH.[22]

Novel treatments in development

Several drugs are under investigation with phase 1,2,3 trials. An oral bradykinin B2 receptor antagonsit (PHA121) that blocks bradykinin within 15 minutes and inhibition lasts at least 12 hours showed effectiveness for both acute and prophylactic treatment in phase 1 clinical tirals.[89]

Garadacimab is a IgG4 type human recombinant monoclonal antibody, that binds to the FXIIa with catalytic site and blocks its proteolytic activity. Garadacimab is administered by subcutaneous manner. In a phase 2 trial, the frequency of attacks was reduced compared with placebo with mean percentage reductions with three doses of garadacimab were 89, 99, and 91 and the drug was well-tolerated.[90] Currently, Garadacimab phase 3 trials is carrying on with 60 participants for prophylactic treatment efficacy and safety.

Other targeted therapies under investigation are ALN-F12 and ARC-F12 and inhibit Factor XII which is developed by using small interfering RNA (siRNA) technology.[91]

Another targeted therapy is IONIS-PKK-LRx which targets plasma prekallikrein and provides downregulation of prekallikrein mRNA synthesis and knock outs the gene encoding prekallikrein by using CRISPR/Cas9 technology.

A one-time intravenous injection of Adeno-associated virüs gene transfer vector inserts an extrachromosomal copy of the SERPING1 gene to induce in vivo production of C1-INH are also in preclinical development in mouse models.[92]

Conclusion

New treatment options for acute attacks and long-term prophylaxis for C1-INH-HAE are developed last years and the avaibility of drugs that can be used oral and subcutaneous route may increase patients quality of life and reduce the need of admission of hospital. New treatments under development such as gene therapy and other targeted therapies promise future for patients.

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The Association of Malignancies with the Clinical Profile of Children with Neurofibromatosis Type 1

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Abstract

Neurofibromatosis type 1 (NF-1) is a significant autosomal dominant disorder with a wide spectrum of clinical findings. These signs (Café au lait spots, bone dysplasia, Lisch nodules) usually start to emerge after the first months of life and most are benign in nature. On the other hand, neoplasms (optic glioma, neurofibroma, malignant peripheral nerve sheath tumor, soft tissue sarcoma, leukemia, breast cancer) are a major cause of morbidity and mortality in NF-1 patients. Cancer risk during lifetime of a NF-1 patient is almost 10 times more than a person without NF-1, but what drives these patients into cancer is still unknown. This study aims to analyze the possible association of clinical findings with malignancies in children with NF-1. Medical records of 55 children with NF-1 who were followed up in a tertiary care pediatric oncology clinic between January 2005 and December 2014 were analyzed. We assessed clinical and demographic characteristics of patients, as well as the NF-1 diagnostic criteria, NF-1 related complications, and malignancies. The NF-1 patients without malignancy were classified in Group 1 while patients with malignancy were in Group 2. Logistic regression analysis was used to determine the risk factors of malignancy in NF-1. The mean age was 7.68±4.65 years. Female sex was dominant in both groups. Café au lait spots were present in all patients. Axillary-inguinal freckling was observed in 76.4% of patients, followed by neurofibromas in 30.9%, Lisch nodules in 29.1%, bone dysplasia in 14.5%, optic gliomas in 23.6%, and a history of first degree relative with NF-1 in 63.6%. Central nervous system (CNS) tumors were present in 40%. Tumors other than CNS tumors were acute myeloid leukemia and schwannoma. None of the diagnostic criteria was a risk factor for predisposing to malignancy by itself. Having >3 criteria was found to be the risk factor for malignancy in NF-1 (OR:5.891, CI 95%: 1.676-20.705, p=0.006). There are no clearly defined risk factors predicting occurance of malignancies in NF-1 at present. However, we found a higher risk of malignancy association in patients who meet more than 3 diagnostic criteria of NF-1.

Keywords: Children, low grade glial tumors, neurocutaneous syndromes, neurofibromatosis

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Introduction

Neurofibromatosis type 1 (NF-1) is one of the most common (NF1 prevalence: 1/2600-4000) genetic disorders and it is placed among the neurocutaneous syndromes.[1–3] It has a broad spectrum of clinical findings which affects several organ systems.[4] NF-1 has an autosomal dominant pattern of inheritance and half of the patients have a de novo mutation. Mutations in the NF-1 gene lead to neurofibromin accumulation which is one of the tumor suppressor proteins.[5] This mutation also provokes proliferation and tumorigenesis in tissues, especially of the neuroectodermal origin. Clinical findings vary among affected individuals due to highly variable clinical expressivity.[6–8] Café-au-lait macules (CALMs), Lisch nodules, and neurofibromas are the most common features of NF-1, and can cause significant morbidity.[4] Manifestations of NF-1 increase with growing age making clinical picture worse at older ages.[6–8]

Highlights

- The clinical manifestations of NF-1 are very heterogeneous.
- The risk of malignancy is markedly increased in patients with NF-1.
- The most frequently detected malignancy is optic glioma in patients with NF-1.

The major cause of mortality in NF-1 are malignant neoplasms.[4] The standardized incidence ratio of the malignancy risk was found 35.6 for children with NF-1 in the Finnish Cancer Registry. Central nervous system (CNS) tumors, malignant peripheral nerve sheath tumor (MPNST), and rhabdomyosarcoma (RMS) are reported malignancies in these studies.[9] Studies of adult NF-1 patients have shown that cutaneous neurofibromas did not differentiate into malignancy, but, MPNSTs are known to develop from pre-existing plexiform neurofibroma.[10,11]

Briefly, life expectancy is 10-15 years lower in NF-1 patients compared to healthy population.[12] Malignancies play a critical role regarding this statement. It is not clear which NF-1 patients develop cancer. In this study, we aimed to identify the clinical features of NF-1 patients that are possibly associated with increased malignancy risk.

Material and Method

Patients Data

We scanned electronic medical records of NF-1 patients followed at Kocaeli University Pediatric Oncology Department between January 2005 and December 2014. Patients registered as neurofibromatosis with the International Classification of Diseases 10 code Q85.0 were listed. Eighty eight patients were tagged with ICD 10 code Q85.0. Inclusion criteria were: age under 18 years, a minimum follow-up time of three months in our center, meeting the National Institutes of Health (NIH) NF-1 Diagnostic Criteria[13] that are detailed in the next sentence, and full access to patients' medical records regarding diagnosis and treatment. NIH diagnostic criteria for NF-1 are six or more CALMs >5 mm in diameter in prepubertal individuals or >15 mm in diameter in postpubertal individuals, two or more neurofibromas of any type or 1 plexiform neurofibroma, axillary or inguinal freckling, optic glioma, two or more Lisch nodules, a distinctive osseous lesion such as sphenoid wing dysplasia or thinning of long bone cortex, and a first-degree relative with NF-1. All patients met the NIH diagnostic criteria. In our center, genetic analysis was not performed on patients who met the diagnostic criteria. Since the study was retrospective, genetic analyzes of the patients were not performed. Fifty-five patients who fulfilled the inclusion criteria were enrolled. Thirty-three patients were not included in the study as they did not fulfill the diagnostic criteria for NF-1 or their medical data were missing.

Demographic findings, diagnostic criteria, clinical findings other than diagnostic criteria, malignancy, and family history were evaluated. Patients were grouped regarding their history of malignancy in two groups, NF-1 patients without cancer (Group 1) and NF-1 patients with cancer (Group 2). All tumors were diagnosed after biopsy except optic glioma which was identified by magnetic resonance imaging. Bone marrow examination was performed in the diagnosis of hematological malignancies. We also evaluated treatment modalities, treatment response, and prognosis of malignancy.

Ethics and Consent to Participate Declarations

All patients/parents signed the consent form and accepted use of their medical records for scientific research.The study was carried out with the permission of Kocaeli University Clinical Researches Ethics Committee (Date: 18.03.2014, Decision No: 14/98).

Statistical analysis

We used IBM SPSS 20.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis. Normality tests were performed for continuous variables. Data were presented as mean±standard deviation for normally distributed continuous variables, median (25-75 percent) for abnormally distributed continuous variables, and proportions for categorical variables. Continuous variables showing normal distribution were compared between groups using Student's t-test, and abnormally distributed variables were compared using the Mann–Whitney U test. Categorical variables were compared using the Chi-square test. Univariate binary logistic regression analysis was performed to identify the risk factors for malignancy in NF 1. Odds ratios were calculated with 95% confidence intervals. A p-value of less than 0.05 was considered statistically significant. The presence of malignancy with NF-1 categories were coded as 0=absent and 1=present. Because there was one significant variable at $p<0.05$ in univariate analysis, we could not analyze the multivariate logistic regression model to identify the independent risk factors. Optic glioma, one of the NF-1 NIH diagnostic criteria, was considered a confounder factor since it is also a malignancy. It was not included in the number of diagnostic criteria in the logistic regression analysis.

Results

Thirty-two patients (58.2%) were female. The mean age at diagnosis was 7.68±4.65 years; the median follow-up duration was 2 (0.25-11.50) years. The mean age at diagnosis of malignancy (n=23) was 8.90±4.22 years. The demographic and clinical characteristics of both groups are summarized in **Table 1**.

The Diagnostic Criteria of Patients

All patients had CALMs. Axillary/groin freckling followed CALMs in 42 (76.4%) patients. Seventeen patients (30.9%) had neurofibromas. Cutaneous neurofibroma and plexiform neurofibroma were observed in 11 (20%) and 10 (18.2%) patients, respectively (Four patients had both cutaneous neurofibroma and plexiform neurofibroma.). Eight (14.5%) patients had bone dysplasia. Congenital tibial pseudoarthrosis was observed in 3 (5.4%) patients; while congenital tibial dysplasia in 2 (3.6%) patients; sphenoid wing dysplasia in 1 (1.8%) patient; ulnar dysplasia in 1 (1.8%) patient and humerus dysplasia in another (1.8%) patient. The other diagnostic criteria are shown in **Table 1**.

Table 1
The comparison of clinical characteristics according to the presence of malignancy

Variables	Group 1 n=32 (100%)	Group 2 n= 23 (100%)	p
Age of diagnosis (year)[a]	7.38±4.61	8.07±4.77	0.61
Duration of follow-up (year)[b]	2.46 (0.25-11.50)	2.00 (0.25-9.90)	0.87
Female	19 (59.4%)	13 (56.5%)	0.83
Cafe au lait spots	32 (100%)	23 (100%)	-
Neurofibroma	8 (25%)	9 (39.1%)	0.26
Lisch nodules	7 (21.9%)	9 (39.1%)	0.17
Freckling	22 (68.8%)	20 (87%)	0.12
Optic glioma	-	13 (56.5%)	-
Bone dysplasia	5 (15.6%)	3 (13%)	1.00
Neurofibromatosis type 1 in family	20 (62.5%)	15 (65.2%)	0.84
Malignancy in family	4 (12.5%)	5 (21.7%)	0.36

[a]mean±SD, [b]median (minimum-maximum), Group 1; the NF-1 patients without malignancy, group 2; the NF-1 patients with malignancy

The frequency of diagnostic criteria was not different between 0-1 age-year, 2-6 age-year and ≥7 age-year groups. No patients had Lisch nodules or bone dysplasia in the 0-1 age-year group. The number of criteria did not correlate with the age at diagnosis of NF-1 (r (55)=0.34; p=0.80).

The Clinical Findings Out of the Diagnostic Criteria

Weight and height percentiles were below the 3rd percentile in 19 (34.5%) and 11 (20%) patients, respectively. Head circumference percentile was above the 90th in 18 (32.7%) patients and was below the 3rd in 1 (1.8%) patient.

Three (5.5%) patients had delayed puberty, and 2 (3.6%) patients had precocious puberty. All patients with delayed puberty had malignancy and 2 of them received chemotherapy (CTX). These tumors were cerebellar astrocytoma in the first patient, cerebral hemispheric glioma in the second, and brainstem and cerebral hemispheric glioma in the third.

Cranial MRI was performed in 51 (92.7%) patients, and 43 (78.2%) of these patients had radiologic abnormalities on MRI. The most common cranial MRI abnormality was unidentified bright objects (UBO) in 40 (72.2%) patients. Excluding UBO and malignancies, other cranial MRI abnormalities were observed in 12 (21.8%) patients (Table 2). Ten (18.2%) patients had an abdominal abnormality and 4 (7.3%) patients had a urinary abnormality on ultrasonography; 2 (3.6%) patients had an echocardiographic abnormality and, 7 (12.7%) patients had endocrinologic disorders (**Table 2**).

Table 2
Abnormalities detected in systemic examinations

Abnormalities	n
Skeletal abnormalities other than diagnostic criteria	20
Scoliosis	10
Pectus excavatum	6
Cubitus valgus	1
Genu valgum	1
Clinodactyly	1
Scoliosis, genu valgum, pectus excavatum, duplication of finger	1
Abnormalities in cranial imaging other than malignancy and UBO	12
Hydrocephalus	4
Asymmetric ventricular volume	3
Aquaduct stenosis	2
Arachnoid cyst	1
Putamen cyst	1
Wallerian degeneration	1
Abnormalities in abdominal ultrasonography*	10
Splenomegaly	6
Hepatomegaly	4
Accessory spleen	3
Endocrinologic abnormalities	7
Hypothyroidism	3
Growth hormone deficiency	2
Type 1 diabetes mellitus	1
Panhypopituitarism †	1
Abnormalities in urinary ultrasonography	4
Increase in renal parenchyma echo	1
Decreased kidney size	1
Hydronephrosis	1
Nephrolithiasis	1
Abnormalities in echocardiography	2
Tricuspid regurgitation	1
Mitral valve prolapse, mitral regurgitation, tricuspid regurgitation	1

* Three patients had more than one abdominal ultrasonography pathology, †Secondary to hypothalamic glioma resection, UBO; unidentified bright objects

We compared patients with and without UBO regarding seizures, intellectual developmental disorders (IDD), and cranial malignancies, and no significant difference was found (**Table 3**). Eleven (20%) patients had seizures. The cause of seizures was febrile convulsion and epilepsy in 5 (9.1%) and 6 (10.9%) patients, respectively. Intellectual developmental disorders were mild in 19 (34.5%) patients, moderate in 5 (10.6%) patients and severe in one (2.1%) patient. Eight patients could not be evaluated in regard to IDD as they were under 6 years of age. Other neurological problems were headache due to CNS tumor in 2 (3.6%) patients, urinary and bowel incontinence in one (1.8%), and hemiplegia in another (1.8%) patient due to plexiform neurofibroma.

Table 3
Evaluation of patients with unidentified bright object

Variables	UBO (+) n=40 (100%)	UBO (-) n=15 (100%)	p
Mean age (years)	7.20±4.03	8.96±5.97	0.30
Patients with seizures	8 (20%)	3 (20%)	1.00
Patients without seizures	32 (80%)	12 (80%)	
Intellectual developmental disorders*			0.85
No	15 (42.9%)	7 (58.3%)	
Mild	15 (42.9%)	4 (33.3%)	
Moderate	4 (11.4%)	1 (8,3%)	
Severe	1 (2.1%)	0 (0%)	
Patients with CNS tumor	18 (45%)	4 (26.7%)	0.35
Patients without CNS tumor	22 (55%)	11 (73.3%)	

CNS: Central nervous tumor, UBO: Unidentified bright object, *Wechsler Intelligence Scale for Children-R was perfomed. Intellectual Developmental Disorders was classified 50-69 intelligence quotient (IQ) as mild, 35-49 IQ as moderate, and 20-34 IQ as severe.

Malignancies of the Patients

Malignancy was detected in 23 (41.8%) patients. Fourteen (60.9%) of these patients were diagnosed with NF-1 during workup for malignancy. The longest duration between diagnosis of NF-1 and malignancy was three years. Three (60%) patients in the 0-1 age group, 4 (25%) patients in the 2-6 age group and 16 (47.1%) patients in the ≥ 7 age group had malignancy (p=0.257).

The malignancy types in the family were brain tumor in 4 patients, breast cancer in one patient, lymphoma in one patient, soft tissue sarcoma in one patient, gastric cancer in one patient, and gastric cancer and renal cell carcinoma in one patient. Two patients had a similar malignancy type as in their families. In these patients, the malignancy type in the family was a brain tumor. One of the patients had optic glioma and the other one had optic glioma, brain stem glioma, cerebral hemispheric glioma, and meningioma.

We evaluated the "NF-1 diagnostic criteria" as possible risk factors for malignancy with univariate logistic regression analysis. None of the diagnostic criteria was a risk factor for malignancy in NF-1 (**Table 4**). We analyzed the number of diagnostic criteria (other than optic glioma) as a predictor of malignancy comparing patients with and without malignancy. To achieve this, patients were stratified in three groups; patients with less than 3 criteria, with 3 criteria and more than 3 criteria. Eleven (20.6%) of patients with less than or with 3 criteria and 12 (76.2%) of patients with more than 3 criteria had malignancy (p=0.004). In univariate regression analysis, having more than 3 criteria was the risk factor for malignancy in NF-1 (OR:5.891, CI 95%: 1.676-20.705, p=0.006).

There was at least one CNS tumor in 22 (40%) patients. Two (3.5%) patients had malignancies other than CNS tumors. Optic glioma was the most prevalent tumor, in 13 (23.6%) patients (**Table 5**). Seven (30.4%) patients with tumors were followed without treatment. Sixteen (69.6%) patients were treated for progressive disease and received CTX. Two of them had primary surgery of the tumor (glioma in the hypothalamus of the first patient, and the other had high-grade glioma in the right temporal lobe) and had adjuvant CTX. Two patients with GBM and brainstem glioma received radiotherapy (XRT).

Table 4
Evaluation of risk factors for malignancy development with univariate logistic regression analysis

Variables	p	OR	95% CI
Follow-up duration (year)	0.79	0.97	0.80-1.19
Male sex	0.832	1.12	0.38-3.33
The presence of neurofibroma	0.266	1.93	0.61-6.14
The presence of freckling	0.127	3.03	0.73-12.60
The presence of bone dysplasia	0.789	0.81	0.17-3.79
The presence of an individual with neurofibromatosis 1 in the family	0.836	0.89	0.29-2.72
The presence of malignancy in the family	0.366	1.94	0.460-8.223
The number of diagnostic criteria >3	0.006	5.89	1.676-20.705

Table 5
Dispersion of malignancies in patients

Tumors	n (%)
Central nervous system tumors	22 (40%)
Glial tumors	22 (40%)
Optic glioma	13 (23.6%)
Cerebral hemispheric glioma	7 (12.7%)
Brainstem glioma	5 (9.1%)
Cerebellar astrocytoma	1 (1.8%)
Meningioma	3 (5.4%)
Non-Central nervous system tumors	2 (3.6%)
Schwannoma	1 (1.8%)
Acute myeloid leukemia	1 (1.8%)

*Five patients had more than one tumor

Eight patients (50%) who received CTX were given carboplatin-vincristine (CV) combination. Carboplatin-vincristine combination was switched to temozolomide in two of these patients (12.5%) due to progressive disease. Five (31.2%) patients with low-grade glial tumors received temozolomide as a primary CTX protocol. In one patient with acute myeloid leukemia, bone marrow transplantation was performed after CTX induction. Only one patient showed an allergic reaction to carboplatin at the last cycle, and the treatment was stopped. Thirteen (81.3%) patients finished treatment while three (18.7%) patients left it with their own will.

