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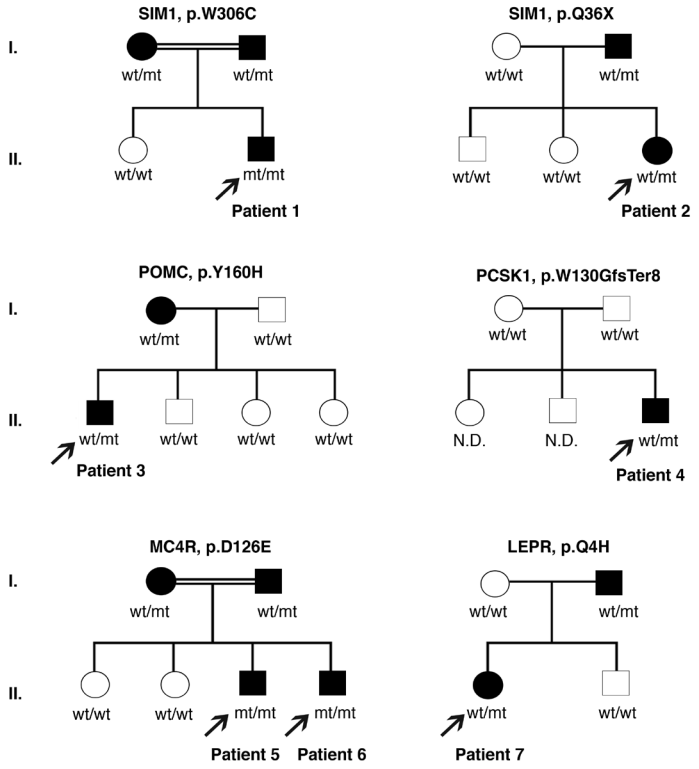

Pedigrees of the families bearing novel variants in obesity related genes. Arrows indicate probands in each family. Genotypes were defined as wild type (wt) or mutant (mt) for corresponding variations

Novel Mutations in Obesity-related Genes in Turkish Children with Non-syndromic Early Onset Severe Obesity: A Multicentre Study

Akıncı A et al.

DOI: 10.4274/jcrpe.galenos.2019.2019.0021

Official Journal of
Turkish Pediatric Endocrinology
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Öğrenmesi kolay[1,2]

Kullanması kolay[1,2]

Taşıması kolay[3,4]

Gigi, ikiz, 4 yaşında, gebelik yaşına göre küçük ağırlıkta doğmuş, ikizi ile birlikte (gebelik yaşına uygun ağırlıkta doğmuş)

Referanslar: 1. Tauber M, et al. Patient Prefer Adherence. 2013;7:455–462. **2.** Rohrer TR, et al. Expert Opin Drug Deliv. 2013;10:1603–1612. **3.** Norditropin NordiFlex® Kısa Ürün Bilgisi ve Kullanma Talimatı. **4.** Data on File: Norditropin NordiFlex™ Development and Comparison to FlexPen®, ID: 000184362. Bagsvaerd, Denmark: Novo Nordisk A/S; 2003.

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Diğer tüm hastalarda IGF-I ölçümü ve bir büyüme hormonu stimülasyon testi gereklidir. Erişkinlik döneminde başlayan BHE: Bilinen hipotalamo-hipofizer hastalıkta, kraniyal ışınlama ve travmatik beyin hasarında belirgin BHE (hipotalamo-hipofizer aksta prolaktin dışında başka bir eksiklik). Akstaki diğer eksiklikler için yeterli replasman tedavisinin başlatılmasından sonra bir provokatif test ile BHE gösterilmelidir. **Kontrendikasyonlar:** Tümör aktivitesi bulgu varlığında; açık kalp cerrahisi, abdominal cerrahi, kazaya bağlı çoklu travma, akut solunum yetmezliği veya benzer durumları takiben akut kritik hastalık komplikasyonları olan hastalarda; somatropine ya da bileşimindeki maddelerden herhangi birisine aşırı duyarlılık durumlarında; kronik böbrek yetmezliği olan çocuklarda renal transplantasyon yapılırken; epifizleri kapanmış çocuklarda kullanılmamalıdır. **Kullanım şekil ve dozu:** Cilt altına enjeksiyon ile (s.c.) kullanılır. Doz hastaya göre ve hastanın tedaviye verdiği yanıt göz önüne alınarak düzenlenmelidir. Genellikle, her gün akşamları ve enjeksiyon yeri değiştirilerek uygulama önerilmektedir. **Genel olarak önerilen doz:** ***Çocuklarda:*** Büyüme hormonu yetersizliği: 0.025-0.035 mg/kg/gün veya 0.7-1.0 mg/m²/gün. Turner Sendromu: 0.045-0.067 mg/kg/gün veya 1.3-2 mg/m²/gün. Kronik böbrek hastalığı: 0.050 mg/kg/gün veya 1.4 mg/m²/gün. Gebelik yasına göre küçük: 0.035 mg/kg/gün veya 1 mg/m²/gün. ***Erişkinlerde:*** Erişkinlerde replasman tedavisi: Doz, hastanın gereksinimine göre belirlenmelidir. Çocukluk döneminde başlayan BHE'si olan hastalarda tedaviye 0.2-0.5 mg/gün dozla başlanması ve sonrasında IGF-I konsantrasyonlarına göre dozun ayarlanması önerilmektedir. Erişkinlikte başlayan BHE hastalarında tedaviye düşük dozla başlanması önerilir: 0.1-0.3 mg/gün. Dozun, hastanın tedaviye verdiği yanıt ve hastanın advers etkiler ile ilgili deneyimleri göz önüne alınarak birer aylık aralıklar ile artırılması önerilmektedir. Serum İnsülin Benzeri Büyüme Faktörü I (IGF-I), doz titrasyonu için rehber olarak kullanılabilir. Doz ihtiyacı yaşa bağlı olarak azalır. İdame dozu kişisel farklılıklar göstermekle birlikte, nadiren 1.0 mg/gün değerinin üzerine çıkar. **Uyarılar/Önlemler:** Tedavisi, her zaman bu konuda bilgi ve deneyimi olan uzman hekimler tarafından yapılmalıdır. Önerilen maksimum günlük doz aşılmamalıdır. Turner Sendromlu hastalarda el ve ayaklarda büyüme artışı gözlenirse, dozun, doz aralığındaki daha düşük bir doza düşürülmesi düşünülmelidir. Kronik böbrek hastalığı olan hastalarda, böbrek fonksiyonları takip edilmelidir. Turner Sendromlu ve SGA'lı çocuklarda tedaviye başlamadan önce ve daha sonra yılda bir kez açlık insülin ve kan glukoz değerlerinin ölçülmesi ve insülin tedavisi almakta olanlarda dozun izlenmesi önerilir. Belirgin diyabet ortaya çıkarsa büyüme hormonu tedavisi uygulanmamalıdır. Aşırı obezite, üst solunum yolu obstrüksiyonu, uyku apnesi öyküsü veya tanımlanamamış solunum enfeksiyonu gibi risk faktörlerinden biri ya da birden fazlası olan Prader-Willi sendromlu hastalarda somatropin tedavisinin başlanması ile ani ölümler bildirilmiştir. İlerleyen hipofiz hastalığı olan hastalarda hipotiroidizm gelişebilir. Şiddetli ve tekrarlayıcı baş ağrısı, görme bozuklukları, bulantı varlığında hasta papil ödemi açısından incelenmelidir. Somatropin tedavisi gören yetişkinlerde veya çocuklarda yeni primer kanser riskinin arttığına dair bir kanıt yoktur. Malign hastalığı tamamen remisyonda olan hastalarda, somatropin tedavisi, relaps oranının artması ile ilişkili bulunmamıştır, ancak bu hastalar relaps açısından somatropin tedavisinin başlangıcından itibaren yakından izlenmelidir. ***Gebelik kategorisi:*** C. Gebelik döneminde somatropin tedavisinin güvenilirliği açısından yeterli kanıt bulunmamaktadır. Somatropinin insan sütüne geçip geçmediği bilinmediğinden emziren kadınlara verileceği zaman dikkat edilmelidir. **Yan Etkiler/Advers Etkiler:** Erişkinlerde periferik ödem, baş ağrısı, parestezi, artralji eklem sertliği ve miyalji görülebilir. Çocuklarda döküntü, artralji, miyalji ve periferik ödem seyrek olarak ve baş ağrısı yaygın olmayan şekilde görülebilir. Lokal enjeksiyon yeri reaksiyonları oluşabilir. Bazı nadir vakalarda benign intrakraniyal hipertansiyon bildirilmiştir. Turner Sendromlu çocuklarda büyüme hormonu tedavisi sırasında el ve ayaklarda büyümenin arttığı bildirilmiştir. **Etkileşimler:** Glukokortikoidler ile birlikte kullanılması büyümeyi inhibe edebilir. Büyüme; gonadotropin, anabolik steroidler, östrojen ve tiroid hormonu gibi diğer tedavilerden de etkilenebilir. **Saklamaya Yönelik Özel Tedbirler:** Açıldıktan sonra: Buzdolabında (2°C-8°C) maksimum 4 hafta saklayınız. Işıktan koruyunuz. Dondurmayınız. 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Papers Only Published with DOI Numbers: Knops NB, Sneeuw KC, Brand R, Hile ET, de Ouden AL, Wit JM, Verloove-Vanhorick SP. Catch-up growth up to ten years of age in children born very preterm or with very low birth weight. BMC Pediatrics 2005 doi: 10.1186/1471-2431-5-26.