In the patients group with malignancy, 9 (39.1%) had stable disease, 4 (17.4%) had a partial response, and 3 (13%) had progressive disease. Seven patients gave up follow up. One of the patients, who received temozolomide and XRT after surgery, died with progressive GBM. The characteristics of patients with malignancy are shown in **Supplemental Table 1**.

Discussion

Our patients had abnormalities of almost all organ systems which showed that clinical heterogeneity of NF-1 is broad in children. The risk of malignancy increases in patients with more than three NIH criteria.

Neurofibromatosis type 1 occurs in childhood and is one of the most common autosomal dominant diseases.[14–16] Since penetrance of NF-1 is 100%, the number and severity of clinical symptoms increase with age. Our results, that, more than half of patients were seven years old and had three diagnostic criteria, were consistent with this fact. However, we did not find

Supplemental Table 1
The characteristics of NF-1 patients with malignancy

Patient no	Tumor	Gender	Age at diagnosis of NF-1 (years)	Age at diagnosis of malignancy (years)	Duration of NF-1 (years)	Duration of tumor (years)	Treatment	Outcome
1	Optic glioma	M	0.33	1.83	3.37	1.87	CTX	Stable disease
2	Optic glioma	M	9.12	10.25	1.71	1.75	Follow-up	Stable disease
3	Optic glioma	F	10.25	10.25	0.58	0.58	CTX	Unfollowed
4	Optic glioma	F	6.75	9.75	6.25	3.25	CTX	Stable disease
5	Optic glioma	M	5.50	5.58	1.66	1.58	CTX	Stable disease
6	Optic glioma	M	8.41	8.50	1.34	1.25	CTX	Partial response
7	Optic glioma	M	0.83	1.41	2.33	1.75	CTX	Partial response
8	Optic glioma	F	3.58	3.58	7.50	7.50	CTX	Progressive disease
9	Optic glioma	F	0.25	3.25	5.41	2.41	CTX	Partial response
10	Optic glioma	F	16.5	16.5	0.58	0.58	Excision+ CTX	Unfollowed
11	Brainstem glioma	F	14.0	14.0	2.00	2.00	CTX	Unfollowed
12	Brainstem glioma	M	11.25	11.25	0.50	0.50	Follow-up	Stable disease
13	Cerebral hemispheric glioma	F	9.75	9.75	0.25	0.25	Follow-up	Unfollowed
14	Cerebral hemispheric glioma	F	7.16	7.24	0.8	0.72	Follow-up	Unfollowed
15	Cerebral hemispheric glioma	F	14.33	14.33	0.50	0.50	Excision+ CTX +RTX	Died
16	Cerebral hemispheric glioma	M	13.91	13.91	1.58	1.58	Follow-up	Progressive disease
17	Cerebellar astrocytoma	F	7.91	9.08	5.92	4.75	CTX	Stable disease
18	Acute myeloid leuchemia	F	7.50	7.50	9.90	9.90	CTX	Stable disease
19	Optic glioma + Brainstem glioma	M	8.08	8.08	2.17	2.17	CTX	Stable disease
20	Optic glioma + menengioma	M	4.00	4.00	2.16	2.16	CTX	Progressive disease
21	Brainstem glioma+ cerebral hemispheric glioma	F	14.50	14.50	0.50	0.50	Follow-up	Unfollowed
22	Cerebral hemispheric glioma + menengioma + schwannoma	M	11.6	11.6	0.58	0.58	Follow-up	Unfollowed
23	Optic glioma + brainstem glioma+ cerebral hemispheric glioma + menegioma	F	7.91	8.91	4.49	3.49	CTX +RTX	Stable disease

CTX: Chemotherapy, NF-1: Neurofibromatosis type 1, XRT: Radiotherapy

a linear correlation between the age of diagnosis and the number of criteria. Café-au-lait macules were one of the primary diagnostic criteria. Café-au-lait macules start to occur after birth, and both, the diameter and the number of CALMs increase with age.[17,18] All of our patients had greater than/equal to 6 CALMs. Previous studies detected cutaneous neurofibroma in more than 80% of patients and plexiform neurofibroma in 30-50% of patients.[18,19] Neurofibromas occur in adolescence and after.[18,19] Few patients (30.9%) in our study were in adolescence or older. Therefore, the frequency of neurofibroma was low in our study. Another classical feature of NF-1 is the dysplasia of long bones in infants.[8] The frequency of bone dysplasia in our patients was similar (14.5%) to literature.[19]

In NF-1, several features other than malignancies vary by age and interest to organ systems.[19] Although our study was held in a pediatric group, we detected several manifestations in almost all organ systems. Unidentified bright object, IDD, and scoliosis were common manifestations of our patients which are not classified in diagnostic criteria.

In our study, UBO was more common than axillary or inguinal freckling, even though UBO is not a diagnostic criterion. Some researchers suggested that UBO can be used as another diagnostic criteria.[20–24] Our result supports this attitude. Although, an association between cognitive disorders and UBO was detected in previous studies,[25] we did not find a relationship between IDD and UBO. Also, CNS malignancies and seizures were not significant in our patients with UBO. Therefore, we do not have an additional recommendation for follow-up in NF-1 patients with UBO. However, patients who were shown to have UBO may be evaluated for NF-1.

The most common malignancies are intracranial tumors in NF-1. Optic glioma is the primary intracranial tumor in NF-1. The frequency of optic glioma was reported as 15-20% in children with NF-14. Varan et al.[26] found intracranial tumors other than optic glioma in 2.3% of NF-1 patients. Almost half of our patients had a malignancy, and we detected optic glioma in 23.6% of them. Besides, the frequency of other intracranial tumors was ten times higher in our study. We think that these results were provided by the help of detailed evaluation of NF-1 patients referred to our pediatric oncology clinic with an intracranial tumor. Schwannoma, meningioma, and acute myeloid leukemia were malignancies other than gliomas in our patients.

A study which evaluated CV efficiency on progressive low-grade glioma showed that event-free survival and tumor response rates were superior in children with NF-1 compared to children without NF-127. Temozolomide did not have a superior effect than CV in regard to survival of patients with low-grade glioma in

the past studies. However, temozolomide has a better tolerance and it is easily administrated.[28] We used CV in 8 and temozolomide in 5 low-grade glioma patients as a primary CTX drug. Carboplatin-vincristine was switched to temozolomide in one patient due to progressive disease. We added RTX to CTX in two patients. One of these had GBM, and the other had a brainstem glioma. The patient with GBM died due to progressive disease. High-grade CNS tumors were reported in a few patients with NF-1, and these patients had a poor prognosis.[29]

In the recent years, several studies investigating risk factors of glioma formation and progression in NF-1 were carried out. Various factors, such as the germline NF1 gene mutation, patient age, patient sex, background genomics (ethnicity/race) and co-existing atopic conditions (eczema, asthma) were investigated.[30–34] Risk factors related to vision loss were defined as female sex, age less than two years, and posterior involvement in optic glioma with NF-1, as well as in optic glioma without NF-1.[35–37] However, optic glioma incidence in NF1 is similar in male and female sex.[38,39] The parental age and the presence of tumors was not correlated in the NF-1 patients.[40] Tabata et al.[19] did a cluster analysis in adults with NF 1 and found positive correlations between spinal neurofibromas and optic gliomas, and also, between optic gliomas and sphenoid wing dysplasia. Furthermore, they reported increasing cutaneous neurofibromas as a risk factor for MPNST.[19] In our study, gender and age were unremarkable in patients with malignancy. We could not find any NIH diagnostic criteria as a risk factor in the univariate analysis of malignancy-risk factors. However, we found that having more than three diagnostic criteria except optic glioma increased risk of malignancy six times.

Retrospective design and limited number of patients in the cohort were the limitations of the study. Also, the study group consisted of patients referred in the pediatric oncology clinic, so patients may not have a homogeneous distribution.

Conclusion

Children with NF-1 have clinical heterogeneity similar to adult patients. Malignancies are the most crucial factor in mortality and morbidity in NF-1. Risk factors for developing malignancy in NF-1 are still unclear. However, we suggest being vigilant about potential malignancy in patients with more diagnostic criteria.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Conflict of Interest: There are no conflicts of interest in connection with this paper, and the material described is not under publication or consideration for publication elsewhere.

Ethics Committee Approval: The study was carried out with the permission of Kocaeli University Clinical Researches Ethics Committee (Date: 18.03.2014, Decision No: 14/98).

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Evaluation of the Renal Function in the Intrauterine Growth Restricted Rats and the Effect of Maternal Glucocorticoids

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Abstract

The purpose of the study was to determine the effect of maternal glucocorticoids on experimental growth retarded rats and the effect of maternal undernutrition in different gestation periods for function of the kidney. This study had two sections. In the first section, 5 groups were formed. 10g/d diet was given in the 1st, 2nd, and 3rd trimester and throughout the pregnancy period. The control group was fed a normal diet. In the second section, 3 groups formed and all the rats take 10 g/d diet throughout pregnancy period. To determine the effect of endogenous glucocorticoids first group was treated with metyrapone, second group metyrapone+dexamethasone and the placebo injected saline until 14 days of pregnancy. The offsprings body and kidney weights were detected in the 0, 3rd and 20th weeks of age. Renal extraction functions and blood pressures from tail detected in the 20th week of age. Urinary excretion and glomerular filtration rate were low in rats that had dietary restriction in the last trimester. The glomerular filtration rates were found to be low in the group that had diet restriction during the whole pregnancy. Blood pressure values were found to be lower in the group that had diet restriction during their pregnancy compared to the control group. Kidney weights were similar in all groups in the first phase. It was observed that renal excretion functions were preserved in the group receiving metyrapone treatment, but there was no statistically significant difference between the results. Low blood pressures were normalized with metyrapone treatment. The kidney sizes at the 20th week of the rats which receiving metyrapone treatment were found to be smaller than those receiving physiological saline solution. Food restriction destroys renal functions but no effects with high blood pressure in adulthood. Glucocortiod exposure in pregnancy may reduce renal development.

Keywords: IUGR, Maternal glucocorticoids, renal excretion functions

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Introduction

Intrauterine growth retardation (IUGR) is a decrease in fetal growth rate due to genetic and environmental effects and the inability of the fetus to reach its genetic potential growth.[1] Maternal undernutrition during pregnancy reduces fetal and placental growth in animals and humans. It has been shown in clinical and experimental studies that maternal malnutrition may cause permanent structural changes in the baby's kidneys, which in turn increases the tendency to adult heart and kidney diseases and may lead to hypertension.[2-5] In fetuses exposed to endogenous and exogenous excessive glucocorticoids during the intrauterine period, HT and many metabolic changes can be observed in advanced ages, in addition to IUGR.[6] This study, it was aimed to evaluate the effects of maternal balanced nutrient restriction on kidney growth, renal excretion functions and blood pressure, and to evaluate the role of excessive maternal glucocorticoid exposure in the intrauterine period.

Highlights

- Intrauterine growth retardation (IUGR) may result in permanent structural changes in kidneys, which in turn increases the tendency to adult heart and kidney diseases and may lead to hypertension
- The effects of maternal balanced nutrient restriction on kidney growth, renal excretion functions, blood pressure the role of excessive maternal glucocorticoid exposure in the intrauterine period remains unclear
- Nutritional restriction during pregnancy, especially during pregnancy and in the last trimester, causes intrauterine growth retardation and deterioration in kidney functions.
- IUGR adversely affects kidney functions, but this effect is independent of the increase in endogenous glucocorticoids.
- Total restriction of nutrients during pregnancy adversely affects intrauterine growth, kidney growth, and kidney excretion functions,
- Total restriction of nutrients during pregnancy does not cause adult blood pressure elevation

Material and Method

All procedures were approved by the local Ethics Committee. All rats received care in compliance with the Principles of the guide for the Care and Use of Laboratory animals. In the study, 45 virgin female Wistar Albino rats were kept in a room with controlled temperature (22±1ºC) and with artificial dark-light cycle (12L:12D). Female rats were mated with male rats for one night. The first day of pregnancy was determined by the demonstration of spermatozoa in the vaginal smears. Pregnant female rats were randomly divided into two groups for the stages of the study and then transferred into individual cages. Maternal weight gains were evaluated during pregnancy.

First Stage

Restricted diet group rats (n=21) were randomly divided into 5 groups. The first group was determined as the control group and fed a normal chow diet. The other groups had a half-restricted diet (10 gr/day feed) at different periods of their pregnancy period. Half restricted diet was given between 0-7th days of pregnancy (first trimester) at the second group of rats. The diet was given between the 8-14th days of pregnancy (second trimester) at the third group of rats and the diet was given between 15-22th days of gregnancy (third trimester) at the fourth group of rats. Fifth group of rats was fed with half restricted diet throughout the pregnancy period. Water was always available ad libitum for all experimental groups. Pregnant mothers gave birth by vaginal delivery on an average of 22 days. Baby rats were evaluated at the 0th, 3rd, and 20th weeks. The weights of the 0-week-old puppies were recorded. After anesthesia (100 mg/g intraperitoneal ketamine) was given, the kidneys were removed and the kidney weights were recorded. Surviving baby rats were allowed to stay with their mothers until they were three weeks old and their weekly weights were recorded. After anesthesia that was given in the 3rd week, the kidneys were removed and their weights were recorded. The remaining baby rats were separated from their mothers from this period onwards and were kept alive until the 20th week by allowing normal food and water intake. In the 20th week, the blood pressures of the baby rats were measured by the tail blood pressure measurement method. One day before the renal hemodynamic evaluation, they were placed in the metabolic cage and their 24-hour urine was collected. A blood sample was taken for biochemical evaluation. After anesthesia was given, the kidneys were removed and their weights were recorded.

Second Stage

The diets of the rats (n=18) in the metyrapone group were restricted by approximately 50% during their pregnancy and were given 10 gr of feed daily. Rats were randomly divided into 3 groups. In the first group, 0,5 mg/ml dose of metyrapone was added to the drinking water and water was available ad libitum between the 1-14th days of pregnancy. In the second group, 0,2 mg/kg dexamethasone (to replace the suppressed maternal glucocorticoids) was injected intraperitoneally at the same time in the morning between the 1-14th days of pregnancy besides metyrapone treatment. In the third group, between the 1-14th days of pregnancy physiological saline solution was injected intraperitoneally. Pregnant rats gave birth by normal vaginal delivery. Postpartum mothers were given a standard laboratory diet. The body weights of some of the baby rats were measured on the first day and after they were anesthetized, their kidneys were removed and kidney weights were measured. When the remaining baby rats were three weeks old, some of the baby rats were anesthetized after their body weights were recorded, then their kidneys were removed and kidney weights were recorded. The remaining group was separated from their mothers and given a standard laboratory rat diet and tap water ad libitum. Weekly body weights were recorded. In the twentieth week, the blood pressures of the baby rats were measured by the tail blood pressure measurement method. One day before the renal hemodynamic evaluation, they were placed in the metabolic cage and 24-hour urine was collected. A blood sample was taken for biochemical evaluation. After anesthesia was given, the kidneys were removed and kidney weights were recorded.

Serum Cr and urine Cr, Na, K levels were studied in the biochemistry laboratory of our hospital using the Konelab60i autoanalyzer device and Thermo Clinical Labsystem (Finland) kits.

Kidney Weight

When the kidneys of the offspring in both the restricted diet group and the metyrapone group were removed at the 1st day, 3rd, and 20th weeks, right and left kidney weights were recorded separately. Fractional kidney weight (FKW) was obtained by dividing the right kidney weight of the offspring by body weight and multiplying by 100. It was expressed as %.

Blood Pressure Measurement

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) of conscious subjects were measured by indirect tail-cuff method (MAY BPHR 9610-PC TAIL-CUFF indirect blood pressure recorder). The blood pressure values obtained were recorded on the computer. The average of the 3 blood pressure values measured from each subject was taken.

Clearance Calculations

Clearance calculations were made using the following formulas.

Urine flow rate (UF) = urine volume/time (µl/min)

Excretion of a substance = urine concentration of this substance x urine flow rate

Sodium excretion (UVNa) was expressed as µmol/min.

Potassium excretion (UVK) was expressed as µmol/min.

Creatinine clearance (ClCr) = creatinine excretion/serum Cr concentration (expressed as ml/min).

Glomerular filtration rate (GFR) was calculated as creatinine clearance (Cr excretion rate divided by serum concentration).

Statistical Analysis

Data were evaluated in Statistical Package for Social Sciences (SPSS) 15.0 and SigmaStat 3.5 Statistical Package Programs. Mean, standard deviation (SD), median, minimum (min), and maximum (max) values were given as descriptive statistics. Kolmogorov-Smirnov and Shapiro Wilk normality tests were used to determine whether the data showed normal distribution. In comparisons involving three or more groups, One-Way Analysis of Variance (One-Way ANOVA) was used for normally distributed variables, and Kruskal Wallis Analysis was used for non-normally distributed variables. Tukey test was used to compare the groups with differences in One Way Analysis of Variance and Dunn Method was used for comparison of groups with differences in Kruskal Wallis Analysis. A p-value of <0.05 was considered statistically significant in all statistical analyses.

RESULTS

Body Weights

When the body weights of the offspring of the rats exposed to food restriction during different gestational periods were compared, it was seen that the lowest body weight was in the rats that were exposed to the restricted diet during pregnancy. Although the first-day body weight was found to be low in the group that received a restricted diet in the last trimester, there was no statistically significant difference when compared with the other groups (**Table 1**). In the comparison of the 3rd-week weights of the restricted diet group rats, the lowest body weight was found in the rats exposed to the restricted diet in the last trimester (**Table 2**). In the 20th week rats, the lowest body weight was found in the group that received a restricted diet in the second trimester. Although the 20th-week body weight of the group that had diet restriction during pregnancy was low, no statistically significant decrease was found (**Table 3**).

In the metyrapone group, which was created to evaluate the effects of endogenous and exogenous steroids, when the body weights of the 1st day, 3rd week, and 20th week were compared, no statistically significant difference was found between the groups (data not shown).

Table 1
1st-day body weights of rats in the restricted diet group.

	Control		0-7th days		8-14th days		15-22nd days		during pregnancy		
	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	p value
BW 1st day (gr)	19	5.09±0.41	34	5.26±0.46	22	5.32±0.53	27	4.97±0.46	25	4.64±0.51[a,b,c]	<0.05

[a] p<0.005; compared to the control group, [b] p<0.005; compared to the group that received a restricted diet between the 0-7th days, [c] p<0.005; compared to the group that received a restricted diet between the 8-14th days

Table 2
Body weights of the restricted diet group rats at the 3rd week.

	Control		0-7th days		8-14th days		15-22nd days		during pregnancy		
	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	p value
BW 3rd week (gr)	13	30.11±3.33[a,b,c]	23	25.18±6.24[b]	15	21.11±2.02	16	19.07±2.51	14	22.13±1.84	<0.05

[a] p<0.005; compared to the group that received a restricted diet between the 8-14th days, [b] p<0.005; compared to the group that received a restricted diet between the 15-22nd days, [c] p<0.005; compared to the group that received a restricted diet during pregnancy.

Table 3
Body weights of rats in the restricted diet group at the 20th week.