Book Chapters: Darendeliler F. Growth Hormone Treatment in Rare Disorders: The KIGS Experience. In: Ranke MB, Price DA, Reiter EO (eds). Growth Hormone Therapy in Pediatrics: 20 Years of KIGS. Basel, Karger, 2007;213-239.

Books: Practical Endocrinology and Diabetes in Children. Raine JE, Donaldson MDC, Gregory JW, Savage MO. London, Blackwell Science, 2001;37-60.

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Tüm temel endikasyonlarda onaylı tek sıvı büyüme hormonu [1-5]

- Kullanım kolaylığı[6]
- Onaylı uzun dönem etkililik[6]
- Uygunluk[7,8]
- Kullanıma hazır sıvı formülasyon[6]
- Kanıtlanmış güvenilirlik[6]
- Yılların biyoteknoloji deneyimi[9]

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▼ **Bu ilaç ek izlemeye tabidir.** Bu üçgen yeni güvenlilik bilgisinin hızlı olarak belirlenmesini sağlayacaktır. Sağlık mesleği mensuplarının şüpheli advers reaksiyonları TÜFAM'a bildirmeleri beklenmektedir. Raporlama yapılması, ilaçın yarar/risk dengesinin sürekli olarak izlenmesine olanak sağlamaktadır. Herhangi bir şüpheli advers reaksiyonu Türkiye Farmakovijilans Merkezi (TUFAM)'ne (www.titck.gov.tr; e-posta: tufam@titck.gov.tr; tel: 0312 218 30 00, 0800 314 00 08; faks: 0 312 218 35 99) ve/veya ilgili firma yetkililerine bildirmeniz gerekmektedir.