	Control		0-7th days		8-14th days		15-22nd days		during pregnancy		
	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	p value
BW 20th week (gr)	7	225.63±59.78	13	233.76±58.23	7	170.58±32.57[a]	8	214.75±44.8	6	190.28±41.47	<0.05

[a] p<0.005; compared to the group that received a restricted diet between the 0-7th days

Kidney Weight

There was no difference between the groups in terms of kidney weights of rats at the 0th, 3rd, and 20th weeks in all groups that in the first stage. The kidney sizes of the offspring of the rats receiving metyrapone treatment at the 20th week were found to be smaller than those receiving physiological saline solution (data not shown).

Renal Excretion Functions

Urinary excretion and glomerular filtration rate (GFR) were low in rats that had a dietary restriction in the last trimester. The glomerular filtration rate was found to be low in the group that had a diet restriction during the whole pregnancy (**Table 4**). In rats with intrauterine growth retardation, renal excretion functions were preserved in those treated with metyrapone, but no statistically significant difference was found between the results (**Table 5**).

Blood Pressure Values

Blood pressure values were found to be lower in the group that had a diet restriction during their pregnancy compared to the control group (**Figure 1**). Low blood pressures were normalized with metyrapone treatment (**Figure 2**).

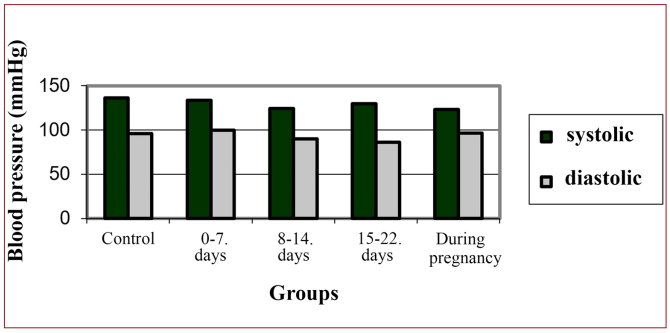

Figure 1. 20th week blood pressures of the restricted diet group rats

Discussion

Many epidemiological pieces of evidences show that the risk of hypertension and coronary heart disease in adults is not only determined by adult lifestyle and environment but also associated with fetal life. Fetal growth pattern (low birth weight) related to malnutrition during pregnancy is associated with elevated blood pressure and increased cardiovascular mortality in childhood.[7-9] Although the growth path of the fetus is primarily determined by genetic factors, it is also guided by the maternal environment.[9] Among the methods that cause fetal growth retardation, the most commonly used method is to reduce the amount of food for the mother, which can be either by reducing the total intake or by reducing the nutritional content such as protein, vitamin A, sodium or iron. In addition, IUGR is created by placental embolization, surgical reduction of placental blood flow, or the use of steroids.[10] In our study, we reduced the amount of maternal nutrition by 50% (10 g/day) to create IUGR. We applied half-nutrition restriction throughout pregnancy or at different periods of pregnancy for one-week periods in order to determine which nutritional deficiency period is more effective on the renal function. No dietary restriction was applied during pregnancy in the control group rats. With this method, the 1st-day body weights of the offsprings in the group that received a restricted diet during pregnancy were significantly lower than the other groups. The offspring of mothers which were fed

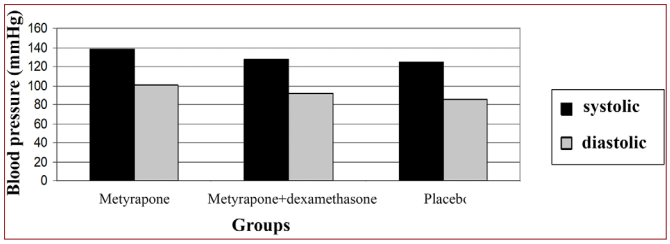

Figure 2: 20th week blood pressures of the metyrapone group rats

Table 4
Renal excretion functions of rats in the restricted diet group at the 20th week.

	Control		0-7th days		8-14th days		15-22nd days		during pregnancy		p value
	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	n	mean±SS	
UF (µl/min)	7	5.32±2.36	13	5.40±5.75	7	3.28±0.86	8	2.67±0.63[a,b]	6	3.03±0.94	<0.05
UVNa (µmol/min)	7	0.42±0.2	13	0.4±0.15	7	0.38±0.053	8	0.39±0.1	6	0.36±0.1	>0.05
UVK (µmol/min)	7	1.30±0.48	13	1.28±0.33	7	1.41±0.21	8	1.29±0.29	6	1.08±0.28	>0.05
GFR (ml/min)	7	1.69±1.01	13	1.16±0.43	7	1.11±0.48	8	0.76±0.30[a]	6	0.84±0.25[a]	<0.05

[a] p<0.005; compared to the control group, [b] p<0.005; compared to the group that received a restricted diet between the 0-7th days

Table 5
Renal excretion functions of 20th week rats in the metyrapone group.

	Metyrapone		Metyrapone+Dexamethasone		Plasebo		p value
	n	mean±SS	n	mean±SS	n	mean±SS	
UF (µl/min)	6	3.99±0.3	11	3.29±1.66	11	3.41±1.9	>0.05
UVNa (µmol/min	6	0.49±0.08	11	0.35±0.16	11	0.34±0.13	>0.05
UVK (µmol/min	6	1.5±0.14	11	1.26±0.45	11	1.18±0.46	>0.05
GFR (ml/min)	6	1.06±0.3	11	0.89±0.35	11	1.02±0.41	>0.05

a restricted diet in the third trimester of pregnancy also tended to have low body weight. Perez et al.[5] also found low weight of the offspring of pregnant rats, which they fed with a diet reduced by 50% in the last two trimesters of pregnancy. Langley-Evans et al.[11] reported that intrauterine growth retardation occurred in the offspring of pregnant rats who underwent protein restriction both during pregnancy and in the third trimester in the intrauterine growth retardation model they created with protein restriction during pregnancy and in 3 different trimesters of pregnancy. Our findings are consistent with the findings of these reports.

It is known that the availability of food influences the rhythm of the HPA axis. Indeed, fasting and food restriction increase HPA axis activity in both humans and rats, leading to secondary adrenal hypertrophy and an increase endogenous steroids.[12] In the metyrapone group, which was created to evaluate the effects of endogenous and exogenous steroids, 50% dietary restriction was applied throughout their pregnancy. There was no statistically significant difference between the groups in the 1st day, 3rd week, and 20th week body weights. This finding suggested that metyrapone treatment had no effect on the weight of baby rats exposed to a restricted diet during the intrauterine period. This result can be interpreted as maternal steroid synthesis, which occurs in response to dietary restriction, does not have a direct effect on the body weights of the offspring. However, Smith and Waddell[13] reported that administration of dexamethasone and carbenoxolone decreased birth weight in rats fed normally during pregnancy, while metyrapone treatment increased birth weight.

In the study by Langley-Evans et al.[11] birth kidney weights of rats exposed to protein restriction between the 8-14th, 15-22nd, and 0-22nd days were detected lower than the control group since it was not statistically significant, so they emphasized that the diet has no negative effect on kidneys. However, in the same study between the 0-7th days, the relative kidney weight of the group that received 9% casein was found to be statistically significantly higher than the control group. In our study, the 1st day kidney weights of rats exposed to an intrauterine restricted diet were lower than the control value; this decrease was more evident in those which were exposed to a restricted diet during pregnancy and in the first trimester. This decrease in kidney weight persisted when corrected for body weight. Our findings are similar to the findings of the study by Langley-Evans et al.[11]

In our study, kidney weight in the 3rd week was found to be low in rats exposed to a restricted diet in the 3rd trimester of pregnancy. However, when corrected for body weight (fractional kidney weight) was higher in these baby rats than in control baby rats. Fractional kidney weights were higher in offspring exposed to restricted diet in the 1st and 2nd trimesters of pregnancy than controls. There might be two reasons for this result. The first is the low body weight of the offspring during this period; the ratio of kidney weight to body weight might be increased. Second, the initially small kidneys might be hypertrophied as a result of a possible decrease in the number of nephrons. We think that these two reasons together contribute to this result.

Intrauterine nutritional deficiency appears to impair kidney development by several mechanisms. It is known that the renin-angiotensin system plays an important role in the normal morphological development of the kidney. Previous studies support that renal renin and angiotensin II mRNA levels are significantly lower in newborns born with intrauterine nutritional deficiency and that suppression of intrarenal RAS may affect nephrogenesis.[14]

Intrauterine nutritional deficiency causes an increase in maternal glucocorticoids and ultimately increases the exposure of the fetus to glucocorticoids. Metyrapone is a potent inhibitor of 11-β hydroxylation of corticosteroids; inhibits corticosterone synthesis from maternal and fetal adrenal glands. In the second part of our study, it was aimed to investigate the effects of maternal glucocorticoids on fetal kidney development in intrauterine nutritional deficiency. For this purpose, endogenous glucocorticoid synthesis was suppressed by giving metyrapone to pregnant rats which received 50% reduced food during pregnancy, and dexamethasone was given externally to another group which glucocorticoid synthesis was suppressed to bring out the effect of suppressed glucocorticoids again. In our study, it was found that metyrapone treatment did not prevent low birth weight in pregnant rats given a restricted diet. In contrast, 1st day absolute kidney weights were better preserved in the offspring of mothers treated with metyrapone and metyrapone+dexamethasone. However, this effect did not persist in advancing periods of life. Since we could not determine the number of nephrons, we cannot comment on the effect of metyrapone treatment on the number of nephrons.[15,16]

Decreased birth weight and kidney weight, high blood pressure, increased albuminuria, low GFR, low Na excretion, decreased fractional sodium excretion and high tissue sodium content were found in fetal programming models that were given both prenatal dexamethasone and maternal protein restriction.[17,18] Although it was not statistically significant, sodium excretion of baby rats exposed to restricted diet during pregnancy tended to be low in our study. Urinary sodium excretion increased with metyrapone therapy (not statistically significant). This finding supports that maternal glucocorticoids reduce sodium excretion.

Systolic blood pressures of puppies born with IUBG tended to be lower than control rats. No difference was found in diastolic blood pressures. In the studies conducted by Langley-Evans et al.[11,19] they found high blood pressure in the offspring of rats fed low protein. While specific nutrient (protein) restriction was applied in these studies, we applied global energy restriction. The difference in our study from these data can be explained by the difference in method. In the study of Holemans et al.[20] in which they performed food restriction, blood pressure was found to be normal. Our findings and those of Holemans K. may indicate that balanced nutrient restriction during pregnancy has less effect on offspring blood pressure than specific nutrient deficiency.

The diastolic blood pressure of the rats treated with metyrapone were higher than the control IUGR rats. This finding can be interpreted as normalization of blood pressure. A possible explanation of this finding may

be metyrapone treatment increased intrarenal renin-angiotensin system (RAS) activity. In studies investigating the interaction between glucocorticoid and intrarenal RAS, there are data that glucocorticoid excess has negative effects on the RAS components of the fetus.[1]

In our study, 24-hour urine of 20-week-old baby rats was collected in metabolic cage and creatinine clearance was calculated. It was determined that the GFR values of the offspring born to mothers which were given a half-restricted diet on the 15-22nd days of pregnancy and during pregnancy (0-22nd days) were statistically significantly lower than the control group. In the second stage of the study, when the rats were evaluated in terms of kidney functions, GFR reduction was more pronounced in the group that received exogenous glucocorticoid therapy in addition to metyrapone. There was no significant effect of metyrapone treatment on GFR. This finding supports that the negative effect of intrauterine growth retardation induced by the dietary restriction on kidney functions is through mechanisms different from the increase in endogenous glucocorticoids.

When evaluated in terms of renal excretion functions, sodium and potassium excretion of baby rats born to mothers which had dietary restrictions during pregnancy were low, although there was no statistically significant difference. It was observed that sodium and potassium excretion returned to normal with metyrapone treatment. We can say that this positive effect of metyrapone treatment on renal sodium and potassium excretion is independent of GFR. Based on this finding, we can conclude that the increase in endogenous glucocorticoids has negative effects on kidney structure as well as negative effects on sodium and potassium transport mechanisms of the kidney. We think that studies at the molecular level are needed to clarify this situation.

Conclusion

In this study, we found that total restriction of nutrients during pregnancy adversely affects intrauterine growth, kidney growth, and kidney excretion functions, but does not cause adult blood pressure elevation. We can say that some of these negative effects are due to the increase in maternal endogenous glucocorticoids that occur in intrauterine nutritional deficiency. Further studies are needed to show the effect of maternal endogenous glucocorticoid increase on kidney structure and functions.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Conflict of Interest: There are no conflicts of interest in connection with this paper, and the material described is not under publication or consideration for publication elsewhere.

Ethics Committee Approval: The study was carried out with the permission of Erciyes University Faculty of Medicine Ethics Committee (Date: 2005, Decision No: 05/159)

Financial Disclosure: The authors have no conflicts of interest to declare.

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Prevalence of Methotrexate Toxicity and Intolerance in Juvenile Idiopathic Arthritis and Possible Risk Factors for Methotrexate Intolerance: A Tertiary Center Experience

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Abstract

Methotrexate has a safe and inexpensive profile and is the first choice of juvenile idiopathic arthritis treatment. Nevertheless, gastrointestinal symptoms are not serious but are common side effects of methotrexate. Sometimes patients may have nausea by thinking of methotrexate and even refuse methotrexate use. In this study, we aimed to define the prevalence of methotrexate side effects in juvenile idiopathic arthritis and the possible risk factors for methotrexate intolerance. Methotrexate intolerance severity score showed a severity of gastrointestinal symptoms. Eighty-seven patients accepted to join the study and answered the questions. The prevalence of adverse events of methotrexate was 64.4% and the rate of gastrointestinal symptoms was 55.2%. Nausea (27.6%) was the most common gastrointestinal symptom. The median methotrexate intolerance severity score was 14.5 (interquartile range: 10-18). However, there was no significant difference in main parameters between tolerant and intolerant groups and no risk factor was observed for methotrexate intolerance. In a conclusion, we observed methotrexate toxicity and intolerance commonly but no associative factors were defined, and, prospective larger studies are necessary to understand and prevent the occurrence of gastrointestinal symptoms.

Keywords: Juvenile idiopathic arthritis, intolerance, methotrexate, nausea

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Introduction

Methotrexate (MTX) has a broad usage in pediatric rheumatic diseases because of its effective, safe, and affordable profile. It is the most commonly selected medication in pediatric rheumatology practice.[1,2] Additionally, MTX is the first and main choice of disease-modifying anti-rheumatic drug (DMARD) for juvenile idiopathic arthritis (JIA).[2] JIA is the most frequent rheumatic disease in childhood and is characterized by arthritis with unknown etiology that is constant for at least 6 weeks in children under 16 years.[3] Despite its many advantages, low dose MTX may lead to adverse effects such as gastrointestinal (GI) symptoms (abdominal pain, nausea, vomiting), hepatotoxicity, rash, and bone marrow suppression. While gastrointestinal symptoms occur frequently, the other symptoms are rare.[1,4-6] MTX toxicity is the occurrence of adverse effects after MTX administration.[4,7] GI symptoms (nausea, vomiting) or fatigue are observed circa 4-36 hours after MTX administration.

Highlights

- There is no prominent risk factor for MTX intolerance in JIA.
- The preventions for MTX intolerance were not enough effective.
- MISS is a practical and easy applicable tool to show MTX intolerance.
- Self-discontinuation due to MTX intolerance is a main reason to interrupt of therapy.

Furthermore, JIA patients meet anticipatory and associative adverse effects of MTX. The anticipatory symptoms occur before MTX administration, such as nausea or abdominal pain by seeing syringe or hospital. The associative symptoms (nausea, vomiting, abdominal pain) happen by thinking, smell, or color of MTX.[7] These anticipatory and associative symptoms are termed MTX intolerance. There is a validated questionnaire for evaluating MTX intolerance in JIA patients. The Methotrexate Intolerance Severity Score (MISS) includes 12 items consisting of abdominal pain, nausea, vomiting, and behavioral changes.[7]

In this study, we aimed primarily to define the prevalence of MTX intolerance and toxicity in JIA patients. The second aim is to identify risk factors for MTX intolerance by comparing JIA patients with and without MTX intolerance.

Materials and Method

Study Population and Design

A cross-sectional descriptive study included JIA patients who were treated with MTX for at least 6 months. One hundred fifty-five patients were listed and the data of patients were collected from electronic files. Three patients did not fulfill the inclusion criteria and the data of ten patients were missing.

For MISS questionnaire, 142 patients were telephoned of which parents of 87 patients accepted the conversation and completed the MISS questionnaire.

The demographic, clinic and laboratory findings at diagnosis [C-reactive Protein (CRP), erythrocyte sedimentation rate (ESR), alanine aminotransferase (ALT)], disease activity, MTX dose, folic acid dose, MTX duration, adverse effects of MTX (abdominal pain, nausea, vomiting, behavioral symptoms, liver enzymes elevation, cytopenia, rash, oral ulcer), accompanying GI disease were collected retrospectively. Juvenile arthritis disease activity score (JADAS) was used to score disease activity. JADAS includes 4 items; the joint count, the physician and the patient's/parent's global assessment, and the inflammatory marker (ESR or CRP).[8] The ethics committee of Ankara City Hospital approved this study. All procedures were conducted according to the principles of the Declaration of Helsinki, and human and animal rights.

Definition of MTX Intolerance

Methotrexate intolerance severity score is used to determine MTX intolerance. MISS is a questionnaire that was developed for MTX intolerance in JIA and includes four components.7 The first three components categorize the abdominal pain, nausea, and vomiting as; after MTX use, anticipatory (before MTX), and associative (reminding of MTX) and the last part consists of behavioral symptoms (restlessness, crying, irritability, refusing) due to MTX induced GI symptoms. The patient should define the severity of symptoms by scoring 1 (mild), 2 (moderate), or 3 (severe). The MISS value ≥6 (maximum score: 36) and at least 1 anticipatory, associative or behavioral symptom showed MTX intolerance.7

Statistical Analysis

Data of the present study were assessed using SPSS (Version 22.0). In this study, quantitative variables were evaluated using the Kolmogorov-Smirnov test, detrended Normal Q-Q Plot, and histogram to define whether they were normally distributed. Normally distributed data were expressed as mean, and standard deviation (SD). Non-normally distributed data were presented as median, interquartile range (IQR). The categorical data were expressed in count and percentage.

A parametric test (Student -t) was used to compare normally distributed independent quantitative variables. If a parametric test was not provided for quantitative parameters, the Mann-Whitney U test was used to compare the independent groups. Differences between categorical data were analyzed using the Chi-square test. P values <0.05 with a 95% confidence interval were considered significant.

Results

Main Characteristics of Patients

Of 87 JIA patients, 61 (70.1%) were female and the median age was 13 (9-17) years. The most common form of JIA was oligoarticular JIA (59.8%) and the median follow-up was 49 (22-73) months. The median duration of MTX use was 24 (16-34) months and the main findings are in **Table 1**.

Table 1
Baseline characteristics of participants

Female, n (%)	61 (70.1%)
JIA subtypes, n (%)	
Oligoarticular JIA	52 (59.8%)
Polyarticular JIA	21 (24.1%)
Systemic onset polyarticular JIA	2 (2.3%)
Enthesitis-related JIA	12 (13.8%)
Age at diagnosis, median (IQR)	7 (3-12) years
JADAS at diagnosis, median (IQR)	21 (18-25)
Age at MTX onset, median (IQR)	9 (4-13) years
Duration of MTX use, median (IQR)	24 (16-34) months
Dose of MTX, median (IQR)	12.5 (10-15) mg/week
Administration of MTX, n (%)	
Oral	19 (21.8%)
Subcutaneous	48 (55.2%)
First oral later subcutaneous	20 (23%)
Folic acid, n (%)	
No	3 (3.4%)
3.75-7.5 mg/week (3 days/week)	75 (86.2%)
7.5-15 mg/week (6 days/week)	9 (10.3%)
Biologic therapy, n (%)	26 (29.9%)
Additional rheumatologic disease, n (%)	4 (4.6%)

JIA: juvenile idiopathic arthritis, JADAS: juvenile arthritis disease activity score, MTX: methotrexate, IQR: interquartile range

Prevalence of MTX Toxicity and Intolerance

In this study, 56 (64.4%) patients had MTX side effects. Of 56 patients, 20 (23%) had purely MTX toxicity, 8 (9.2%) had purely MTX intolerance and 28 (32.2%) had both of them together. GI toxicity occurred in 39 (44.8%) patients after MTX administration and nausea was the most common adverse effect (**Table 2**). MTX intolerance was observed in 36 patients who had MISS ≥6 and, all of them had GI symptoms and nausea as the most frequent symptom (27.6%) (**Table 2**). The median value of the MISS questionnaire was 14.5 (10-18) and detailed results of the MISS questionnaire are summarized in **Table 3**. We observed MTX intolerance in the first 6 months of treatment (**Figure 1**). The MTX reminders such as a syringe, yellow color, and thinking or talking about MTX might cause intolerance. The thinking of MTX was the most common trigger of symptoms (**Table 2**). Self- discontinuation of MTX occurred in 14 (16.1%) patients due to MTX intolerance. In some cases, we tried to prevent intolerance by changing the dose or administration way. Use of folic acid or anti-emetics were other preventions (**Table 2**).

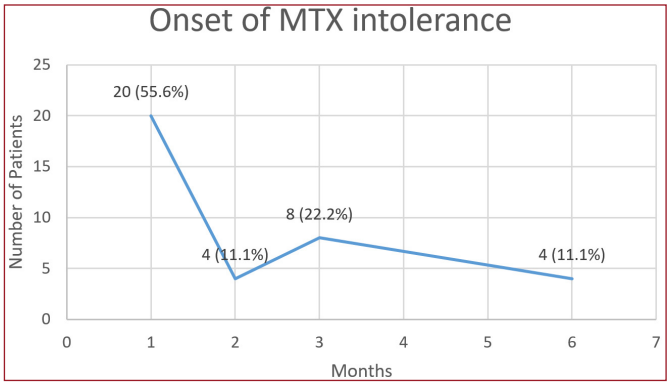

Figure 1. Distribution of MTX intolerance onset
MTX, methotrexate

Table 2
The prevalence of MTX toxicity and intolerance, the results of the MISS questionnaire

MTX toxicity, n (%) (after MTX)	**48 (55.2%)**
GI symptoms	39 (44.8%)
Abdominal pain	3 (3.4%)
Nausea	38 (43.7%)
Vomiting	23 (26.4%)
Liver enzymes elevation	16 (18.4%)
Oral ulcers	4 (4.6%)
Cytopenia	3 (3.4%)
MTX intolerance, n (%) (before MTX)	**36 (41.4%)**
Abdominal pain	0
Nausea	24 (27.6%)
Vomiting	11 (12.6%)
Fatigue	4 (4.6%)
MISS (Range: 0-36), n (%)	
MISS (0)	38 (43.7%)
MISS (1-5)	13 (14.9%)
MISS (6-36)	36 (41.4%)
Triggers of MTX intolerance, n (%)	**30 (34.5%)**
Doctor office	6 (6.9%)
Syringe/tablet	18 (20.7%)
Yellow color	9 (10.3%)
Talking about MTX	20 (23%)
Thinking about MTX	25 (28.7%)
Preventions of MTX intolerance, n (%)	**24 (27.6%)**
Reducing MTX dose	4 (4.6%)
Changing route of MTX administration	12 (13.7%)
Stopping MTX	3 (3.4%)
Elevation of folic acid	3 (3.4%)
Antiemetic drugs	2 (2.3%)

MTX, methotrexate; GI, gastrointestinal; MISS, methotrexate intolerance severity score

Table 3
The prevalence and severity of gastrointestinal and behavioral symptoms due to MTX intolerance

	No symptom	Mild symptom	Moderate symptom	Severe symptom
Abdominal pain, n (%)				
after MTX	84 (97.3%)	-	2 (2.3%)	1 (1.1%)
anticipatory	87 (100%)	-	-	-
associative	87 (100%)	-	-	-
Nausea, n (%)				
after MTX	51 (58.6%)	6 (6.9%)	13 (14.9%)	17 (19.5%)
anticipatory	65 (74.7%)	-	12 (13.8%)	10 (11.5%)
associative	78 (89.6%)	-	3 (3.4%)	6 (6.9%)
Vomiting, n (%)				
after MTX	65 (74.7%)	3 (3.4%)	5 (5.7%)	14 (16.1%)
anticipatory	76 (87.4%)	2 (2.3%)	5 (5.7%)	4 (4.6%)
Behavioral symptoms, n (%)				
restlessness	55 (63.2%)	3 (3.4%)	13 (14.9%)	16 (18.4%)
crying	64 (73.6%)	3 (3.4%)	6 (6.9%)	14 (16.1%)
irritability	67 (77%)	2 (2.3%)	3 (3.4%)	15 (17.2%)
refusal of MTX	54 (62.1%)	3 (3.4%)	8 (9.2%)	22 (25.3%)

MTX, methotrexate

Evaluating Risk Factors for MTX Intolerance

The main characteristics of tolerant and intolerant patients were compared to define probable factors that might cause MTX intolerance. There was no significant relation between MTX intolerance and gender, JIA subgroups, laboratory parameters at diagnosis (AST, ALT, CRP, ESR, CBC), JADAS at onset, age at diagnosis, age at MTX onset, the dose of MTX and duration of MTX use. There was no significant difference in the main parameters for tolerant and intolerant groups (**Table 4**).

Table 4
Comparing tolerant and intolerant patients to define risk factors

	MTX tolerant	MTX intolerant	p value
Gender, n (%)			0.238*
Girls	33 (37.9%)	28 (32.2%)	
Boys	18 (20.7%)	8 (9.2%)	
JIA subtypes, n (%)			0.378*
Oligoarticular JIA	28 (32.2%)	24 (27.6%)	
Polyarticular JIA	14 (16.1%)	9 (10.3%)	
Enthesitis-related JIA	3 (3.4%)	9 (10.3%)	
Age at diagnosis, median (IQR)	7 (2-12)	8 (5-13)	0.31**
JADAS at diagnosis, median (IQR)	17.4 (20.9-25.2)	18.4 (21.1-24.6)	0.67**
Age at MTX onset, median (IQR)	8 (3-12)	9 (5.25-13.75)	0.286**
Duration of MTX use, median (IQR)	23 (16-29)	27.5 (15-48)	0.165**
Dose of MTX, median (IQR)	13.2 (10-15)	12.5 (10-18.75)	0.962**
Administration of MTX, n (%)			0.151*
Oral	14 (16.1%)	5 (5.7%)	
Subcutaneous	31 (35.6%)	17 (19.5%)	
Oral → subcutaneous	6 (6.9%)	14 (16.1%)	
Folic acid, n (%)			0.389*
No	1 (1.1%)	2 (2.3%)	
3.75-7.5 mg/week (3 days/week)	46 (52.9%)	29 (33.3%)	
7.5-15 mg/week (6 days/week)	4 (4.6%)	5 (5.7%)	
Biologic therapy, n (%)	16 (18.4%)	10 (11.5%)	0.814*

MTX, methotrexate; JIA, juvenile idiopathic arthritis; JADAS, juvenile arthritis disease activity score; IQR, interquartile range, *Chi-Square test, **Mann Whitney U test

Discussion

In the current study, we focused on the prevalence of MTX toxicity and intolerance in JIA, the results of the MISS questionnaire, and the probable risk factors for MTX intolerance. Among 87 JIA patients, we found that 55.2% had MTX toxicity and 41.4% had MTX intolerance with MISS of 6 and over. Nausea was the most common symptom before and after MTX. The remembering or thinking of MTX was the most frequent trigger of MTX intolerance. Additionally, we observed no significant association between MTX intolerance and other parameters.

Juvenile idiopathic arthritis is the most common rheumatic disease in childhood and might have devastating results without effective treatment. MTX is a central point of medical therapy of JIA because of its safety and effectiveness.[1,2,5] Nevertheless, MTX can have rarely serious side effects like bone marrow suppression or elevation of liver enzymes. On the other hand, MTX-induced GI symptoms were quite common in clinical practice.[4,7] The clinicians usually ignore GI symptoms of MTX, because there are not many DMARDs options for children.

In this study, 48 (55.2%) patients had MTX-induced GI symptoms; 9 (10.3%) patients suffered from GI problems before MTX, 12 (13.8%) patients had problems after MTX and 27 (31.1%) patients had GI symptoms both before and after MTX. We observed MTX intolerance in 36 (41.4%) patients. In adult studies, the prevalence of MTX intolerance was between 11 and 39.5%.[9-12] Two studies about JIA reported MTX intolerance of 51% and 42%.[7,13] A current study that compared MTX intolerance between adults and children reported MTX intolerance of 64% in children and 10% in adults.[14] All our patients with MTX intolerance had nausea (41.4%). Among the patients, 12.6% had vomiting and nobody reported abdominal pain before MTX. The rate of MTX-induced nausea in JIA was reported between 21-73%.[7,15] MTX-induced nausea was the most common reason for self-discontinuation. In our study, 14 (16.1%) of 36 patients with MTX intolerance discontinued MTX. The self-discontinuation due to MTX intolerance was reported between 10-67% in studies.[15-17] Because of the different methods and populations of studies, the range of self-discontinuation was broad.

Methotrexate intolerance severity score was a beneficial tool to show MTX intolerance and all participants of this study readily completed the questionnaire. The MISS questionnaire provided gradual MTX intolerance objectively and we found that the median score of the MISS questionnaire was 14.5 (IQR: 10-18).

In this study, all MTX intolerance cases occurred in the first 6 months of MTX treatment and 20 (55.6%) of 36 patients had symptoms in the first month. According to studies, it occurred within the first year of treatment.[7,13] A current study reported MTX intolerance of 25% within 6 months and 30% within the first year.[18]

The rare and serious side effects of MTX were persistent cytopenias and elevations of liver enzymes. These adverse events were reported in 5-9%.[19,20] We observed an 18.4% elevation of liver enzymes, and 3.4% cytopenia. None of them was persistent and the laboratory parameters were normalized after a pause of MTX dose for a few weeks. In studies, recommendations for MTX intolerance were focused on preventing nausea before its occurrence and early use of anti-emetics was suggested.[15,20] The known countermeasures were not enough to prevent MTX intolerance, such as folic acid and changing the dose of MTX. Current studies investigated the effects of acupressure points, coffee/dark chocolate, and behavioral treatments.[20] We observed no decrease in nausea after folic acid or dose changing.

The causes and pathogenesis of MTX intolerance are still unknown and a few studies reported no prominent risk factors for MTX intolerance like ALT, thrombocyte, and creatinine.[21] Franova et al.[18] defined no potential predictors for MTX intolerance. In this study, we found no difference in clinical and laboratory parameters of tolerant and intolerant patients and, we observed no distinctive demographic, clinical, and laboratory findings that might be associated with MTX intolerance.

A cross-sectional design, single-center experience, relatively small number of patients, and filling MISS questionnaire were the main limitations of this study.

Conclusion

The predictive possible risk factors of MTX intolerance and prevention of MTX intolerance before occurrence in JIA are not available. The MISS questionnaire is suitable for defining MTX intolerance objectively. MTX intolerance is an important reason for self-discontinuation. There is no effective solution for MTX intolerance. However, MTX is still the first option for DMARDs in JIA and it is necessary for prospective studies on a larger number of patients to impede MTX intolerance.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Conflict of Interest: There are no conflicts of interest in connection with this paper, and the material described is not under publication or consideration for publication elsewhere.

Ethics Committee Approval: This study was performed in line with the principles of the Declaration of Helsinki. The Ethics Committee of Ankara City Hospital (Date 08.06.2022/E2-22-1928) granted approval.

Financial Disclosure: The authors have no conflicts of interest to declare.

Informed Consent: Informed consent was obtained from the parents of the patients.

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Virtual Touch Tissue Imaging and Quantification Elastography in Determining the Effects of Chronic Kidney Disease on Tendons in Pediatric Patients

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Abstract

The aim of this study is to determine the possible elasticity changes of the quadriceps, patellar, and Achilles tendons using the Virtual Touch Tissue Imaging and Quantification (VTIQ) Elastography method in children with chronic kidney disease. VTIQ elastography measurements of the quadriceps, patellar, and Achilles tendons were performed in children with end-stage renal disease and the healthy control group. Tendon stiffness values of the patient and the control group were compared. Twenty children with end-stage renal disease and 13 healthy children were included in the study. The mean age was 13.05±3.3 years, 12.31±3.2 in the patient group and healthy control group, respectively. Age did not show a statistically significant difference between the patient and control group. The median duration of dialysis was 2.0 (1-9) years. Duration of the dialysis showed a positive correlation with shear wave velocity (SWV) of the musculotendinous junction (MTJ) of the right Achilles tendon (r=0.81 and p=0.001). Parathormone levels showed a positive correlation with SWV of MTJ of the right Achilles tendon (r=0.62 and p=0.03). There was no statistically significant difference in tendon stiffness values of right quadriceps, patellar tendons, Achilles MTJ, Achilles midtendinous (MIDT) area, left quadriceps, patellar tendons, Achilles MTJ, Achilles MIDT area between the patient and control group (p=0.93, p=0.42, p=0.21, p=0.67, p=0.55, p=0.19, p=0.08, p=0.89, respectively). Tendon stiffness values did not differ in children with CKD compared to healthy children. Nevertheless, further long-time follow-up studies are needed to reveal the relation between tendon stiffness and chronic kidney disease.

Keywords: Child; chronic kidney diseases; elastography; tendons; ultrasonography

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Introduction

Chronic kidney disease (CKD) means progressive loss of kidney function in course of time. It is related to significant health complications, growth impairment, and decreased life expectancy in childhood.[1,2] Spontaneous tendon rupture is one of the complications of chronic renal failure[3,4] Secondary hyperparathyroidism, corticosteroid use, fluoroquinolone use, amyloidosis, chronic acidosis, chronic inflammation, and malnutrition are the predisposing factors for this complication.[5] The quadriceps, patellar, and Achilles tendons are mostly affected.[3,6] If not treated or missed, this complication may result in disability. To reduce the morbidity and the economic load on the healthcare expenditure, early detection, and early medical management are essential.

Ultrasonography (US) is more helpful in diagnosing tendon diseases than clinical examination.[7] Low cost, no radiation exposure, accessibility, portability, ease of use, and real-time capability are the advantages of the US. However, conventional US is not sufficient to evaluate biomechanical characteristics of the tendons. Ultrasound elastography (UE) is a method that helps to recognize pathologic conditions via determining tissue elasticity.[8] It has been used in the musculoskeletal system to evaluate muscle elasticity, shoulder bursitis, lateral epicondylitis, spondyloarthritis, and rotator cuff disease.[9]

Highlights

- Tendon stiffness values did not differ in children with CKD compared to healthy children
- Duration of the dialysis and PTH levels showed a positive correlation with shear wave velocity (SWV) of the musculotendinous junction (MTJ) of the right Achilles tendon
- Further long-time follow-up studies are needed to reveal the relation between tendon stiffness and chronic kidney disease.

The two UE methods are real-time elastography and shear wave elastography (SWE). Manual compression is used in real-time elastography whereas an acoustic beam is used in SWE. Virtual Touch Imaging Quantification (VTIQ) and Virtual Touch Quantification (VTQ) are the SWE techniques that directly measure tissue stiffness. VTIQ has a superior diagnostic performance compared to VTQ with a smaller region of interest (ROI) and multiple-point measurement.[10]

The goal of this study was to determine the possible elasticity changes of the quadriceps, patellar, and Achilles tendons using the VTIQ method in children with CKD.

Material and Method

Study Population

This is a cross-sectional study consisting of thirty-three volunteers. Twenty patients with end-stage chronic renal disease who were on dialysis and thirteen control subjects admitted to our institution between January 2020 and March 2021 were included. The control group was formed from randomly selected healthy children who presented to the ultrasonography unit. The exclusion criteria for patient and control groups were as follows: previous trauma or surgery of lower extremity and history of systemic inflammatory diseases. Shear wave velocity (SWV) of bilateral quadriceps, patellar, and Achilles tendons were examined in all volunteers. Duration time of dialysis and parathormone levels were noted for the patients. This study was approved by Erciyes University ethics committee (date:22.05.2019, decision no: 308). Written informed consent was obtained from the parents of all individuals.

Examination Technique

Ultrasound elastography examinations were performed using a Siemens S 3000 (Siemens Healthcare, Erlangen, Germany) ultrasound device. A 9L4 probe and VTIQ technique is used. A single pediatric radiologist with 15 years of experience in ultrasonography and 3 years of experience in elastography performed the sonographic examinations.

Quadriceps tendons and patellar tendons were evaluated in a supine position while the knees were in flexion at approximately 45°, keeping the foot on the floor. The measurements of quadriceps and patellar tendons were obtained from the midtendinous (MIDT) area. Evaluation of the Achilles tendons were performed in prone position while hanging the feet down the end of the table. The measurements of Achilles tendons were obtained from the musculotendinous junction (MTJ) and midtendinous (MIDT) area. All tendons were evaluated in the longitudinal plane. Compared to the axial plane, the longitudinal plane was more appropriate to evaluate a larger area. The size of ROI specified by the manufacturer was 1.5×1.5 mm. Conventional B-mode ultrasound guided the positioning of the ROI. Four valid measurements were performed for each localization (**Figure 1, 2**). Then, a mean value of the SWV (expressed in m/s) was obtained.

Statistical Analysis

Statistical analyses were performed by SPSS IBM Statistics Version 22.0 (SPSS Inc, Chicago, IL, USA). Shapiro–Wilk test was used for testing normality. Variables were presented as mean±SD or median (range). The comparison of two groups distributed normally and the comparison of two groups not distributed normally, were performed by independent samples t-test and Mann–Whitney U test, respectively. Chi-square test was used to compare categorical variables. Pearson correlation was used for normally distributed data whereas Spearman correlation was used for non-normally distributed data. Reliability measurements which are expressed as intraclass correlation coefficient (ICC) were performed for the elastography measurements of each localization. Differences were regarded as significant at $p<0.05$.

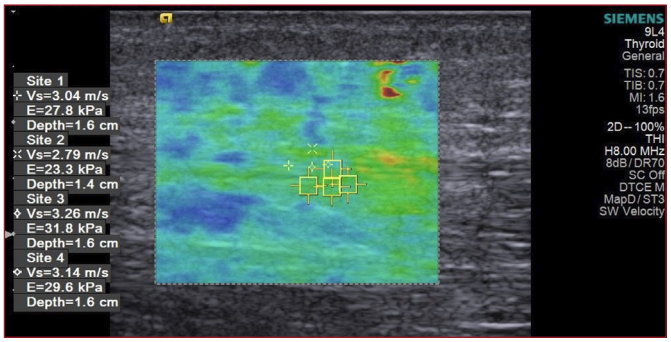

Figure 1. VTIQ measurements of MTJ of Achilles tendon in a 16 years old boy with CKD.