OMNİTROPE® 5 mg (15 IU)/1.5 mL ve 10 mg (30 IU)/1.5 mL ve OMNİTROPE® 15 mg (45 IU)/1,5 mL SC enjeksiyon için çözelti içeren kartuş **Etkin madde:** OMNİTROPE® 5 mg kartuş 5 mg Somatropin'e eşdeğer (15 IU) 1.5 mL, OMNİTROPE® 10 mg kartuş, 10 mg Somatropin'e eşdeğer (30 IU) 1.5 mL, OMNİTROPE® 15 mg Somatropin'e eşdeğer (45 IU) 1,5 mL çözelti içerir. Somatropin rekombinant DNA teknolojisi ile *Escherichia coli*'den üretilmiştir. **Terapötik endikasyonlar:** İnfantlar, çocuklar ve adolesanlar: Büyüme hormonunun (GH) yetersiz salınımından kaynaklanan büyüme bozuklukları, Turner sendromu ile ilişkili büyüme bozuklukları, kronik böbrek yetmezliği ile ilişkili büyüme bozuklukları, -2 standart sapma (SD) altındaki doğum ağırlığı ve/veya doğum boyu olan 4 yaşında veya daha sonraki yaşlarda büyümede geri kaldığı gösterilen (son yıl süresince uzama hızı (HV) SDS < 0), gestasyonel yaşa göre (SGA) küçük doğmuş kısa boylu çocuklardaki (mevcut uzunluk standart sapma oranı (SDS) < -2.5 ve parental ayarlanmış SDS < -1) büyüme bozuklukları, Prader-Willi sendromunda (PWS), büyüme ve vücut kompozisyonunun düzeltilmesi için. Yetişkinler: Belirgin bir büyüme hormonu yetmezliği olan yetişkinlerde replasman tedavisi için. **Pozoloji:** Çocuklarda büyüme hormonunu salgılama yetersizliğine bağlı büyüme bozukluğu: Genel olarak günlük 0.025–0.035 mg/kg (günlük 0.7–1.0 mg/m²) tavsiye edilmektedir. Daha yüksek dozlar da kullanılmıştır. Prader-Willi Sendromu olan çocuklarda büyüme ve vücut kompozisyonunun düzeltilmesi için: Genel olarak günlük 0.035 mg/kg (günlük 1.0 mg/m²) tavsiye edilmektedir. Günlük doz 2.7 mg'ı aşmamalıdır. Turner sendromuna bağlı büyüme bozukluğu: Günlük 0.045 – 0.050 mg/kg (günlük 1.4 mg/m²) tavsiye edilmektedir. Kronik böbrek yetmezliğine bağlı büyüme bozukluğu: Günlük 0.045 – 0.050 mg/kg (1.4 mg/m²) doz tavsiye edilmektedir. Gestasyonel yaşa göre küçük doğmuş (SGA) çocuklarda / adolesanlarda büyüme bozukluğu: Genellikle en son uzunluğa ulaşılıncaya kadar günlük 0.035 mg/kg (günlük 1 mg/m²) tavsiye edilmektedir. Yetişkinlerde büyüme hormonu yetersizliği: Günlük 0.15 – 0.3 mg gibi düşük bir dozla tedaviye başlanmalıdır. Doz, IGF-1 konsantrasyonuna göre belirlenen bireysel hasta gereksinimlerine göre aşamalı olarak artırılmalıdır. **Uygulama sıklığı ve süresi:** OMNİTROPE® günde bir defa, akşamları uygulanır. Büyüme hormonu eksikliği tedavisi normal olarak, uzun süreli tedavi gerektirir. Dozaj ve tedavi süresi hastanın tedaviye vereceği yanıta göre bireyselleştirilmelidir. **Uygulama şekli:** Subkütan enjeksiyon şeklinde uygulanır. Yalnızca OMNİTROPE® 5 mg (15 IU)/1.5 mL ile kullanım için özel olarak geliştirilmiş bir enjeksiyon aracı (enjeksiyon kalemi) olan SurePal™ 5; OMNİTROPE® 10 mg (30 IU)/1.5 mL ile kullanım için özel olarak geliştirilmiş bir enjeksiyon aracı (enjeksiyon kalemi) olan SurePal™ 10; OMNİTROPE® 15 mg (45 IU)/1.5 mL ile kullanım için özel olarak geliştirilmiş bir enjeksiyon aracı (enjeksiyon kalemi) olan SurePal™ 15 ile uygulanmalıdır. **Özel popülasyonlara ilişkin ek bilgiler: Böbrek yetmezliği:** Kronik böbrek yetmezliği durumunda, tedaviye başlamadan önce böbrek fonksiyonları normalin %50'sinin altında olmalıdır. Büyüme bozukluğunu doğrulamak için tedavinin başlatılmasından önce bir yıl süreyle büyüme takip edilmelidir. Bu dönem süresince böbrek yetmezliği için konservatif tedavi (asidoz, hiperparatiroidizm ve beslenme durumu kontrolünü içeren) uygulanmalı ve tedavi süresince sürdürülmelidir. Bu dönem süresince böbrek yetmezliği için konservatif tedavi (asidoz, hiperparatiroidizm ve beslenme durumu kontrolünü içeren) uygulanmalı ve tedavi süresince sürdürülmelidir **Karaciğer yetmezliği:** Karaciğer fonksiyon bozukluğu olan hastalarda somatropin klerensinde azalma görülebilmektedir, ancak bu durumun klinik önemi bilinmemektedir. **Pediyatrik popülasyon:** Somatropin dozu ve uygulama takvimi her hastaya göre bireysel olarak ayarlanmalıdır. Epifiz füzyonu oluşmuşsa tedaviye devam edilmemelidir. Büyüme hormonu tedavisine yanıt zamanla azalma eğilimi gösterir. **Kontrendikasyonlar:** Somatropin veya herhangi bir yardımcı maddeye karşı aşırı duyarlılık. Somatropin; tümör aktivitesine dair herhangi bir bulgu olduğu zaman kullanılmamalıdır ve tedaviye başlamadan önce anti-tümör tedavisi tamamlanmış olmalıdır. Somatropin epifizleri kapanmış çocuklarda büyümenin uyarılması için kullanılmamalıdır. Açık kalp ameliyatı, abdominal cerrahi, multiple kaza travması, akut solunum yetmezliği veya benzer durumlar gibi akut kritik hastalığı olanlarda somatropin ile tedavi uygulanmamalıdır. Özel kullanım önerileri: Eğer somatropin almakta olan bir kadın oral östrojen tedavisine başlarsa, serum IGF-1 seviyelerinin yaşa göre uygun aralıkta muhafaza edilebilmesi için somatropin dozunun arttırılması gerekli olabilir. Büyüme hormonu, T4'ün T3'e tiroid bezi dışındaki dönüşümünü artırabilir, bu durum serum T4 seviyesinde azalma ve serum T3 konsantrasyonlarında bir artışla sonuçlanabilir. Malign hastalıkların tedavisine sekonder olarak görülen bir büyüme hormonu yetmezliği varsa malignitenin nüks belirtilerine dikkat edilmesi tavsiye edilmektedir. Şiddetli ve yinelenen baş ağrısı, görme problemleri, mide bulantısı ve/veya kusma durumlarında papillödemi için fundoskopinin yapılması tavsiye edilmektedir. Eğer papillödemi varlığı doğrulanırsa, iyi huylu intrakraniyal hipertansiyon tanısı düşünülmelidir ve eğer uygunsa büyüme hormonu tedavisi kesilmelidir. OMNİTROPE® 5 mg (15 IU)/1.5 mL içeren form her mL'de 9 mg benzil alkol içerir. Benzil alkol varlığından dolayı prematüre bebekler ve yeni doğanlara uygulanmamalıdır. Bebeklerde ve 3 yaşına kadar olan çocuklarda toksik reaksiyonlara ve anaflaktoid reaksiyonlara sebebiyet verebilir. **Gebelik ve laktasyon Genel tavsiye Gebelik kategorisi**: C **Gebelik dönemi:** OMNİTROPE® gebelik döneminde kullanılmamalıdır. Emziren kadınlarda somatropin içeren ürünlerle yapılan klinik çalışma yoktur. Emzirmenin durdurulup durdurulmayacağına ya da OMNİTROPE® tedavisinin durdurulup durdurulmayacağına/ tedaviden kaçınılıp kaçınılmayacağına ilişkin karar verilirken, emzirenin çocuk açısından faydası ve OMNİTROPE® tedavisinin emziren anne açısından faydası dikkate alınmalıdır. **İstenmeyen etkiler:** Büyüme hormonu yetmezliği olan hastalar, ekstrasellüler hacim eksikliği ile karakterizedir. Somatropin ile tedaviye başlandığı zaman bu hacim hızla düzelmektedir. Yetişkin hastalarda periferal ödem, ekstremitelerde tutukluk, artralji, miyalji ve parestezi gibi sıvı retansiyonu ile ilgili advers etkiler yaygın olarak meydana gelmektedir ve kendiliğinden veya doz azaltılması ile birlikte hafifler. Çocuklarda bu gibi advers etkiler yaygın değildir. Somatropin, hastaların yaklaşık %1'inde antikor oluşumuna neden olmaktadır. Bu antikor oluşumu klinik değişikliklere neden olduğu saptanmamıştır ve bağlanma kapasitesi düşüktür. Somatropinin serum kortizol seviyelerini büyük olasılıkla taşıyıcı proteinleri etkilemek suretiyle ya da artan hepatik klirens yoluyla azalttığı rapor edilmiştir. Klinik önemi sınırlı olabilir ancak kortikosteroid replasman tedavisi, tedaviye başlamadan önce optimize edilmelidir. Somatropinle tedavi gören büyüme hormonu yetmezliği olan çocuklarda seyrek ya da çok seyrek olarak lösemi vakaları meydana geldiği rapor edilmiştir ve bu durum pazarlama sonrası deneyimde yer almıştır. Beyin ve kafaya radyasyon uygulaması gibi hazırlayıcı faktörler olmaksızın lösemi riski artışına dair bir kanıt bulunmamaktadır. GH ile tedavi edilen çocuklarda femur başı epifiz kayması ve Legg-Calvé-Perthes hastalığı raporlanmıştır. Femur başı epifiz kayması, endokrin bozukluklar halinde daha sık meydana gelmektedir ve boy kısalığı halinde Legg-Calvé-Perthes daha sıktır. Pazara erişim sonrası deneyimde, her ne kadar nedensel bir ilişki gösterilemese de somatropin ile tedavi edilen Prader-Willi sendromlu hastalarda seyrek olarak ani ölüm raporlanmıştır. Somatropin ile tedavi edilirken bu patolojilerin daha sık olup olmadığı bilinmemektedir. Azalan insulin duyarlılığı nedeniyle hiperglisemi somatropine ait sınıf etkisi olduğu düşünülebilir. Ayrıntılı bilgi için TITCK onaylı KUB bakınız. **Doz aşımı ve tedavisi:** Akut doz aşımı başlangıçta hipoglisemiye ve daha sonra hiperglisemiye neden olabilir. Uzun süreli doz aşımı, yüksek doz insan büyüme hormonunun bilinen etkileri ile uyumlu belirti ve bulgularla sonuçlanabilir. **Farmakokinetik özellikler:** Emilim: Sağlıklı gönüllülerde ve büyüme hormonu yetersizliği olan çocuklarda subkütan olarak uygulanan somatropinin biyoyararlanımı yaklaşık %80'dir. Sağlıklı yetişkenlerde 5 mg/1,5 mL OMNİTROPE®'un subkütan enjeksiyonundan sonra Cmaks ve tmaks değerleri sırasıyla 72 ± 28 mikrogram/L ve 4 ± 2 saattir. Sağlıklı yetişkenlerde 10 mg/1,5 mL OMNİTROPE®'un 5 mg subkütan enjeksiyonundan sonra Cmaks ve tmaks değerleri sırasıyla 74 ± 22 mikrogram/L ve 3,9 ± 1,2 saattir. Sağlıklı yetişkenlerde 15 mg/1,5 mL OMNİTROPE®'un 5 mg subkütan enjeksiyonundan sonra Cmaks ve tmaks değerleri sırasıyla 52 ± 19 mikrogram/L ve 3,7 ± 1,2 saattir. Eliminasyon: Büyüme hormonu yetersizliği olan yetişkinlerde intravenöz uygulamadan sonra somatropinin ortalama terminal yarı ömrü yaklaşık 0,4 saattir. Ancak, OMNİTROPE® 5 mg/1,5 mL ve 10 mg/1,5 mL'nin subkütan uygulamasından sonra 3 saatlik, OMNİTROPE® 15 mg/1,5 mL'nin subkütan uygulamasından sonra ise 2,76 saatlik bir yarı ömre ulaşılmıştır. Gözlenen farklılığa subkütan uygulamayı takiben enjeksiyon bölgesinden yavaş emilimin neden olması muhtemeldir. **Raf ömrü:** OMNİTROPE® 5 mg (15 IU)/1.5 mL için 24 ay, OMNİTROPE® 10 mg (30 IU)/1.5 mL için 18 ay, OMNİTROPE® 15 mg (45 IU)/1.5 mL için 18 aydır. İlk kullanımdan sonra raf ömrü: İlk kullanımdan sonra kartuş enjeksiyon kaleminin içinde kalmalıdır. Açıldıktan sonra buzdolabında (2°C - 8°C) saklanması koşulu ile 28 gün içerisinde kullanılmalıdır. Dondurulmamalıdır. Orijinal enjeksiyon kaleminin içerisinde ışıktan korunarak saklanmalıdır. **Saklamaya yönelik özel tedbirler:** Açılmamış kartuş: Buzdolabında (2°C - 8°C) saklanmalı ve taşınmalıdır. Dondurulmamalıdır. Orijinal ambalajında ışıktan korunarak saklanmalıdır. **Ambalajın niteliği ve içeriği:** Bromobütil tıpası ve alüminyum çek-çıkar kapağı olan renksiz, 1.5 mL'lik Tip I kartuş ile ambalajlanır. **KDV DAHİL PERAKENDE SATIŞ FİYATI:** OMNİTROPE® 5 mg (15 IU)/1.5 mL SC enjeksiyon için çözelti içeren kartuş 313,71 TL KDV Dahil (19.02.2019), OMNİTROPE® 10 mg (30 IU)/1.5 mL SC enjeksiyon için çözelti içeren kartuş 570,21 TL KDV dahil (19.02.2019), OMNİTROPE® 15 mg (45 IU)/1,5 mL SC enjeksiyon için çözelti içeren kartuş 905,77 TL KDV dahil (19.02.2019). **RUHSAT SAHİBİ:** Sandoz İlaç San. ve Tic. A.Ş. Suryapı & Akel İş Merkezi Rüzgarlıbahçe Mah. Şehit Sinan Eroğlu Cad. 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Achieving Optimal Short- and Long-term Responses to Paediatric Growth Hormone Therapy

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Abstract

It is over sixty years since the first administration of human growth hormone (GH) to children with GH deficiency, and over thirty years since recombinant human GH has been available for treatment of GH deficiency and a wider range of non-GH deficiency disorders. From a diagnostic perspective, genetic analysis, using single gene or Sanger sequencing and more recently next generation or whole exome sequencing, has brought advances in the diagnosis of specific causes of short stature, which has enabled therapy to be targeted more accurately. Genetic discoveries have ranged from defects of pituitary development and GH action to abnormalities in intracellular mechanisms, paracrine regulation and cartilage matrix formation. The strategy of GH therapy using standard doses has evolved to individualised GH dosing, depending on diagnosis and predictors of growth response. Evidence of efficacy of GH in GH deficiency, Turner syndrome and short children born small for gestational age is reviewed. The importance of critical assessment of growth response is discussed, together with the recognition and management of a poor or unsatisfactory growth response and the organisational issues related to prevention, detection and intervention regarding suboptimal adherence to GH therapy.

Keywords: Paediatrics, short stature, growth hormone therapy, growth hormone deficiency, Turner syndrome, small for gestational age

Introduction

Human pituitary-derived growth hormone (GH) has been in use to promote growth in short children for more than 25 years, until it was halted in 1985 due to recognition of the association with Creutzfeldt-Jakob disease (1). The first recombinant human GH received approval for paediatric clinical use for growth promotion in 1985, from both the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Initially, the approval was specifically for children with GH deficiency (Figure 1), but over time GH has been licensed for use in a number of non-GH deficiency growth disorders, including chronic renal insufficiency, Turner syndrome and short stature related to birth size in small for gestational age (SGA) children. The non-GH deficiency disorders of Noonan syndrome and idiopathic short stature have also received approval for GH use from the FDA, but not the EMA, and short stature due to *SHOX* gene haploinsufficiency and Prader-Willi syndrome (PWS) have received approval for some GH formulations in some countries (2,3,4).

The capacity to secrete endogenous GH and the sensitivity to administered GH vary greatly, both within and among these disorders (5). Thus, there is a continuum whereby GH secretion is very low and responsiveness to treatment is high in patients with severe GH deficiency, in contrast to those with severe GH resistance (Laron syndrome) where GH secretion is high and response to administered GH is very low or non-existent (5,6,7). Conditions in-between include PWS and GH

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neurosecretory dysfunction, with good GH sensitivity but diminished secretion and idiopathic short stature, chronic renal insufficiency and SGA, with slightly reduced or normal GH secretion and variable GH responsiveness. When GH sensitivity is decreased, particularly in patients with SGA, Turner syndrome and idiopathic short stature, higher or pharmacological GH doses are generally required (5,8).