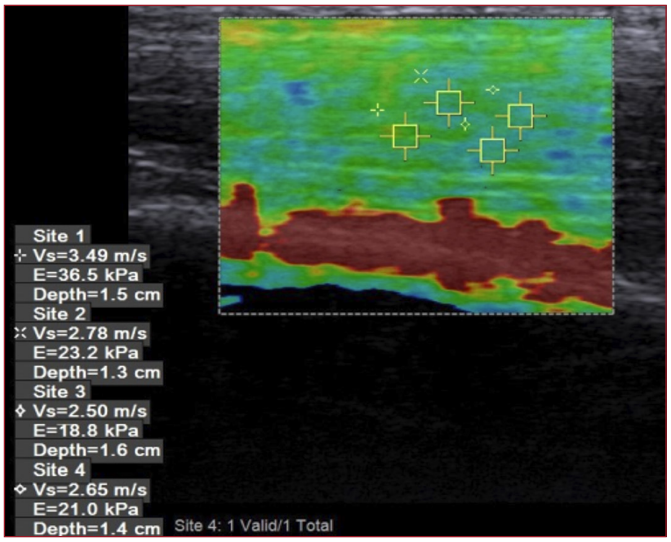

Figure 2. VTIQ measurements of MTJ of Achilles tendon in a 14 years old healthy boy.

Results

Demographic, clinical and laboratory characteristics of the volunteers were given in **Table 1.** There were 20 and 13 children in the patient group and control group, respectively. Age and gender did not show a statistically significant difference between the patient and control group (p=0.41 and p=0.52). Correlation analysis was performed for right and left sided tendons (**Table 2**). The duration of dialysis showed a high positive correlation with SWV of MTJ of the right Achilles tendon (r= 0.85 and p<0.001) but was not correlated with SWV of other locations. Parathormone levels showed a high positive correlation with SWV of MTJ of the right Achilles tendon (r=0.62 and p=0.03) but were not correlated with SWV of other locations. Shear wave elastography measurements of right and left, quadriceps MIDT, patellar MIDT, Achilles MTJ, Achilles MIDT for all volunteers was given in **Table 3.** The intraobserver agreement expressed as intraclass correlation coefficient (ICC) was perfect for each location. ICC values are shown in **Table 4**.

Table 1
Demographic, clinical and laboratory characteristics of patient and control groups

	Patient group N=20	Control group N=13	p
Age (years)	12 (6-17)	11 (9-18)	0.41
Gender (male/female)	7/6	13/7	0.52
Duration of dialysis (years)	2.0 (1-9)		-
PTH (pg/mL)	435±267	-	-

PTH; parathormone, The parameters are presented as median (range) for age and duration of dialysis and mean±SD for PTH levels

Table 2
Correlation analysis between SWV of tendons and age, duration of dialysis and PTH levels.

	Age	Duration of dialysis	PTH
SWVs of right Q MIDT	-0.346 0.057	0.192 0.445	-0.321 0.210
SWVs of right P MIDT	-0.146 0.424	-0.181 0.458	-0.266 0.285
SWVs of right A MTJ	0.060 0.779	0.812 0.001	0.616 0.033
SWVs of right A MIDT	0.171 0.357	-0.117 0.645	-0.117 0.656
SWVs of left Q MIDT	0.033 0.862	0.210 0.403	0.137 0.599
SWVs of left P MIDT	0.116 0.534	-0.124 0.623	-0.301 0.241
SWVs of left A MTJ	0.157 0.453	0.465 0.128	0.425 0.169
SWVs of left A MIDT	-0.214 0.248	0.242 0.333	0.151 0.562

The first line is r value, the second line is p value for all parameters. SWV, Shear wave velocity; PTH, parathormone

Table 3
Shear wave elastography measurements of right and left, Q MIDT, P MIDT, A MTJ, A MIDT

	Patient group	Control group	p
SWVs of right Q MIDT (m/s)	3.25±0.94	3.22±0.41	0.93
SWVs of right P MIDT (m/s)	3.12±1.01	3.40±0.85	0.42
SWVs of right A MTJ (m/s)	3.96±1.51	3.32±0.87	0.21
SWVs of right A MIDT (m/s)	4.75±1.15	4.91±0.80	0.67
SWVs of left Q MIDT (m/s)	3.22 (2.26-5.56)	3.05 (2.53-3.59)	0.55
SWVs of left P MIDT (m/s)	2.61±0.76	2.92±0.47	0.19
SWVs of left A MTJ (m/s)	4.19±1.26	3.47±0.69	0.08
SWVs of left A MIDT (m/s)	4.68±1.31	4.63±0.58	0.89

The parameters are presented as mean±SD for SWVs of right and left tendons except for left Q MIDT of the patient and control group. SWV of the left Q MIDT of the patient and control group was presented as median (range). Q; quadriceps, P; patellar, A; Achilles, MTJ; musculotendinous junction, MIDT; midtendinous area

Table 4
Intraobserver reliability measurements

Localization	ICC	95% CI	p
Right Q MIDT	0.94	0.84-0.98	<0.001
Right P MIDT	0.95	0.87- 0.98	<0.001
Right A MTJ	0.95	0.88-0.99	<0.001
Right A MIDT	0.94	0.84-0.99	<0.001
Left QMTJ	0.94	0.86–0.98	<0.001
Left P MIDT	0.95	0.89-0.99	<0.001
Left A MTJ	0.93	0.83-0.98	<0.001
Left A MIDT	0.89	0.74-0.97	<0.001

Q; quadriceps, P; patellar, A; Achilles, MTJ; musculotendinous junction, MIDT; midtendinous area, ICC; interclass correlation coefficient

Discussion

In the current study, the tendon stiffness values of the children with end-stage renal disease and healthy children were evaluated. There was no statistically significant difference in tendon stiffness between the groups. Duration of dialysis and parathormone levels showed a positive correlation with SWV of right MTJ of Achilles tendons whereas no correlation was found with SWV of other tendons.

Elastographic studies on the tendons with different pathologic conditions reveal a wide variety of discrepancies in results. In some studies, it is reported that a tendon with pathologic changes is softer than the normal tendon.[11-15] Turan et al.[11] reported that the Achilles tendons of patients with ankylosing spondylitis were softer than the healthy group. De Zordo et al.[12] evaluated the stiffness of Achilles tendon in patients with chronic Achilles tendinopathy and reported evident softening. Dirrichs et al.[13] evaluated Achilles, patellar or epicondylar tendons in patients with chronic tendon pain and reported that SWVs was lower in symptomatic tendons compared to those in asymptomatic ones. Chen et al.[14] evaluated ruptured Achilles tendons and reported softening compared with the healthy tendons. In a study comparing quadriceps tendons of the patients with chronic hemodialysis and healthy individuals, softening and thinning are reported in the patient group.[15] On the other hand, opposed results are reported in some studies.[16-18] Mutlu et al.[16] evaluated the Achilles tendons and performed the measurements in proximal, middle, and distal thirds of the tendons in patients with CKD. They reported that there was an increased stiffness for all 3 parts in the patient group compared to healthy individuals. Caglar et al.[17] evaluated the Achilles tendon in patients with CKD in hemodialysis and healthy individuals, using VTIQ. They reported that tendon stiffness values were higher in the distal third of the Achilles in patients, compared to healthy controls. Zhang et al.[18] reported that the patellar tendons of the athletes with unilateral tendinopathy had higher stiffness measurements compared to both the controls and unaffected tendons of the athletes.

Using SWE, Coombes et al.[19] evaluated SWV of insertional Achilles tendons and patellar tendons in patients with tendinopathy and healthy individuals. They reported lower tendon stiffness in Achilles whereas higher tendon stiffness in patellar tendons in the patient group compared to healthy controls. Hekimoglu et al.[20] evaluated Achilles tendon elasticity in patients with CKD in hemodialysis and healthy individuals by using SWE. No statistically significant difference was reported between the groups in their study. In contribution with Hekimoglu et al.[20] there was no significant difference in SWE measurements between the patient and control groups in the current study. They performed the measurements only in the middle part of the tendon and stated that as a reason for the statistically insignificant difference. In the current study, the measurements of the Achilles tendons were performed in two localization (musculotendinous junction and midtendinous area). However, still, there was no statistically significant difference between groups. Hekimoğlu et al.[20] reported that there was no association between the duration of hemodialysis and mean stiffness values of Achilles tendons. Unlike their results, in the current study, the duration of the dialysis showed a very high positive correlation with SWV of the musculotendinous junction of the right Achilles tendon. Also, parathormone levels showed a high positive correlation with the SWV of right Achilles at the musculotendinous junction. Kural et al.[21] reported that the SWV measurements of the Achilles of the patients with chronic kidney disease on hemodialysis and the people with renal transplant showed significant differences when compared with healthy people. In their study the median duration of dialysis was 12 years (range, 5-20 y) for the patients with CKD and 8 years (range, 5-16 y) for transplant group. The median duration of dialysis was 2 years (range, 1-9 y) in the current study. Based on these findings, the relatively short duration of dialysis in our patient group may be responsible for the insignificant difference. If the duration time was longer, significant differences might be obvious in tendon stiffness between groups. Thus, further long-time follow-up studies are needed to reveal the relation between tendon stiffness and CKD.

Age did not show a correlation with the SWV of the tendons in the current study, in contribution with Wakker et al.[22] and Fu et al.[23] who studied the normal SWV values of Achilles tendons in healthy individuals.

The discrepancy between the results of all these studies may be explained by several factors. One of them is the various ultrasound elastography methods and manufacturers used for measurements. For example, Turan et al.[11] and De Zordo et al.[12] used compression elastography while Dirrichs et al.[13], Chen et al.[14], Zhang et al.[18], Coombes et al.[19] used SWE. Trottmann et al.[24] in their study, used two different manufacturers for ultrasound elastography measurements and compared the values. They stated that the measurement values showed statistically significant differences. Another factor is the nonuniformity of examination protocols. Aubry et al.[25] compared the SWV of the Achilles tendons under variable tightness, they reported that the highest velocities were found in dorsiflexion of the ankle and maximum plantarflexion gave the lowest values. De wall et al.[26] evaluated the Achilles tendon in three positions; 15° plantar flexion, neutral, and 15° dorsiflexion. In several studies,[15-17,22] the Achilles tendon was evaluated in the prone position, with feet hanging down from the side of the table. In contribution with them, in the current study, the measurements of the Achilles were performed in the prone position, feet hanging down the end of the table. Breda et al.[27] evaluated the patellar tendons in passive extension. Dickson et al.[28] evaluated the quadriceps and patellar tendons in a supine lying or seated position in 30° flexion. Quadriceps and patellar tendons were evaluated in 45° flexion in the current study similar to the study of Dickson et al. SWV is affected by the plane of acquisition due to anisotropy. The tendons are anisotropic organs. Therefore, SWV values vary according to the angle at which the ultrasound elastography waves pass through the tendon fibers. The mean SWV was reported to be higher in the sagittal plane compared with the axial plane.[23]

The relation between secondary hyperparathyroidism and the tendon rupture mechanism is controversial. Shiota et al.[29], reported that the cause of the tendon rupture was osteolytic bone resorption at the tendon insertion site and tendon structure was not affected. Nevertheless, Terai et al.[30], reported that vascular calcification increased in CKD with secondary hyperparathyroidism. Kurtoglu et al.[31], reported intratendinous calcific nodules in Achilles tendons in

a patient with hyperparathyroidism associated with parathyroid adenoma. Supporting Terai et al.30 and Kurtoglu et al.[31], parathormone levels correlated positively with tendon stiffness values of Achilles MTJ, in this study.

There are some limitations in the current study. One of them is the relatively small study population. Another one was that, we did not perform a histological analysis because it wasn't possible to achieve histologic samples as there was no indication. Another limitation was the duration of dialysis, which was relatively short to see the effects of hyperparathyroidism on tendon elastography properties.

However, this study is the first to evaluate the elastic properties of the tendons in the pediatric age group with CKD, to the best of our knowledge.

Conclusion

Tendon stiffness values were not statistically different in children with CKD compared to healthy children. A relatively short duration of the dialysis in the volunteers may be responsible for the result. Further long-time follow-up studies are needed to reveal the relation between tendon stiffness and CKD.

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Evaluation of Clinical and Genetic Characteristics of Primary Ciliary Dyskinesia

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Abstract

Primary ciliary dyskinesia (PCD) is a clinically and genetically heterogeneous condition characterized by defective motile cilia activity. There is no "gold standard" diagnostic test currently available. In this article, we summarize the clinical and genotypic features of 25 children with PCD who received therapy at a single location in Turkey. This study was done between October 2020 and July 2022 as a retrospective cohort study in which the medical records of Turkish and refugee patients with PCD were reviewed regarding their medical history, clinical and radiologic findings, and genetic data. We evaluated the outcomes of 25 patients whose genetic results were reported to be associated with known PCD genes. The mean age of patients with PCD was 10.5 (±5). PICADAR scores ranged from 2 to 10, with the mean score being 6.1 (± 2.2). Age at diagnosis was shown to be moderately negatively correlated with PICADAR. (r;-0,502, p;0,01).16% DNAH5 within four patients, 16% with CCDC40 in four patients,12% with DNAAF2 in three patients, 8% with DNAH11 in two patients, 8% with TTC25 in two patients, 8% with DNAAF4 in two patients, 8% with CCNO in two patients 4% with DYNC2H1 in one patient, 4% with DNAI1 in one patient, 4% with ARMC4 in one patient,4% with RSPH4A in one patient, 4% with HYDIN in one patient,4% with CCDC65 in one individual from each PCD gene. The association between the phenotype and genotype of PCD patients in the southeast Anatolian region of our nation was explored for the first time in this study. Additionally, PCD patients with PIBO were reported for the first time with CCNO defects. Genotype and phenotype studies will help us determine the prognosis of patients in the future. These findings should increase our knowledge of PCD pathogenic pathways, hence enhancing early illness diagnosis, disease treatment, and prognosis.

Keywords: Primary ciliary dyskinesia, genetic, genotype, phenotype

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Introduction

Primary ciliary dyskinesia (PCD) is a clinically and genetically heterogeneous condition characterized by defective motile cilia activity.[1] It is a rare disorder often inherited in an autosomal recessive and X-linked disease pattern.[2] Patients often seek therapy for persistent wet cough, sinusitis, bronchiectasis, otitis media, and infant respiratory distress, with approximately fifty percent of patients presenting situs inversus.[1] There is no "gold standard" diagnostic test currently available. There are unique guidelines for determining the diagnosis of PCD individuals with distinct clinical characteristics.[3,4] They suggested that diagnosis needed access to a variety of technically challenging approaches, such as nasal nitric oxide (nNO), high-speed video analysis (HSVA), transmission electron microscopy (TEM), and genetic testing.[5] However, reaching all of these diagnostic methods may not be possible. Despite identifying more than 40 disease-causing genes, 20% to 30% of patients with a confirmed PCD diagnosis have no known genetic etiology.[6] Patients may be diagnosed with PCD using improved genetic testing tools, and novel genes can be found.

Highlights

- The relationship between the phenotype and genotype of patients with PCD in the southeast Anatolian area of our country was investigated for the first time.
- PCD patients with PIBO were reported, for the first time, with CCNO genetics.
- Genotype and phenotype studies will help us determine the prognosis of patients in the future. These findings should increase our knowledge of PCD pathogenic pathways, enhancing early illness diagnosis, disease treatment, and prognosis.

This article summarizes the clinical and genotypic features of 25 children with PCD who received therapy at a single location in Turkey. Additionally, we examined the correlation between PICADAR score, clinical, radiological, and laboratory features of patients with PCD.

Material and Method

This study was done between October 2020 and July 2022 as a retrospective cohort study in which the medical records of Turkish and refugee patients with PCD were reviewed regarding their medical history, clinical and radiologic findings, and genetic data.

Over the last two years, our department has followed up with 38 PCD patients. All patients underwent genetic testing. Six patients with situs inversus and a Primary Ciliary Dyskinesia Rule (PICADAR) score ≥5 had normal genetic testing results, and the genetic results of seven patients have not yet been released. Therefore, these 13 patients were excluded from the study. Patients carrying 25 pathological genetic homozygote alleles for primary ciliary dyskinesia were enrolled in the study.

At their clinical follow-up, we recorded the age at diagnosis, PICADAR score, weight z-scores, height z-scores, BMI z-scores, duration of symptoms, history of birth (term or preterm), persistent wet cough, an abnormal situs, an abnormal heart, a history of pneumonia, chronic sinusitis, chronic otitis, bronchiectasis, persistent rhinitis, recurrent wheezing, hearing loss, hospitalization in the neonatal period, prior infections, parental consanguinity, duration of symptoms, pulmonary function test (PFT) results, echocardiographic finding, radiological evaluation, and sputum microbiology result.

PICADAR score estimates the probability of PCD. It contains seven predictive factors that may be employed in any patient with a persistent wet cough, including full-term gestation, chest symptoms in the neonatal period, admission to a neonatal care unit, chronic rhinitis, ear complaints, situs inversus, and a congenital heart abnormality. For a cut-off score of ≥5, the tool's sensitivity and specificity were 0.90 and 0.75, respectively, to diagnose PCD.[7] Patients were suspected of PCD if their Primary Ciliary Dyskinesia Rule (PICADAR) score was ≥5. All patients' PICADAR scores were determined at admission. The Spirometry PFTs were conducted in line with the norms of the American Thoracic Society.[8] The measures of forced expiratory flow in 1 second (FEV1) %, forced vital capacity (FVC)%, and forced expiratory flow (FEF) 25-75% are stated. Before the test, the patients were informed about the techniques. PFT was not applied to children under six and non-cooperative children. It was applied to over six years of age and cooperative children. BMI z scores were recorded at the time of their most recent clinical follow-up. The BMI was computed by dividing weight in kilos by the square of height in meters. The "z" score for BMI for age was derived using the Centers for Disease Control and Prevention software.

Genetic Analysis

Genomic DNA extraction was performed according to the manufacturer's instructions (Maxwell RSC Blood DNA kit, Promega, USA) using the Maxwell RSC Instrument (Promega, USA). 30 µl of Proteinase K (PK) Solution was added into a 200 µl blood sample. 300 µl of Lysis Buffer was added to the blood and PK mix and incubated at 56°C for 20 minutes. After this step, each blood lysate sample was transferred to the cartridges. At the end of the assay in the instrument, 50 µl of DNA was eluted. The concentration of DNA was determined spectrophotometrically by measurement of the absorbance at 260/280 nm using a Nanodrop 1000 apparatus (Thermo Fisher Scientific). The concentration of DNA samples for libraries was determined using Qubit 3.0 (Thermo Fisher Scientific). The sequencing libraries for exome sequencing were prepared according to the Twist Human Core Exome Kit protocol (Twist Bioscience, USA). Paired-end 150 bp spread sequencing was performed on a NovaSeq system (Illumina, USA). Whole-exome sequencing

(WES) was performed for all the affected patients with a suspected history of PCD. Nova Seq data was then uploaded to the Sophia DDM (Sophia Genetics-Lausanne-Switzerland) platform and made ready for analysis. In Silico prediction databases, by consensus, stated that the changes were "destructive". The variants were evaluated using the American College of Medical Genetics (ACMG) 2015 criteria.[9]

Statical Analysis

Statistical analysis was performed using SPSS (Statistical Package for Science Studies) version 22.0 for Windows. Firstly, descriptive statistics were performed with the data obtained. Then, the Shapiro-Wilk test was used to test whether the variables were normally distributed. Characteristic data are presented as number (%) for categorical variables and mean±SD or median (minimum-maximum) for continuous variables, where appropriate. The differences between groups were compared using the independent sample T-test and Mann-Whitney U tests for numerical values. Correlations between data not normally distributed were examined using Spearman's correlation test, and correlations between normally distributed data were analyzed using Pearson's correlation test. All tests were two-tailed, and p-values less than 0.05 were considered statistically significant in all cases.