The differences in sensitivity to administered GH in children with growth failure arising from different conditions means that treatment management varies according to factors including the diagnosis, gender and age of the patient at GH initiation. An Advisory Board was convened in Dubai in December 2017, by Merck Serono Middle East FZ-LLC, Dubai, United Arab Emirates, with the aim of addressing the issues of the short- and long-term management of paediatric GH therapy for children with growth failure due to different conditions. This article reports the discussions and conclusions of the Advisory Board meeting.

The Genetic Basis of Short Stature

Human adult height is a polygenic trait in which the additive genetic contribution to normal variation is reported to be approximately 80% (9,10). While it is polygenic overall, multiple monogenic defects in genes coding for proteins with key functions in GH secretion and action have been identified to be associated with growth failure. At least eight genes have been identified in which individual abnormalities were associated with isolated GH deficiency and at least 19 genes where mutations resulted in combined pituitary hormone deficiency, with several related to specific syndromes (11,12). There were also 10 genes reported to be associated with GH resistance or insulin-like growth factor-1 (IGF-1) insensitivity. However, genome-wide association studies have indicated that only a minority of genes related to adult height are directly associated with the GH-IGF-1 axis and many genes act through other pathways (10).

Bone growth in the epiphyseal growth plate is influenced by many factors, including cytokines, nutritional status, other hormones such as thyroxine, glucocorticoids and gonadotropins, various paracrine factors within the extracellular matrix, and intracellular proteins (13,14). Next-generation sequencing has also shown that many genetic disorders that were previously thought to be only associated with skeletal dysplasia can present as dominant forms of apparent idiopathic short stature. These include, for example, abnormalities in the gene for the retinoic acid degrading enzyme *CYP26C1* (15), coding and non-coding regions of the short-stature homeobox-containing gene *SHOX* (16,17), the *ACAN* gene coding for the growth plate extracellular matrix proteoglycan aggrecan (18,19), the natriuretic peptide receptor-B gene *NPR2* (20,21,22) and the gene encoding Indian Hedgehog (*IHH*) (23).

These studies have led to a new paradigm (Opinion Box) in which regulation of the epiphyseal plate is recognised to be pivotal to human linear growth, with disorders of the GH-IGF-1 axis making a less important contribution to height (13). The distinctions between idiopathic short or tall stature and skeletal dysplasias have become blurred and it is now understood that a genetic mutation can lead to a spectrum of phen. Gain of function mutations of various genes can lead to tall stature, whereas mild polymorphisms that modulate function and/or expression may result in low normal height.

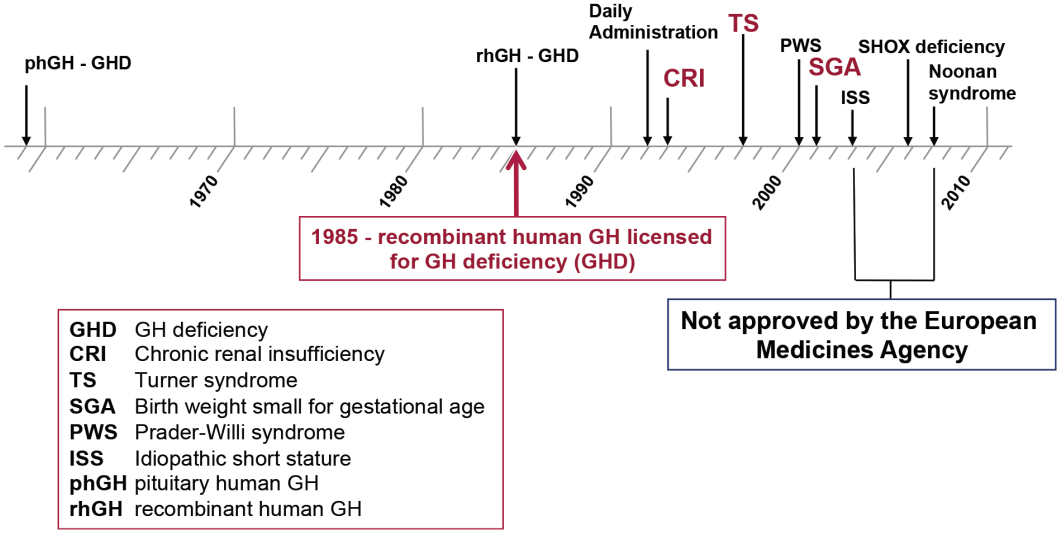

Figure 1. Growth disorders with approval for growth hormone therapy from the United States Food and Drug Administration and the European Medicines Agency

Opinion Box. Consensus opinions of the advisory board

1. Regulation of the epiphyseal growth plate is pivotal to human growth; disorders of the GH-IGF-1 axis are less prevalent than genetic disorders caused by abnormalities of genes involved in intracellular mechanisms, paracrine regulation and cartilage matrix formation, shown by studies using next-generation sequencing.

2. A formal mathematical model of growth prediction is not used in many countries; however, predictive factors should be used to tailor GH treatment and individualise GH dose. Alternatively, tailoring can be performed according to serum IGF-1 level.

3. Initiating GH at a young age is a key factor for a good response, irrespective of the cause of short stature, and whenever possible GH treatment should start when the patient is pre-pubertal.

4. Assessment of the growth response at the end of year one of GH therapy is a responsibility of the clinician; if a poor growth response is documented, consideration of poor adherence to GH therapy should be included in further management.

GH-IGF-1: growth hormone-insulin-like growth factor-1

Mutations of genes that are not critical for growth, and mutations that are heterozygous or merely impair function of the gene product, can lead to isolated short stature, while mutations that cause severe loss of function affect critical genes causing skeletal dysplasias concomitant with growth failure (13,24). Therefore, testing for genetic defects in order to provide a diagnosis needs to be very specific and directed (25,26,27,28), although there are also arguments in favour of a hypothesis-free approach, using growth-specific whole exome sequencing-based gene panels (29,30,31).

GH Therapy for GH Deficiency and Non-GH Deficiency Causes of Short Stature

GH Therapy: Aims and Growth Response

For paediatric patients who receive exogenous GH for short stature, the therapy must be effective and safe. The induced catch-up growth should increase the height standard deviation score (SDS), with the aim of achieving an adult height SDS close to the genetic target height SDS, based on mid-parental height (2,32). Reports have suggested an association of excessive IGF-1 levels with adult morbidity, with the results that the aim of therapy for patients with initially low serum IGF-1 SDS is to treat with a GH dose that results in an IGF-1 increase to within the normal range (33,34). However, in patients with non-GH-deficient causes of growth failure, and initially normal IGF-1 SDS, elevated IGF-1 levels associated with higher GH doses may be required to achieve an acceptable height gain (34,35,36). GH treatment should also be as patient-friendly as possible, with appropriate injection devices and low injection volumes (37). Additionally, GH treatment should be cost-effective (34,38,39), providing sufficient efficacy with the lowest dose, which requires a personalised approach to therapy (40,41).

A number of different measurements have been used to assess growth response to GH treatment (42). These include the change in height SDS and height velocity at yearly intervals after starting GH therapy. Catch-up growth may also be determined from change in height over the first two years of GH treatment or using mathematical models of height SDS (43). However, there is no consensus on the definition of a good indicator of response and, if a relatively low cut-off level is taken, more than 50% of patients may be defined as poor responders (6,42,44). There may be multiple factors that affect response, such as concomitant disease, unanticipated GH insensitivity or poor adherence with the treatment (43). Nevertheless, this depends to a large extent on the correct diagnosis, because the aetiology has a major impact on the response to paediatric GH therapy (45).

Prediction of Response

A number of models have been devised to predict the growth response to GH therapy for various aetiologies (41,46,47,48,49,50,51). Using such models, the mean predicted height velocity with a standard dose of 0.3 mg/kg/week is approximately 2 cm/year greater for children with GH deficiency, compared with children born SGA and girls with Turner syndrome. The factors that influence the prediction of the first-year growth response for patients with GH deficiency, Turner syndrome and SGA are shown in Table 1. The primary influence for children with GH deficiency is severity of the condition, whereas the primary influence for girls with Turner syndrome and children born SGA is the dose of GH per kg body weight per week (6,52,53).

Predictive models can also be used for long-term growth response (54,55,56). Factors that influence adult height are shown in Table 2, including the calculated variation explained by all predictive factors combined for each diagnostic cause (54,57,58,59). Height at GH initiation and mid-parental target height has an impact on adult height for patients in each of the diagnostic categories. The first-year response to GH treatment was strongly correlated with gain to adult height and, therefore, change in height SDS should be formally assessed at the end of year one of GH therapy (58,59,60). These predictive factors can be used to tailor GH treatment even if a formal mathematical model is not used (Opinion Box). Alternatively, GH dose can be individualised by adjusting the dose according to serum IGF-1 level (41).

GH Therapy in Specific Diagnoses

GH Deficiency

GH deficiency may either be isolated or occur together with deficiency of other pituitary hormones; patients with multiple pituitary hormone deficiencies will need replacement of additional hormones at appropriate levels over time, which could have a further impact on their growth rate. The severity of the GH deficiency, determined from the peak GH concentration seen on provocation testing, has a strong influence on the response rate. Children with more severe disease and very low stimulated GH peak have a greater response to GH therapy (44,46,61). Initiation of GH at as young an age as possible (Opinion Box) is also a key factor for a good response in GH-deficient patients and GH treatment should start when the patient is still pre-pubertal (45,57).

Turner Syndrome

Age at GH initiation is also strongly negatively correlated with growth response in girls with Turner syndrome (62,63). The genetic variability of girls with Turner syndrome results in marked differences among patients of different phenotypic characteristics (64). Dysmorphic features, such as webbed neck, cubitus valgus, shortening of the $4^{th}/5^{th}$ metacarpal and lymphoedema, may often be identified early, particularly if the chromosomal abnormalities include defects of the *SHOX* gene (17). While growth failure with

Table 1. Predictive factors of the first year growth response to growth hormone treatment in patients with different causes of growth failure

	GH deficiency	Turner syndrome[a]	Small for gestational age
Maximum stimulated GH peak	1 (–)	-	-
Age at onset	2 (–)	2 (–)	2 (–)
Height SDS-target height SDS[b]	3 (–)	5 (–)	4 (+)
Body weight SDS	4 (+)	3 (+)	3 (+)
GH dose, weight-based	5 (+)	1 (+)	1 (+)
Birth weight SDS	6 (+)	-	-

[a]Other predictors for patients with Turner syndrome are oxandrolone treatment 4 (+) and number of injections/week 6 (+); [b]Target height calculated from mid-parental height. (+) and (–) indicate whether influence is positive or negative. Adapted with permission from Loftus et al (51).

GH: growth hormone, SDS: standard deviation score

Table 2. Factors predictive of near adult height with growth hormone treatment in patients with short stature due to various causes

		GH deficiency	Idiopathic short stature	Turner syndrome	Small for gestational age
Predictors	R^2 (variance in growth explained by combined factors)	0.60	0.64	0.67	0.70
Age	GH start	–	–	–	
	Puberty start			+	
	GH duration	+			+
Birth	Weight (BW) or length (BL)	+ (BW)			+ (BL)
Height	GH start	+	+	+	+
	Mid-parental height	+	+	+	+
GH therapy	Dose or number of injections	+		+	
GH secretory capacity	Peak stimulated GH concentration	–			
First-year response	Change in height SDS or index of responsiveness	+	+	+	+
Silver-Russell syndrome					–

(+) and (–) indicate whether influence is positive or negative. Data for growth hormone deficiency, idiopathic short stature, Turner syndrome and small for gestational age are, respectively, derived from Ranke et al (54), Ranke et al (57), Ranke et al (58), and Ranke et al (59).

GH: growth hormone, SDS: standard deviation score

decreased adult height is reported to occur in at least 95% of Turner syndrome cases, diagnosis and GH initiation is often delayed (64,65). Appropriate screening criteria, particularly using country-specific reference standards, can enable better and more efficient identification of short stature and earlier initiation of GH therapy (65). Dose of GH is also an important influence on growth in Turner syndrome and the recommended dose is generally around 50 µg/kg/day (64), which is higher than for patients with GH deficiency (3,35).

Short Stature Related to SGA

For children born SGA, approximately 90% will show catch-up growth within the first 2-3 years of life (66,67). However, GH therapy may be required for those children who do not show catch-up growth and can also improve body composition and metabolic health (67,68,69). Efficacy of GH treatment is greater if started at an early age (66) and height SDS was shown to be increased to a greater extent when patients at start of treatment were younger than four years compared with those over four years (70). However, treatment before four years of age is not recommended and not approved in Europe (3). The dose approved by the EMA is 33 µg/kg/day, although higher doses are approved by the FDA (3,71). While higher doses have been reported to provide better short-term efficacy, they are associated with supraphysiological IGF-1 levels and are, therefore, not recommended (71). Additionally, the increase in height gain with a higher dose was mainly in the short-term catch-up period and there was little additional benefit of a higher dose over the longer term at adult height (72).

Management of Poor Growth Response

Principles of Management

The recognition of a poor growth response to GH treatment is an important part of management of children with growth failure. However, a poor growth response is reported surprisingly frequently, particularly in children with Turner syndrome or born SGA, but the reported incidence depends to a large extent on the criteria used (42). The principles for management of a poor response are summarised in Figure 2A. To prevent occurrence of a poor response, the diagnosis of the cause of growth failure must be correct and the dose of GH administered should be based on that diagnosis, preferably with a prediction of the anticipated response (41). Growth must be determined accurately and the response at the end of the first year should be assessed; if the response is insufficient the primary diagnosis should be ascertained and the dose checked to ensure appropriateness (6). If the response is very low, then discontinuing GH treatment should be considered, particularly for non-GH deficiency diagnoses. However, there is no consensus on the cut-off of a low or very low response (6). Our preference is to use the change in height SDS in pre-pubertal children and consider a first-year change < 0.3 SDS to be insufficient. A poor response following confirmation of the diagnosis may require an increase in GH dose, although this should be within the approved label.