The study was conducted according to the ethical norms and standards of the Declaration of Helsinki, and ethical approval was obtained from the local ethics committee.

Results

We evaluated the outcomes of 25 patients whose genetic results were reported to be associated with known PCD genes. The mean age of patients with PCD was 10.5 (±5). The mean age of the patients at the time of diagnosis was 9.2 (±4.9) years. Sixteen (64%) patients were female, and nine (36%) were male. There were 16 (64%) consanguineous marriages involved. There were eight (%32) immigrants among the patients.

PICADAR scores ranged from 2 to 10, with the mean score being 6.1 (±2.2). Age at diagnosis was shown to be moderately negatively correlated with PICADAR. (r;-0.502, p;0.01).The PICADAR score of five patients (20%) was less than five, whereas the score of twenty (80%) patients was more than five. The mean age at diagnosis of patients with a PICADAR score of >5 was 8 (±4.6), and for those with a PICADAR score of < 5, the mean age at diagnosis was 14.4 (±1.5). Patients with high and low PICADAR scores were compared. The age at diagnosis of patients with high PICADAR score was significantly lower (p=0.01). When the height z scores, weight z scores, and BMI z scores of those with low and high PICADAR scores were compared, no statistically significant difference was found (p>0.05).

The mean age of patients with situs inversus was 9 (min:1-max:16). The mean age of patients without situs inversus was 10 (min:4-max:16) years. The age at diagnosis of patients with and without situs inversus was compared. There was no statistically significant difference between them (p=0.330). When the height z scores, weight z scores, and BMI z scores of those with situs inversus and without situs inversus were compared, no statistically significant difference was found (p>0.05).

In terms of symptoms, most of the patients %96 (n=24), had previous pneumonia and wet cough. The other symptoms;44% (n=11) of patients had prenatal respiratory distress, 56 % (n=14) had recurrent sinusitis, 20% (n=5) had recurrent otitis, 16 % (n=4) had hearing impairment, 60% (n=15) had situs inversus,4 % (n=1), had clubbing, and 12 % (n=3) had congenital cardiac abnormalities (atrial septal defect, patent ductus arteriosus, mitral regurgitation and pulmonary hypertension). According to their prior medical histories, %, 8 (n=2) of the patients who had a lobectomy and 4% (n=1) had bronchiolitis obliterans (**Table 1**). Bronchiectasis was diagnosed on computed tomography (CT) in 72% (n=18) of the patients. Among these patients, 50% (n=9) had bronchiectasis in one lobe ,38.8% (n=7) had bronchiectasis in two lobes, 10.5% had bronchiectasis in three lobes (n=2), Before the PCD diagnosis, lobectomies were performed on two patients. The most prevalent microorganisms in sputum culture were *Haemophilus influenzae* (36%) (n=9), *Staphylococcus aureus* (12%; n=3), methicillin resistance *Staphylococcus aureus* (4%; n=1), *Streptococcus pneumonia* (4%; n=1), and *Pseudomonas aeruginosa* (4%; n=1) (**Table 2**).

Nineteen children older than six years were given pulmonary function testing. Decrease of forced expiratory volume in the first second (FEV1) (mean (±SD): 65.7% (19.8) predicted) and forced vital capacity (FVC) (mean (±SD): 65.6% (19.5) predicted) were observed in these children. Additionally, forced expiratory flow at 25 percent and 75 percent of the pulmonary volume (FEF25-75) was (mean (±SD): 73.7% (19.5) predicted).

In our study, 24 patients were aged two years and over, and their BMI was calculated. The mean BMI Z-score was 1.03 (±0.82). The mean height Z-score and weight Z-score of the patients were, respectively, -0.74 (±0.92) and -1.02 (1.1). There was a no correlation between BMI z-score and FEV1, FVC and FEF25-75 of patients (respectively: r;-0.42, p;0.864; r;-0.123, p;0.616; r;-0.16, p;0.513).

The following pathogenic variations were found through genetic analysis:16% DNAH5 within four patients, 16% with CCDC40 in four patients,12% with DNAAF2 in three patients,8% with DNAH11 in two patients, 8% with TTC25 in two patients,8% with DNAAF4 in two patients,8% with CCNO in twopatients4% with DYNC2H1 in one patient, 4% with DNAI1 in one patient, 4% with ARMC4 in one patient,4% with RSPH4A in one patient, 4% with HYDIN in one patient,4% with CCDC65 in one individual from each PCD gene (**Table 1** and **Table 3**).

Table 1
Primary ciliary dyskinesia patients' demographics and clinical traits as determined by genetic testing.

	DNAH5 (n=4)	CCDC40 (n=4)	DNAAF2 (n=3)	DNAH11 (n=2)	TTC25 (n=2)	DNAAF4 (n=2)	CCNO (n=2)	DYNC2H1 (n=1)	DNAI1 (n=1)	ARMC4 (n=1)	HYDIN (n=1)	RSPH4A (n=1)	CCDC65 (n=1)
Age of diagnosis, median (min-max) age	9 (2-17)	6.5 (1-11)	12 (6-14)	9.5 (8-11)	15.5 (16-15)	15.5 (16-15)	4.5 (1-8)	4	3	6	9	8	14
Female/Male n	3/1	2/2	2/1	0/2	1/1	2/0	2/0	1/0	1/0	0/1	1/0	1/0	0/1
Refugee	1	0	1	0	1	1	2	0	0	1	0	1	0
Consanguineous (%)	3 (75%)	4 (100%)	2 (66.6%)	1 (50%)	1 (50%)	1 (50%)	2 (100%)	0	1 (100%)	0	1 (100%)	0	1 (100%)
PICADAR score (min-max)	7 (6-9)	9 (8-9)	6 (5-8)	3.5 (2-5)	2,5 (2-3)	4,5 (3-6)	6 (5-7)	6	7	10	6	5	3
Situs inversus, n (%)	4 (100%)	4 (100%)	2 (66.6%)	0	0	1 (%50)	1 (%50)	0	1 (100%)	1 (100%)	1 (100%)	0	0
Recurrent sinusitis n (%)	2 (50%)	1 (25%)	2 (66.6%)	1 (%50)	1 (%50)	1 (%50)	2 (100%)	0	1 (100%)	1 (100%)	0	1 (100%)	1 (100%)
Recurrent otitis, n (%)	0	1 (25%)	1 (33,3%)	1 (%50)	0	0	0	0	1 (100%)	1 (100%)	0	1 (100%)	0
Neonatal respiratory distress,n (%)	2 (50%)	3 (75%)	2 (66.6%)	1 (%50)	0	1 (%50)	0	1 (100%)	0	1 (100%)	0	1 (100%)	0
Term/preterm n	4/0	4/0	3/0	2/0	2/0	2/0	2/0	0/1	1/0	1/0	1/0	1/0	1/0
Hearing impairment n (%)	0	1 (25%)	1 (33,3%)	0	0	0	0	0	0	1 (100%)	0	1 (100%)	0
Congenital heart defect, n(%)	0	0	1 (33,3%)	0	0	0	1 (%50)	1 (100%)	0	0	0	0	0
Clubbing, n (%)	0	0	0	0	0	0	1 (%50)	0	0	0	0	0	0
Bronchiolitis obliterans n (%)	0	0	0	0	0	0	1 (%50)	0	0	0	0	0	0
History of lobectomy, n (%)	0	0	1 (33,3%)	1 (%50)	0	0	0	0	0	0	0	0	0
Bronchiectasis n (%)	3 (75%)	3 (75%)	2 (66.6%)	2 (100%)	2 (100%)	2 (100%)	1 (50%)	0	0	1 (100%)	0	1 (100%)	1 (1005)

Table 2
Relationship between genetic findings and growth, pulmonary function tests, and the presence or absence of bronchiectasis.

	DNAH5 (n=4)	CCDC40 (n=4)	DNAAF2 (n=3)	DNAH11 (n=2)	TTC25 (n=2)	DNAAF4 (n=2)	CCNO (n=2)	DYNC2H1 (n=1)	DNAI1 (n=1)	ARMC4 (n=1)	HYDIN (n=1)	RSPH4A (n=1)	CCDC65 (n=1)
Height z score median (min-max)	-1.37 (-0.14) -(-2.32)	-1.0 (-0.68) -(-1.66)	-0.39 (-0.57)- (-0.22)	-0.06 (-0.56) -(0.43)	-0.815 (-1.24) -(-0.39)	0.51 (0.48 -0.54)	-0.78 (-2.1) -(0.58)	-1.29	-1.77	0.46	0.48	-0.77	-1.44
Weightz score median (min-max)	-2.1 (-3.05) -(-0.81)	-1.7 (-3.34) -(0.03)	-0.27 (-0.24)- (-0.31)	-0.57 (-1.3)- (0.21)	-0.2 (-1.29) -(0.82)	-0.59 (-0.97) -(-0.21)	-1.35 (-1.87.) -(0.83)	-0.36	-.242	-1.23	1.43	-3.22	-1.33
BMI z score	-1.8 (-3)-(-0.85)	-0.35 (-0.48)- (-0.13)	-1.05 (-1.99)- (-0.11)	-0.78 (-1.73)- (0.17)	0.12 (-0.8) -(1.04)	-1.03 (-0.57) -(-1.5)	-0.87 (-2.8.) -(1.06)	0.92	-1.7	-2.6	1.6	-4.51	-0.77
FEV1% median (min-max)	68.5 (61-76)	70 (60-80)	78.5 (69-88)	78.5 (69-88)	82.5 (82-83)	72.5 (50-95)	19	-	-	68	80	56	57
FVC% median (min-max)	77 (57-77)	56 (53-89)	77.5 (66-89)	77.5 (66-89)	86 (84-88)	69.5 (45-94)	23	-	-	71	82	55	50
FEEF25-75% median (min-max)	68.5 (68-69)	86.5 (78-95)	82.5 (70-95)	82.5 (70-95)	88.5 (82-95)	78.5 (57-100)	16	-	-	79	98	73	81
Microbiology of Mucus													
H. influenza, n	2	1	-	1	1	1	1	-	-	1	-	-	1
S. Aures n	-	-	1	-	1	-	-	1	-	-	-	-	-
P. aeruginosa, n	-	-	-	-	-	-	-	1	-	-	-	-	-
S. pneumonia n	-	-	-	1	-	-	-	-	-	-	-	-	-
No Growth n	2	3	-	-	-	-	-	-	-	-	-	-	-
Bronchiectasis n (%)	3 (75%)	3 (75%)	2 (66.6%)	2(100%)	2 (100%)	2 (100%)	1 (50%)	0	0	1 (100%)	0	1 (100%)	1 (100%)

Table 3
Classification comprehensive of identified variations in primary ciliary dyskinesia genes.

Patinets no	Sex	Gene	Transcript ID	Variant: Coding (HGVS nomenclature c.)	Variant: Protein (HGVS nomenclature p.)	Type	Zygosity	Reference Genome
1	F	CCD40	NM_001243342	c.940-1G>C	p.(?)	Splice-site	Homozygous	GRCh37/hg19
2	M	DNAH5	NM_001369	c.5579del	p.(Asn1860Ilefs*11)	frameshift	Homozygous	GRCh37/hg19
3	M	CCD40	NM_001243342	c.940-1G>C	p.(?)	splice_acceptor_-1	Homozygous	GRCh37/hg19
4	F	DYNC2H1	NM_001080463 NM_001080463	c.10366G>A c.2789T>C	p.(Gly3456Ser) p.(Leu930Pro)	Missense missense	Compound heterozygous	GRCh37/hg19 GRCh37/hg19
5	M	DNAH11 HYDIN	NM-001277115 NM_001270974	c.13008G>A c.7603C>T	p.(Trp4336*) p.(Arg2535Cys)	Nonse missense	Homozygous Homozygous	GRCh37/hg19 GRCh37/hg19
6	F	DNAAF4	NM_130810	c.583del	p.(Ile195*)	nonsense	Homozygous	GRCh37/hg19
7	F	CCD40	NM-017950	c.940-1G>C	p.(?)	splice_acceptor_-1	Homozygous	GRCh37/hg19
8	M	ARMC4	NM_018076	c.2528dupT	p.(Leu843Phefs*52)	frameshift	Homozygous	GRCh37/hg19
9	F	DNAH5 DNAH1	NM-001369 NM_015512	c.5747G>A c.1784A>G	p.(Trp1916*) p.(Lys595Arg)	Nonsense missense	Homozygous Homozygous	GRCh37/hg19 GRCh37/hg19
10	M	CCD40	NM-17950	c.2931_2944 dup	p.(asp982Glysf*50)	frameshift	Homozygous	GRCh37/hg19
11	M	TTC25	NM-031421	c.716G>A	p.(Trp239*)	nonsense	Homozygous	GRCh37/hg19
12	M	CCDC65	NM-033124	c.718C>T	p.(Arg240*)	nonsense	Homozygous	GRCh37/hg19
13	F	DNAAF2	NM_018139	c.1199_1214dup	p.(Gly406Argfs*90)	frameshift	Homozygous	GRCh37/hg19
14	F	RSPH4A	NM_001010892	c1351c>A	p.(Gln451Lys)	missense	Homozygous	GRCh37/hg19
15	F	TTC25	NM_031421	c.1079C>A	p.(Ser360*)	nonsense	Homozygous	GRCh37/hg19
16	F	DNAH5	NM_001369	c.6423_6424del	p.(Cys2141*)	nonsense	Homozygous	GRCh37/hg19
17	F	CCNO	NM_021147	c.842C>T	p.(Ser281Phe)	missense	Homozygous	GRCh37/hg19
18	F	DNAAF2	NM-001083908	c.1595A>G	p.(Glu532Gly)	missense	Homozygous	GRCh37/hg19
19	F	DNAAF4	NM_130810	c.583del	p.(Ile195*)	nonsense	Homozygous	GRCh37/hg19
20	M	DNAH11	NM-001277115	c.13008G>A	p.(Trp4336*)	Nonse missense	Homozygous	GRCh37/hg19
21	F	HYDIN	NM-001277115	c.13008G>A	p.(Trp4336*)	Nonse missense	Homozygous	GRCh37/hg19
22	M	DNAAF2	NM-001083908	c.1214_1215ins16	p.gly406Argfs*89	frameshift	Homozygous	GRCh37/hg19
23	F	DNAI1	NM_015512	c.138del	p.Ala47Profs	nonsense	Homozygous	GRCh37/hg19
24	F	DNAH5	NM_001369	c.5579del	p.(Asn1860Ilefs*11)	frameshift	Homozygous	GRCh37/hg19
25	F	CCNO	NM_021147	c.842C>T	p.(Ser281Phe)	missense	Homozygous	GRCh37/hg19

Discussion

In this study, the relationship between the phenotype and genotype of patients with PCD in the southeast Anatolian area of our country was investigated for the first time. More genetic diversity was found in our study than in two earlier studies published in our country.[9,10] Recent migration in this region might have contributed to a rise in genetic diversity. And also, PCD patients with PIBO were reported for the first time with CCNO genetics.

In our study, the mean age of patients at diagnosis was 9.2 years. In published research in our country, the age at diagnosis was found to be 8.3 years, while in another study, it was found to be nine years.[10,11] Our study's mean age was similar to those of previous studies. Studies from different countries show that PCD patients are diagnosed at different ages.[12-14] This difference may be related to regional and international variations in diagnostic capabilities. Patients will be diagnosed earlier as a result of the improvement of diagnostic techniques and their increased accessibility. Now that genetic testing is available in our location, we can diagnose patients earlier.

The percentage of consanguineous marriages among parents in our study was 64%. In two different studies conducted in our country, the rate of consanguineous marriage was 64% and 80.4%, respectively.[10,11] Studies conducted in other countries found between 13-19%.[15,16] This ratio is observed to be significantly higher in our country. People should be educated to minimize consanguineous marriage. PCD incidence may be decreased.

The clinical signs of PCD in children are quite heterogeneous. Some patients may show signs in early infancy, while others may show symptoms later in life. This varies based on the pathogenic effects of the patient's genes. PCD is characterized by a daily productive wet cough, recurrent lung infections, chronic rhinosinusitis, and chronic middle ear inflammation.[17] In contrast to other causes of respiratory distress in term newborns (e.g., transient tachypnea of the newborn—TTN), which often manifest during the first few hours of life, the majority of PCD patients are healthy immediately after delivery but develop respiratory distress at 12–24 hour of life.[17] 50% of PCD patients have situs abnormalities, and 10-12% have congenital heart diseases.[18] The clinical results of our group paralleled those of other studies. At the first examination, most patients (%96) had a history of persistent wet cough and recurrent lung infections. 60% of the patients had situs anomalies. Meanwhile, no laterality defects were observed in patients with DNAH11, TTC25, DYNC2H1, CCDC65, and RSPH4A mutations, as in previous reports.[10,19] 12% of patients also had congenital heart disease. Compared to other studies, we found a lower rate of respiratory symptoms (44%) in newborns.[20] This may be due to our patients' different genetic pathogenic variant outcomes.

Our research shows that patients with a high PICADAR score were diagnosed much earlier. However, there was no correlation between situs inversus and age at diagnosis. Similar to our study, Asfuroglu et al.[21] discovered a significant correlation between the PICADAR score and the age of diagnosis. In contrast to this investigation, there was no association between

situs inversus and the age of diagnosis. A patient with a high PICADAR score will have more complaints and make more frequent clinic visits. Therefore, early diagnosis is possible. However, if the patient has situs inversus and is asymptomatic, the patient's visits to the doctor and referrals to pediatric pulmonology for further diagnosis are decreased. Another reason there was no significant difference between situs inversus and the age at diagnosis in our study may be because there was no pediatric pulmonologist in the region before, and no method could be used for diagnosis in this region. In the last two years, the use of genetic diagnosis methods has increased the diagnosis of patients.

In our study, 72% of the patients had bronchiectasis at the time of diagnosis. 50% had bronchiectasis in one lobe, 38.8% had bronchiectasis in two lobes, and 10,5% had bronchiectasis in three lobes. Markus et al.[22] showed that 56% of pediatric patients had bronchiectasis. In another study, Bronchiectasis was detected in 80.4% of our patients.[9] Different research reports varying rates. This may be due to the patients in the study having different genes. Before the PCD diagnosis, lobectomies were performed on two patients because of bronchiectasis. In most situations, lung surgery is not suggested for PCD; lobectomy may be considered in cases with localized illness resistant to conservative treatment.[23] However, these two patients did not have refractory lung disease. Before deciding on a lobectomy, the cause of bronchiectasis should be investigated beforehand in patients with bronchiectasis.

The most common pathogens are *H. influenzae*, *S. aureus*, *Moraxella catarrhalis*, and *S. pneumonia*.[24] The most prevalent germ discovered in 36% of our patients were *H. influenzae*, which is consistent with findings from earlier investigations. One of our patients (DYCN2H1) had pseudomonas growth even though she was younger. It was linked to having a history of being born early and in the hospital for a long time in the intensive care unit.