Adherence to GH Therapy

The responsiveness of an individual patient to GH therapy can be determined from the difference between observed and predicted gain in height or height velocity (47). If responsiveness is reduced, while the diagnosis is deemed correct and presence of concomitant disease ruled out, then poor adherence to the treatment regimen should be considered (Opinion Box) (6,43,47,73). Monitoring of adherence should begin as soon as the treatment is initiated because some patients may not take the medication right from the start or renew their prescriptions, particularly if there is a lack of perceived need (74,75,76,77). While measurements of IGF-1 SDS may give an indication of adherence with GH, it is often not determined routinely and may not provide a definitive answer, because changes in concentration depend on multiple factors (77,78). Success of any therapy is dependent on good adherence and increased adherence may have a greater impact on health than improvements in specific medications. In the case of GH therapy, poor adherence has been shown to be associated with impaired clinical outcomes and reduced growth response (43,79,80,81).

Methods for assessing adherence have generally been poor, frequently relying on reporting by patients or carers, but have indicated that up to 82% of patients may miss at least some doses of GH (43,73,77). A strategy for prevention and management of adherence is outlined in Figure 2B. For effective management of poor adherence to GH, the paediatric endocrinologist or specialist nurse needs to learn techniques of non-judgemental motivational discussion. This requires time and organisation, knowledge of common issues affecting adherence at different treatment periods and ability to structure discussion with open questions, with emphasis on pre-GH treatment education. The same healthcare professional should discuss adherence at each outpatient visit. The strategy also involves addressing the choice of injection device and facility to identify adherence issues. New techniques of electronic monitoring are improving this process and provide important feedback data on evidence of sub-optimal adherence, which may not be available from self-reported data from patients and caregivers, clinical history or auxological measurements.

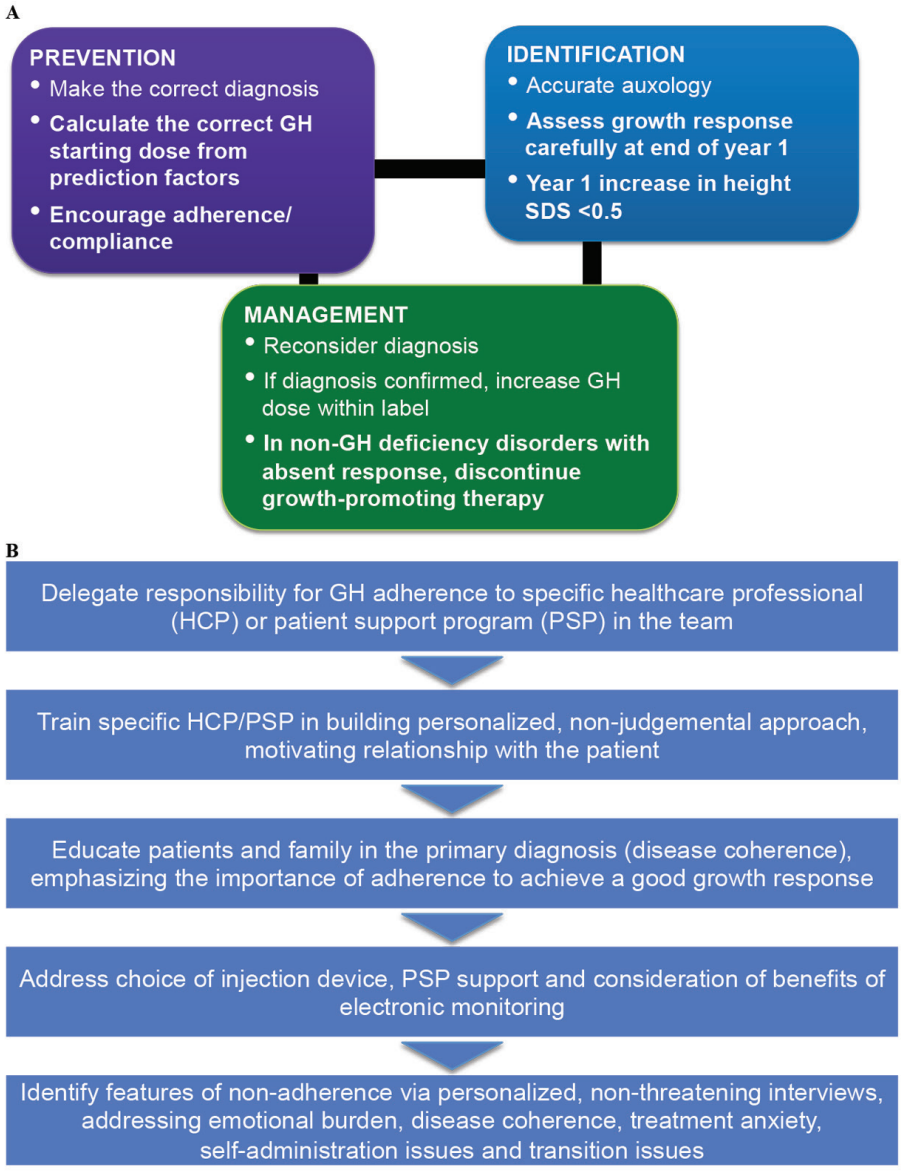

Figure 2. Management principles and strategy for patients with (A) a poor growth response and (B) non-adherence to GH therapy for short stature

Electronic monitoring of GH injections is enabled through use of the easypod™ injection device and Easypod Connect© system, which is the only such solution currently approved and with published information (43,73). The device facilitates administration of a pre-set dose of GH, automatically records injection times and doses, and provides the patient with information such as number of doses remaining (82). The injection information can be downloaded at any time via Easypod Connect by healthcare personnel, which enables distance monitoring, with less need for frequent face-to-face visits. Thus, healthcare personnel are able to address issues of non-adherence with treatment at an early stage before any decrease in GH efficacy. Studies to date have indicated good acceptance of the device and high levels of adherence over several years (83,84,85). Studies with the device have also shown a significant correlation of high adherence with improved outcomes (43,85).

Manipulation of Puberty for Added Growth Advantage

Due to frequent delays in diagnosis of patients with short stature, GH treatment is often initiated close to, or even after, the start of the pubertal growth spurt. Studies have generally indicated that the growth response is greater if GH is started at a younger age, and particularly at the pre-pubertal stage, irrespective of the cause of short stature (45,57). While an increase in GH dose during

puberty has been suggested, there are no clinical studies that have shown a convincing beneficial effect on adult height. Therefore, delaying puberty to allow exogenously administered GH to act for a longer period has been suggested as a strategy to improve overall linear growth (86). Oxandrolone administration has been examined in boys with constitutional delay of growth and idiopathic short stature, but had no significant effect on adult height (86,87). However, addition of oxandrolone to GH therapy has been studied in girls with Turner syndrome and provided approximately 3 cm of extra adult height gain (86,88).

Several small, off-label studies showed that delaying puberty with a gonadotropin-releasing hormone agonist (GnRHa) could increase adult height in children with idiopathic short stature or born SGA, but the effect was modest, not considered clinically significant and outweighed by adverse effects (89,90). When GH initiation is delayed and is close to or during puberty, adding a GnRHa may delay puberty and potentially prolong GH effectiveness. Although such combination treatment is not licensed, GnRHa added to GH has been examined in several studies in children with growth failure due to various different causes (86). The combination of a GnRHa with GH was reported to result in a significant increase in adult height in patients with GH deficiency (91,92) and also in those with idiopathic short stature (86,93) and born SGA (94). However, the GnRHa treatment has negative effects on body composition and, while these effects are reversible after GnRHa cessation, the effects on bone mineral density are of concern and could increase fracture risk (93,95,96).