Our study found no correlation between growth and nutrition and lung function in patients with PCD. Maglione et al.[24] did a long-term study and found no statistically significant link between the first FEV1 and FVC Z scores and the first BMI Z scores. They also found that the BMI and spirometry were stable during follow-up. The results of our study were similar to those of Maglionin et al.[24] Because PCD pulmonary disease is milder than diseases such as CF, it may not be associated with nutrients and growth.

There is no diagnostic gold standard for PCD. However, there are particular protocols to establish the diagnosis, ideally at a younger age.[4,25] Approximately 65-70% of PCD patients may be diagnosed using next-generation sequencing technologies, contributing to early diagnosis and management. Genetic testing cannot diagnose around 30% of patients, and a negative genetic test does not rule out PCD.[26] Genetic testing was used as a diagnostic method for the patients in our study. Even though six individuals in our research had PICADAR scores >5, situs inversus, and respiratory symptoms, a genetic diagnosis was impossible. We found that in our study, 80% of the patients could be diagnosed genetically, but only 20% could not be conclusively identified. For the diagnosis of PCD, further diagnostic procedures are required. DNAH5, CCDC40, and DNAAF2 were the most prevalent mutations in our study's area. This result was comparable to the genetic frequency discovered in the studies by Emiralioglu et al.[10] and Hornef et al.[27] As distinct from Emiralioglu et al.'s[10] study, we observed DNAAF4, DYNC2H1, CCDC65, and DNAAF2 alleles in our region.

In our study, one patient had PIBO, a previously undiagnosed sign of PCD that was recognized based on clinical symptoms, chest high-resolution CT scans, and lung function. The high-resolution CT scan revealed mosaic perfusion and air trapping (**Figure 1**, **Figure 2**), and the lung function test showed that the tiny airways were significantly obstructed. She had a poor clinical course. She needs oxygen and has a clubfoot. She was underweight, had retarded growth, and had poor lung function. Previously reported in a PCD patient with a DNHA1 mutation.[19] Our patients' mutation is different. This is the first reported case of PCD with PIBO in Turkey. The genetic result of this patient is CCNO. We know that in previous studies, CCNO more commonly presented severe respiratory symptoms.[19,28]

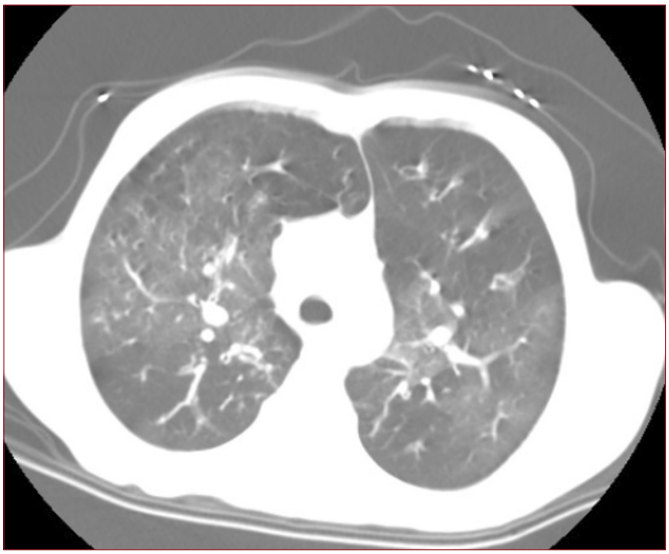

Figure 1. A axial view of HRCT of the lungs shows a mosaic attenuation pattern and air trapping.

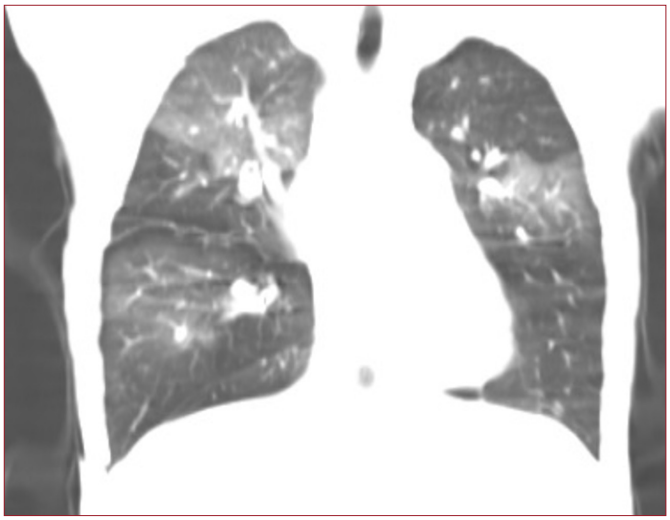

Figure 2. A coronal view of HRCT of the lungs shows a mosaic attenuation pattern and air trapping.

Conclusion

The data presented here are inconsistent with previous findings in Turkey describing the clinical and genetic spectrum of PCD. In contrast to prior research published in Turkey, we found DNAAF4, DYNC2H1, CCDC65, and DNAAF2 alleles in our location. This study reported PCD patients with PIBO for the first time with CCNO. Genotype and phenotype studies will help us determine the prognosis of patients in the future. These findings should increase our knowledge of PCD pathogenic pathways, hence enhancing early illness diagnosis, disease treatment, and prognosis.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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

Ethics Committee Approval: The study was carried out with the permission of the Gaziantep University Faculty of Medicine Clinical Research Ethics Committee (Decision No:2022/ 226).

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Informed Consent: All patients signed the free and informed consent form.

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Bacteriological Profile and Antimicrobial Resistance Pattern Among Healthcare-Associated Infections in a Pediatric Intensive Care Unit

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Abstract

Healthcare-associated infections (HAIs) are a global public health issue with clinical and socioeconomic consequences. These infections are important indices for the quality of healthcare services which are serious complications that should be addressed in pediatric intensive care units (PICUs). This study aimed to retrospectively examine the bacterial HAIs, the frequency of isolated pathogen microorganisms, the areas of infection, and the antibiotic susceptibility recorded in the surveillance system in our Pediatric Intensive Care Unit in five years between 01.01.2015 and 31.12.2019. In the study period, 1593 patients were admitted to PICU, and 244 HAIs were detected in 141 patients. A bacterial pathogen was isolated in 190 HAIs of the 99 patients. In those episodes, Gram-negative microorganisms were most commonly seen (160/190). *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella spp.* were the most common bacteria. *Enterococcus spp.* and coagulase-negative staphylococci were the most common Gram-positive microorganisms. The mortality rate of a bacterial HAI was 40.4%. There was no resistance against vancomycin in Gram-positive microorganisms. The resistance rate against methicillin was 100% in coagulase-negative staphylococci and 50% in *S. aureus* strains. The cumulative rate of carbapenem resistance was found as 76.1% in *Pseudomonas aeruginosa*, 45.2% in *Klebsiella spp.* and 0% in *Escherichia coli*. In 2019, the resistance rate against colistin in *Klebsiella spp.* and *Pseudomonas aeruginosa* were 46.2% (6/13) and 20% (1/5), respectively. The resistance rate against carbapenem and colistin was 81.1% and 0% in *Acinetobacter baumannii*. It was observed that the use of carbapenem before an infection episode increased significantly, and the rate of carbapenem resistance reached 100% over the years in *Pseudomonas aeruginosa* and *Klebsiella spp.* A significant proportion of the isolates were multidrug-resistant strains, significantly threatening survival. Implementation of effective preventive strategies to combat the emergence of antibiotic resistance is urgently needed.

Keywords: Healthcare-associated infections, antibiotic resistance, pediatric, intensive care, mortality

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Introduction

Healthcare-associated infections (HAIs), also known as nosocomial infections or hospital-acquired infections, are the most important complications encountered in children, especially in pediatric intensive care units where critically ill patients are hospitalized. HAIs are an important source of morbidity and mortality worldwide.[1–4] In addition to morbidity and mortality, it prolongs hospital stay, increases antibiotic use and the risk of multi-antibiotic resistance to pathogens, and increases care costs.[1,5,6] The microorganisms isolated as causative agents in pediatric intensive care units and their antibiotic susceptibility and following the changes that occur over the years guide in planning the empirical antibiotic treatment on a case-by-case basis and ensuring the rational use of antibiotics in the unit. With this study, we aimed to examine the characteristics of bacteria isolated as the causative agent of HAIs in our pediatric intensive care unit over five years and the changes in our antibiotic use during the same period.

Highlights

- The most common bacterial HAI agents were Gram-negative bacteria
- The most Gram-negative bacteria were resistant to commonly used broad-spectrum antibiotics such as cefepime, piperacillin-tazobactam, carbapenem, and colistin
- It was observed that 40.4% of the patients diagnosed with bacterial HAI died due to infection
- The resistance to piperacillin-tazobactam, carbapenem, and levofloxacin increased over the years in *Klebsiella spp.* and *P. aeruginosa*. The carbapenem resistance of these two agents was 100% in 2019

Material and Method

The study was carried out by retrospectively examining the information of patients who were hospitalized in the pediatric intensive care unit between 01.01.2015 and 31.12.2019 and were followed up for more than 48 hours and diagnosed with HAI in Erciyes University Pediatric Intensive Care Unit. For the study, an application was made to the Erciyes University Clinical Research Ethics Committee on 12.06.2019, and ethics committee approval was obtained with decision no. 2019/437. The hospitalization-discharge information required for our study was obtained from the hospital data processing unit, the information about the clinical follow-up period of the patients, daily follow-up notes, and surveillance notes made by the hospital infection control committee (antibiotics used before infection, isolated agent and antibiotic resistance profile). Patients hospitalized and diagnosed with HAI clinically and laboratory during the follow-up period and bacteria isolated in sterile field cultures (blood, urine, , cerebrospinal fluid) ,tracheal aspirate and/or wound cultures were included in the study. The study did not include HAI episodes in which culture positivity was not detected. Isolated cathater tip culture positive results excluded from the study. The diagnosis of HAI was made based on the American Center for Disease Control (CDC) 2015 diagnostic criteria.[7] A bacterial HAI was defined as the isolation of a microorganism from a culture of sterile regions (blood, urine, tracheal aspirate, cerebrospinal fluid) or wound cultures of patients who developed signs of infection beyond 48 hours of hospital admission with or without local or systemic symptoms. Endotracheal aspirate cultures considered for colonization were not found to be significant and were not included in the study. Epidemics seen in the unit during the study period were also included in the study if they met inclusion criteria. If the same microorganism was isolated from another infection site and there is clinical evidence that the bloodstream infection is secondary to another infection site, only the culture result of the primary site was evaluated in the records. Some of the patients included in the study had more than one underlying chronic disease. Among the existing diseases, the one with the most clinical importance was selected and included in the study as the primary underlying chronic disease. Patients who died within 14 days of an HAI diagnosis were considered HAI-related mortality. Antibiotic treatments for the cases when the HAI episode developed were also examined. Piperacillin-tazobactam and combined antibiotic regimes, carbapenem, quinolone, and colistin treatments were defined as broad-spectrum antibiotics. Prolonged antibiotic use was also considered as antibiotic use longer than ten days in the last 30 days. Resistance results that were given as intermediate in the evaluation of culture antibiograms were accepted as resistant in our study.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences version 25 (IBM Corp., Armonk, NY, USA). The median (interquartile range-IQR) value of the non-parametric numerical data in the study group was calculated, and the categorical data were given as a percentage (%).

Results

Characteristics of the Cases and Episodes of HAIs

During the five years between January 2015 and December 2019, 1593 patients were hospitalized in our PICU. Two hundred forty-four attacks were detected in 141 patients diagnosed with HAI. Among these attacks, 190 HAI attacks of 99 cases with positivity in a sterile field and/or wound bacterial cultures were included in the study. Demographic and clinical characteristics of 99 cases diagnosed with bacterial HAI are shown in **Table 1**. The median age of the cases was ten months, and 56% were male. There was no underlying disease in 16% (16/99) of the cases. The most common chronic diseases were neurological (39.4%), cardiovascular (19.2%), malignancies (6.1%), respiratory (5.1%) and metabolic (5.1%) disorders, respectively. Infection-related mortality was 40% (40/99) in cases with bacterial HAI.

Table 1
Demographic and clinical features of the patients with a bacterial healthcare-associated infection in the pediatric intensive care unit

Variables	N=99
Age, month, median (IQR)	10 (33)
Sex, male n (%)	56 (56.6)
Underlying chronic diseases, n (%)	
None	16 (16.2)
Neurological	39 (39.4)
Cardiovascular	19 (19.2)
Malignancies	6 (6.1)
Respiratory	5 (5.1)
Metabolic	5 (5.1)
Primary immunodeficiencies	4 (4.0)
Renal	1 (1.0)
Others	4 (4.0)
Mortality, n (%)	40 (40.4)

While 61.1% (116/190) of the agents were isolated from blood culture, 29.5% (59/190) from tracheal aspirate, 5.3% (10/190) from urine, 3.2% (6/190) from wound, 0.5% (1/190) were isolated from cerebrospinal fluid, and again 0.5% (1/190) from peritoneal fluid cultures. The number of episodes by year was 47, 28, 46, 28, and 41 in 2015, 2016, 2017, 2018, and 2019, respectively. The characteristics of 190 bacterial HAI attacks in 99 cases are shown in **Table 2**.

Table 2
Type of specimen with positive results, isolated organisms, and characteristics of the infection episodes

Variables	N=190
Type of specimen, n (%)	
Blood culture	116 (61.1)
Endotracheal aspirate	56 (29.5)
Urine culture	10 (5.3)
Wound swap	6 (3.2)
CSF	1 (0.5)
Peritoneal fluid	1 (0.5)
Number of episodes by year, n (%)	
2015	47 (24.7)
2016	28 (14,7)
2017	46 (24.2)
2018	28 (14.7)
2019	41 (21.6)
Isolated organisms, n (%)	
Gram-negative	**160 (84.2)**
*Klebsiella spp.**	38 (20)
Escherichia coli	11 (5.8)
Pseudomonas aeruginosa	49 (25.8)
Acinetobacter baumannii	38 (20)
Stenotrophomonas maltophilia	7 (3.7)
Serratia marcescens	10 (5.3)
*Enterobacter spp.***	4 (2.1)
Alcaligenes faecalis	3 (1.6)
Gram-positive	**30 (15.8)**
Enterococcus spp.†	21 (11.1)
CoNS	5 (2.6)
Staphylococcus aureus	2 (1.1)
Streptococcus pneumoniae	1 (0.5)
Leuconostoc spp.	1 (0.5)
Prolonged antibiotic usage in the previous 30 days	159 (83.7)
Broad spectrum antibiotic usage	64 (33.7)
Mortality, n (%)	**40 (21.1)**
Gram-negative infections	34 (21.25)
Gram-positive infections	6 (20)

**Klebsiella spp.*: *Klebsiella pneumoniae*, *Klebsiella oxytoca*
***Enterobacter spp.*: *Enterobacter aerogenes*, *Enterobacter cloacae*
†*Enterococcus spp.*: *Enterococcus faecium*, *Enterococcus faecalis*
Abbreviations: CoNS, Coagulase-negative staphylococci; CSF, cerebrospinal fluid

Gram-negative bacteria constituted the majority of bacteria isolated, and a total of 160 episodes of HAI due to Gram-negative bacteria were seen (84.2%). The most commonly isolated agents were *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Acinetobacter baumannii*, and *Escherichia coli*, and their ratios among all agents were 25.8%, 20%, 20%, and 5.8%, respectively. Enterococci (21/190, 11.1%) were the most common cause among Gram-positives, followed by coagulase-negative staphylococci (5/190, 2.6%).

For all episodes evaluated, the rate of antibiotic use longer than ten days in the last 30 days was 83.7% (159/190), while the use of broad-spectrum antibiotics before the attack was 33.7% (64/190). When we analyzed the mortality rates according to the agents, the mortality rates due to Gram-positive and Gram-negative HAI episodes were similarly 20% and 21.5%, respectively.

We looked at the distribution of bacteria isolated as HAI agents yearly. The most common bacteria were *P. aeruginosa* in 2015, 2016, 2017, and 2018, and *Klebsiella spp.* in 2019. In **Figure 1**, the yearly distribution percentage of the bacteria is given by specifying the number of detection in the boxes. Considering the distribution of other agents, Serattia marcescens clustered in 2017, and HAIs originating from *A. baumannii* were generally among the top 3 every year.

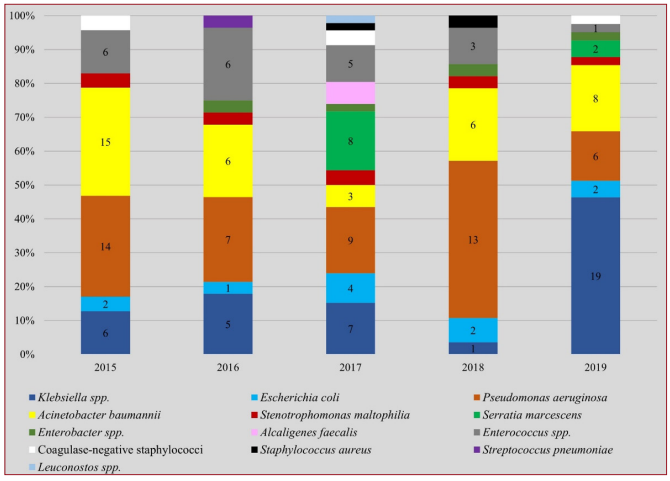

Figure 1. Distribution of the bacterial pathogens by year

Antibiotic Susceptibility Analyses of Bacterial Pathogens

The cumulative rates of antibiotic resistance were analyzed separately for Gram-negative and Gram-positive bacteria. The highest resistance rate was seen against ceftazidime (76.5%) and cefepime (71.9%) in *Klebsiella spp.* On the other hand, *E. coli* had high resistance to levofloxacin (100%) and cefepime (40%). Piperacillin-tazobactam and meropenem resistances in *P. aeruginosa* were 54.2% and 76.1%, respectively. While colistin resistance was not observed in *A. baumannii*, amikacin resistance was 50%, and gentamicin resistance was 81.6%. There was no resistance for trimethoprim/sulfamethoxazole (TMP-SMX) in *S. maltophilia* but 14.3% for levofloxacin. Meropenem resistance was 12.5% in *S. marcescens* strains. Cumulative antibiotic resistance rates of Gram-negative bacteria are shown in **Table 3**.

Table 3
Antibiotic resistance rates of selected Gram-negative bacteria

Antibiotic	*Klebsiella spp.** (n=38)		*Escherichia coli* (n=11)		*Pseudomonas aeruginosa* (n=49)		*Acinetobacter baumannii* (n=38)		*Stenotrophomonas maltophilia* (n=7)		*Serratia marcescens* (n=10)	
	R/(R+S)	R %	R/(R+S)	R %	R/(R+S)	R %	R/(R+S)	R %	R/(R+S)	R %	R/(R+S)	R %
Amikacin	3/35	8.6	1/11	9.1	16/46	34.8	18/36	50	N/A	N/A	1/10	10
Gentamicin	11/20	55	1/3	33.3	19/42	45.2	31/38	81.6	N/A	N/A	6/9	66.7
Ampicilin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ceftriaxone	4/8	50	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cefotaxime	15/24	62.5	3/9	33.3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ceftazidime	13/17	76.5	N/A	N/A	23/49	46.9	N/A	N/A	0/3	0	5/8	62.5
Cefepime	23/32	71.9	4/10	40	16/44	36.4	N/A	N/A	N/A	N/A	7/8	87.5
Ciprofloxacin	15/33	45.5	4/7	36.4	10/47	21.3	30/38	78.9	N/A	N/A	7/10	70
Levofloxacin	7/11	63.6	2/2	100	5/15	33.3	20/27	74.1	1/7	14.3	5/8	62.5
Piperacillin-tazobactam	18/38	47.4	2/10	20	26/48	54.2	N/A	N/A	N/A	N/A	3/10	30
Meropenem	14/31	45.2	0/10	0	35/46	76.1	30/37	81.1	N/A	N/A	1/8	12.5
Imipenem	5/27	18.5	0/7	0	32/44	72.7	27/33	81.8	N/A	N/A	N/A	N/A
Colistin	6/19	31.6	0/3	0	1/21	4.8	0/37	0	N/A	N/A	N/A	N/A
Tigecycline	5/17	29.4	0/3	0	N/A	N/A	0/35	0	N/A	N/A	1/5	20
Fosfomycin	2/3	66.7	0/3	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TMP-SMX	12/20	60	0/1	0	N/A	N/A	28/36	77.8	0/7	0	2/8	25

**Klebsiella spp.*: *Klebsiella pneumoniae*, *Klebsiella oxytoca*, Abbreviations: N/A, not available; TMP-SMX, trimethoprim/sulfamethoxazole

The most common Gram-positive agent was enterococci, and vancomycin resistance was not observed. High-dose gentamicin and ampicillin-sulbactam resistances were 50% and 25%, respectively. Although methicillin resistance was 100% and 50% in coagulase-negative staphylococci and *S. aureus*, glycopeptide resistance was not observed in any of them. *Streptococcus pneumoniae*, which grew in only one peritoneal fluid sample during the study period, was sensitive to penicillin. The resistance rates of Gram-positive agents are shown in **Table 4**.

In our study, we also examined the change over the years besides the cumulative rate of antibiotic resistance. For this purpose, we evaluated the rates of resistance to broad-spectrum antibiotics year-based in the three most common Gram-negative bacteria (*P. aeruginosa*, *Klebsiella spp.*, and *E. coli*), **Figure 2**. The resistance to piperacillin-tazobactam, carbapenem, and levofloxacin increased over the years in *Klebsiella spp.* and *P. aeruginosa*. The carbapenem resistance of these two agents was 100% in 2019. The resistance to cefepime and levofloxacin in *P. aeruginosa* was 100% in 2019, and the resistance to piperacillin-tazobactam was 80%. It was observed that resistance rates in *E. coli* did not increase in 2019, unlike others.

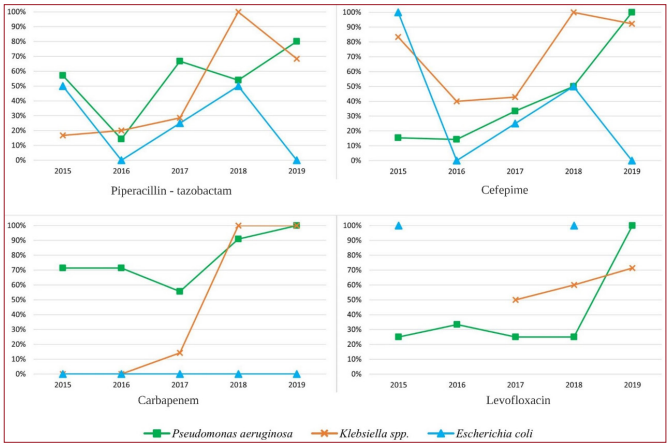

Figure 2. Trends of broad-spectrum antibiotic resistance rates by years in selected Gram-negative bacteria

Evaluation of antibiotic use in PICU

During the study period, treatments of the cases at the onset of the bacterial HAI attack were also evaluated. The use of cefepime, piperacillin-tazobactam, carbapenem, quinolone, and colistin between 2015 and 2019 was examined (**Figure 3**). It has been determined that the use of carbapenems in cases has increased gradually since 2016. While carbapenem was used in

Table 4
Antibiotic resistance rates of Gram-positive bacteria

Antibiotic	*Enterococcus spp.** (n=21)		CoNS (n=5)		*Staphylococcus aureus* (n=2)		*Streptococcus pneumoniae* (n=1)	
	R/(R+S)	R %	R/(R+S)	R %	R/(R+S)	R %	R/(R+S)	R %
Penicillin G	N/A	N/A	N/A	N/A	N/A	N/A	0/1	0
Gentamicin	7/14	50	3/4	75	1/2	50	N/A	N/A
Ampicilin/sulbactam	5/20	25	5/5	100	1/2	50	N/A	N/A
Clindamycin	N/A	N/A	5/5	100	1/2	50	N/A	N/A
Meticilin	N/A	N/A	5/5	100	1/2	50	N/A	N/A
Cefotaxime	N/A	N/A	N/A	N/A	N/A	N/A	0/1	0
Ciprofloxacin	5/21	23.8	N/A	N/A	N/A	N/A	N/A	N/A
Teicoplanin	0/21	0	0/5	0	0/2	0	N/A	N/A
Vancomycin	0/21	0	0/5	0	0/2	0	N/A	N/A
TMP-SMX	N/A	N/A	3/4	75	0/2	0	1/1	100

**Enterococcus spp.*: *Enterococcus faecium*, *Enterococcus faecalis*, Abbreviations: CoNS, Coagulase-negative staphylococci; N/A, not available; TMP-SMX, trimethoprim/sulfamethoxazole

17.9% of cases during any bacterial HAI attack in 2016, this rate increased to 19.6% in 2017, 28.60% in 2018, and 43.9% in 2019. Similarly, colistin usage gradually increased from 2.2% to 14.6% starting in 2017. During the same period, the use of cefepime and piperacillin-tazobactam declined gradually. While quinolones were not used before a bacterial HAI attack in 2016 and 2017, they were used at 14.3% and 12.2% in 2018 and 2019, respectively.

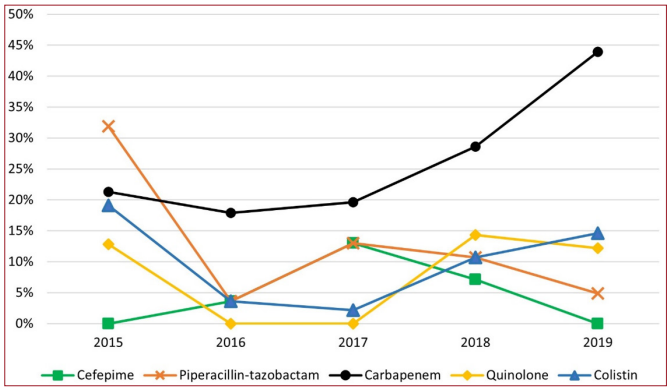

Figure 3. The rate of use of broad-spectrum antibiotics before an infection episode by years

Discussion

HAI is common in developed and developing countries. The World Health Organization (WHO) reported that 8.7% of hospitalized patients developed HAI in a study conducted in 55 hospitals located in 4 regions (Europe, Eastern Mediterranean, Southeast Asia, and Western Pacific).[1] The PICU environment has a high rate of HAI (up to 23%) due to frequent invasive procedures and use of medical devices (central lines, endotracheal tubes) and patient factors (immature immune system, immune deficiencies).[8] In our study, 8.8% of inpatients had at least one HAI attack, and the rate of those who had a bacterial HAI was 6.2% among all patients hospitalized in the PICU.

Patients with various underlying diseases are hospitalized in intensive care units. These underlying diseases may also pose a risk for the development of infection. In our study, neurological and cardiovascular disorders were the most common underlying diseases. It should be kept in mind that the patients in this group will have a high risk of bacterial HAI.

The two most frequent forms of HAI are catheter-associated bloodstream infections (CA-BSI) and pneumonia.[5,9,10] Other HAI encountered in the PICU include surgical-site infections and catheter-associated urinary tract infections. In a study conducted in the USA, the incidence of HAI types was found to be 28% for bloodstream infections, 21% for ventilator-associated pneumonia, and 15% for urinary tract infections.[2] The incidence of HAI types in a national point prevalence study conducted by Kepenekli et al.[4] including 50 pediatric intensive care units; ventilator-associated pneumonia was reported in 55%, bloodstream infection in 27%, and urinary tract infection in 7%.Compatible with other studies, bacterial agents were most commonly isolated from blood, tracheal aspirate, and urine in our study.

Our study observed Gram-negative bacteria more frequently during the five years. In a 5-year prospective and multicenter study from Spain, in the evaluation of the causative microorganism of 99 patients diagnosed with HAI and culture growth; 63 (63.6%) were Gram-negative, 19 (19.2%) Gram-positive, and 17 (17.2%) resulted in fungal positivity.[3] The most common microorganisms isolated in a study from Italy were respectively; Gram-negative (54%), Gram-positive (32%), fungal (7%), and viruses (7%).[11] In our study, the most common Gram-negative agents were; *P. aeruginosa*, *A. baumannii*, and *Klebsiella spp*. In a study from a PICU in Brazil, Gram-negative bacteria were most frequently observed, and *A. baumannii* and *Klebsiella pneumoniae* were reported most frequently among them.[12] We did not evaluate fungal agents in our study, but the frequency of Gram-negative bacteria was similar to the studies in the literature.[13,14] In addition, increasing the rates of Gram-negative bacteria, mainly *P. aeruginosa*, *A. baumannii*, and *Klebsiella spp.*, draws attention. It was seen that the rate of Gram-positive microorganisms in bloodstream infections decreased over the years. We think the sending of control cultures caused this by excluding false positivity after the first growth of coagulase-negative staphylococci.

In our study, it was observed that 40.4% of the patients diagnosed with bacterial HAI died due to infection. The mortality rate was similar in Gram-negative and Gram-positive infections. In a 5-year retrospective study conducted in the pediatric intensive care unit of Adana Numune Training and Research Hospital, the mortality rate was 9%.[6] In a 3-year retrospective study in Israel, mortality was 6% for patients admitted to the pediatric intensive care unit, while mortality was 52% for those who developed bloodstream infections.[15] Various studies from Turkey have mortality data ranging from 2.4% to 27.6%.[16,17] Our mortality rate was found to be higher compared to other studies from Turkey. This may be because patients needing intermediate intensive care are being followed up in the wards, and patients who come with more severe clinics are taken to the pediatric intensive care unit. In addition, patients with chronic neurological disorders, cardiovascular diseases, and postoperative cardiovascular surgery are followed up mostly in our PICU. A large proportion of the patients we follow have a history of prolonged hospitalization due to their underlying chronic diseases. All these factors increase the risk of developing HAI and the associated mortality.

Resistance rates of bacterial pathogens detected in intensive care units are gradually increasing. This leads to treatment failure and increases in mortality. It is considered one of the biggest global health threats, expected to result in 10 million attributable deaths by 2050.[5] Tackling antibiotic resistance has become a priority for the WHO. While studies conducted in the past 15-20 years have targeted Gram-positive agents, recent developments show that Gram-negative bacteria have come to the fore.[18] Increased antibiotic resistance among Gram-negative microorganisms, especially in intensive care units, has limited treatment options.[13]

In our study, most Gram-negative bacteria were resistant to commonly used broad-spectrum antibiotics such as cefepime, piperacillin-tazobactam, carbapenem, and colistin. We also found that carbapenem resistance was widespread among Gram-negative bacteria. The WHO report on pathogen prioritization in 2017 declared extended-spectrum beta-lactamase *Enterobacteriaceae* (especially *Klebsiella spp.*), carbapenem-resistant *A. baumannii*, and *P. aeruginosa* as priority pathogens according to the spectrum of resistance to antimicrobial agents.[19] These top three multidrug resistance bacteria (*Klebsiella*, *Acinetobacter*, and *Pseudomonas*) acquired from PICUs worldwide were the same in recent studies.

Different prevalence rates are reported for carbapenem resistance in Gram-negative bacteria. Carbapenem resistance rates reported in *K. pneumoniae* strains are 60% in India, 36% in Italy, and over 15% in some South American countries such as Argentina and Brazil.[20] In a study from Turkey between 2013 - 2016, the rate of carbapenem resistance was found as 100% in *Acinetobacter* spp., 62.5% in *Pseudomonas* spp., 50% in *E. coli*, 36.7% in *Klebsiella spp.*, 33.3% in *Enterobacter spp.*, and 25% in *Serratia spp.*[21] In the CAESAR 2018 report, ertapenem resistance was reported as 43%, and imipenem/meropenem resistance was reported as 38% in *K. pneumoniae* isolates obtained from blood and cerebrospinal fluid samples from Turkey.[22] In our study, it was observed that carbapenem resistance increased from 10% to 100% in the *Klebsiella spp*. group in a short period of 3 years. For *P. aeruginosa*, this ratio increased from 59% to 100% within three years.

Among Gram-positive organisms, antimicrobial resistance has remained consistent. Methicillin resistance has not changed significantly. Vancomycin sensitivity was analyzed in *Enterococcus*, none of which had shown resistance. Although positive cases were detected by surveillance of vancomycin-resistant enterococci (VRE), it was important that vancomycin resistance was not observed in strains causing invasive infection. It would be appropriate to evaluate the use of linezolid in the empirical treatment of infections within this framework.

Since infections occur in a large proportion of PICU patients, the use of broad-spectrum antibiotics is very common in this population. The largest point prevalence study so far, including 38 PICUs (both general and cardiac) in 23 countries, revealed antibiotic usage in 56% of PICU patients, of which the vast majority were treated with parenteral antibiotics and 50% with combination therapy.[23] There is a serious relationship between antibiotic use and drug-resistant microorganisms. In case-control studies, it has been observed that piperacillin-resistant *P. aeruginosa* develops resistance to fluoroquinolones after prolonged antibiotic use, as well as resistance to the antibiotic used or other antibiotics of the same class.[24] Colonization with carbapenem-resistant Gram-negative in the gut microbiome has been documented to occur after only very brief exposure to these antibiotics.[25] In our study, long-term and broad-spectrum antibiotic use rates were high at the onset of an HAI episode. When we look at the five-year period, an increase in the use of carbapenem, colistin, and quinolones has also attracted attention. On the other hand, while the incidence of Gram-negative agents is increasing, the options to treat these agents are rapidly decreasing. The reduction of resistant pathogens is associated with the rational use of antibiotics. In critically ill children, inappropriate antibiotic prescribing ranges up to 60% (mainly overly broad spectrum and wrong dosage), and as such, the PICU represents a major target environment for antibiotic stewardship programs (ASPs).[26]

There are some limitations of our study. In our study, other important risk factors for HAI, such as the presence of a catheter, ventilator support, and length of stay, were not examined. Possible associations between these risk factors and HAI episodes were not the subject of this study. Fungal infections were also excluded. Since the local microbiological data were obtained from the records of the infection control committee, an evaluation of the minimum inhibitory concentration (MIC) values could not be made. Another limitation was that it was a single-center study. It should not be forgotten that there may be different trends in other centers, and each center should approach by examining and criticizing their own epidemiological characteristics.

Conclusion

In our pediatric intensive care unit, like in the world, the most common bacterial HAI agents were Gram-negative bacteria. It was shown that antibiotic resistance increased rapidly in Gram-negative agents, especially in *Klebsiella spp*. It is thought that the use of broad-spectrum antibacterials, both in our unit and in the world, has increased over the years, and this may also contribute to the development of resistance. Effective treatment of frequent ICU isolates is paramount to preventing multidrug resistance. For this, it is necessary to prevent unnecessary antibiotic use and develop and implement antibiotic stewardship strategies

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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

Ethics Committee Approval: The Erciyes University Clinical Research Ethics Committee approved this study (date: 12.06.2019, number: 2019/437).

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Informed Consent: Because of the retropective nature of the study, no informed consent was obtained from the parents of the patients.

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Letter to the Editor

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Pollakiuria Due to Constipation in a Girl: LUTS or PEDUF?

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Letter to the Editor

Dear Editor,

The bladder and rectum are originated from common embryological origin in the cloaca and are innervated by sacral 2-4 parasympathetic nerves. Similarly, the external anal and urethral sphincters are innervated by the pudendal nerve originating from the sacral 2-4 Onuf nuclei. It has been shown that there is an autonomic reflex interaction between the lower parts of the bladder and the gastrointestinal tract.[1,2] As a result of bladder stimulation with constipation, the problem of urinating frequently and in small amounts occurs. It is defined as the lower urinary tract symptoms (LUTS, lower urinary tract symptoms) or unusual daytime urinary frequency (PEDUF, extraordinary daytime urinary frequency).[1,3] In this article, we would like to emphasize the importance of combined evaluation of children with voiding disorder.

A five-year-old girl presented with complaints of constipation, loss of appetite, and frequent urination for 2 months. While he was in the examination room, it was observed that he urinated every 5 minutes and urinated in a very small amount. It was learned from her history that her mother had type 2 diabetes and Hashimoto's thyroiditis, and her grandmother had type 1 diabetes. He earned toilet training at night at the age of 3. He had severe constipation (Bristol categories 1). Her physical examination was unremarkable with height 108.5 cm (73 p), weight 21 kg (91 p) and body mass index 17.84. Basics urine and metabolic panels were normal. A kidney urinary bladder ultrasound was also normal. She was diagnosed as lower urinary tract symptoms associated with constipation and given lactulose (Duphalac suspension) 5 ml twice a day. Two weeks later, her bladder symptoms gradually decreased and finally disappeared.

Voiding dysfunction (VD) is a common problem in children with an observed rate of 9.5%-19.2% in our country.[4,5] VD may accompany with constipation.[6]

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A series of pathogenesis has been suggested for the formation of VD symptoms such as pollakiuria in children with constipation.[1] The first is that the rectum and bladder are affected by the same neuropathology. Secondly, it is explained that a problem in a system affects the neighboring organ, that is, the accumulation of stool causes urine from the bladder. Third, prolonged constipation and changes in pelvic floor muscles can cause pelvic organ prolapse and urinary incontinence. In childhood, the Rome 3 criteria are used for constipation over the age of four. Accordingly, 2 or fewer stools per week, the presence of a large fecal mass in the rectum, or the stool diameter is large enough to block the toilet can be counted.[7] Our case's stool every 3-4 days is consistent with the diagnosis of constipation. Frequent urination, which is not excessive, draws attention in patients. Differential diagnoses should be made with polydipsia, diabetes, polyuria, urinary tract infection, nephrogenic diabetes insipidus, and viral syndromes.[1] Those were ruled out in our patient and finally, a diagnosis of LUTS or PEDUF was made based on anamnesis, physical examination, and laboratory findings. Apart from constipation, PEDUF can also be observed in patients with a tic disorder, obsessive-compulsive disorder, and Tourette's syndrome.[3] These problems should also be taken into account in the treatment of cases.

Lactulose is recommended for the treatment of constipation as the first line of treatment.[8] It is resistant to the enzyme lactulose disaccharidase, which is a disaccharide and is broken down into lactic, acetic, and formic acid, which stimulates osmotic and intestinal movements by saccharolytic bacteria in the large intestine. It was observed that the complaints of pollakiuria started to decrease with lactulose treatment in our patient. We think that pediatricians should consider VD as a common disease of the bowel and bladder and plan treatment strategy based on this knowledge.

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