Conclusions

GH therapy for growth failure due to causes with approval for use to promote growth in short children can induce clinically beneficial short-term and long-term gains in height. The sensitivity and responsiveness to GH treatment are increased in children with GH deficiency compared with children with non-GH deficiency disorders, such as Turner syndrome or SGA. GH therapy should be individualised for each patient, based on the diagnosis and factors predicting growth response, such as age, severity of GH deficiency and deficit from genetic target height. A formal assessment of the response after the first year of GH therapy is recommended, with calculation of the gain in height SDS. The gain should ideally be compared with the individualised prediction; alternatively, cut-off levels of first-year height SDS change of 0.3 or 0.5 have been suggested as lower limits of an acceptable capacity for catch-up growth.

Good adherence to GH therapy is essential to achieve optimal short- and long-term responses, although management of poor adherence generally requires time and organisation. Novel techniques of electronic monitoring are helpful and can provide data that demonstrate when adherence is reduced, which may not be detectable from the patient's history or auxological observations. In some clinical situations, such as GH deficiency, SGA with short stature at onset of puberty and idiopathic short stature, addition of a GnRHa (for a minimum of two years) to GH therapy can increase adult height gain, but adverse effects should be carefully monitored and a positive benefit-risk ratio has not been formally assessed by regulatory authorities; these combinations were used in clinical trials only and are not included in any GH therapy label at present.

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Authorship Contributions

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ORIGINAL ARTICLE

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Novel Mutations in Obesity-related Genes in Turkish Children with Non-syndromic Early Onset Severe Obesity: A Multicentre Study

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What is already known on this topic?

Non-syndromic, early onset, severe obesity is usually a result of mutations in a single gene (monogenic), such as *SIM1, POMC, PCSK1, MC4R, LEP* and *LEPR*, that directly influence the leptin-melanocortin pathway which regulates satiety.

What this study adds?

We identified six different novel variants within five obesity-related genes *(SIM1, POMC, PCSK1, MC4R* and *LEPR)* in seven of 105 childrens with early onset severe obesity in a Turkish population.

Abstract

Objective: Non syndromic monogenic obesity is a rare cause of early onset severe obesity in the childhood period. This form may not be distinguishable from other forms of severe obesity without genetic analysis, particularly if patients do not exibit any physical abnormalities or developmental delay. The aim of this study was to screen 41 different obesity-related genes in children with non-syndromic early onset severe obesity.

Methods: Children with severe (body mass index-standard deviation score > 3) and early onset (< 7 years) obesity were screened by next-generation sequencing based, targeted DNA custom panel for 41 known-obesity-related genes and the results were confirmed by Sanger technique.

Results: Six novel variants were identified in five candidate genes in seven out of 105 children with severe obesity; two in *SIM1* (p.W306C and p.Q36X), one in *POMC* (p.Y160H), one in *PCSK1* (p.W130G fs Ter8), two in *MC4R* (p.D126E) and one in *LEPR* (p.Q4H). Additionally, two previously known variations in *MC4R* were identified in four patients (p.R165W in three, and p.V166I in one).

Conclusion: We identified six novel and four previously described variants in six obesity-related genes in 11 out of 105 childrens with early onset severe obesity. The prevalence of monogenic obesity was 10.4% in our cohort.

Keywords: Early, onset, severe obesity, novel mutations

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Introduction

Common forms of obesity are caused by a combination of environmental and behavioral factors, together with an underlying genetic predisposition to obesity. The etiology of childhood obesity is multifactorial. Non syndromic early-onset severe obesity is usually monogenic, while other forms of obesity are polygenic and occur due the cumulative effect of multiple susceptibility genes which regulate energy intake and expenditure. It has been reported that non-syndromic monogenic obesity is very rare, not exceeding 7% of childhood obesity cases (1,2,3). However, this ratio varies with ethnic characteristics and the proportion of consanguineous couples within any given population. To date mutations in several genes which cause the development of early-onset, severe obesity in children have been described. However, with advances in genetic testing, more genetic causes of obesity continue to be identified. Most of these genes, such as *LEP, LEPR, SIM1, POMC, PCSK1* and *MC4R*, are involved in the central regulation of satiety via the leptin-melanocortin signaling pathway. Therefore variants in any of these genes cause overt changes in food intake, body weight and energy expenditure and are also associated with some forms of neuroendocrine and immune dysfunction (4,5,6).

Syndromic obesity is usually diagnosed clinically with features such as hyperphagia, early-onset severe obesity, developmental delay or other findings caused by defects in the responsible gene. However, in some types of monogenic obesity, it may not be possible to diagnose the underlying genetic defect solely on the basis of clinical findings. For example, mutations in *MC4R* lead to the most prevelant form of monogenic obesity and, because the clinical features resemble those found in exogenous obesity, differential diagnosis can only be confirmed by detection of genetic variants (4,5,6,7). With the exception of leptin deficiency due to leptin gene mutations, treatment options are limited in early-onset severe obesity. Newly available, targetted drugs will offer a novel therapeutic option for those patients with monogenic obesity due to *MC4R* or *POMC* dysfunction (8,9). Consequently, genetic testing should be advocated in children with early onset severe obesity as they may be suitable candidates for current or promising new drugs such as MC4R agonists. The present study, has been conducted to assess the variants of 41 different obesity-related genes in Turkish children with non-syndromic early onset severe obesity.

Methods

The study population was selected from among severe obese patients referred to our center at İnönü University, Malatya, Turkey, for genetic analysis from different centers in geographically diverse parts of Turkey. Inclusion criteria for children and adolescents were obesity onset at less than seven years of age and a body mass index-standard deviation score (BMI-SDS) >3. Patients taking any drugs or followed up with any specific endocrine disorders, such as Cushing syndrome or hypothyroidism, and those with syndromic features were not included in the study. The study protocol was approved by the regional Ethical Committees (Malatya Clinical Research Ethics Committee, 21.01.2018, no: 2018-20), and informed consent was obtained from the parents of all children before their participation.

Anthropometric Measurements

All patients were examined in the morning after an overnight fast. Height and weight were measured by experienced nurses from the pediatric endocrinology outpatient clinic. BMI was calculated as body weight in kilograms divided by the square of the height in meters. BMI and BMI-SDS were calculated using age and gender specific percentiles of Turkish children from established reference data (10).

DNA Preparation

Genomic DNA was isolated from peripheral blood mononuclear cells using the QiAamp DNA Blood Mini Kit (cat. no. 51106, Qiagen, Hilden, Germany). DNA purity and quality was confirmed by agarose gel electrophoresis. DNA concentration was measured by Qubit (Life Technologies, Singapore). Before the library preparation, appropriate dilution was made for each sample.

Next Generation Sequencing

Sequencing libraries were prepared according to the manufacturer's instructions using CDHS-1346Z-901 QIASeq™ Targeted DNA Custom Panel (ref. no. 333525, Qiagen, Hilden, Germany) that includes all exomes with 10bp exon-intron junctions of 41 target genes (*DYRK1B, LEP, LEPR, MC4R, NR0B2, POMC, UCP3, ADRB2, ADRB3, AGRP, MC3R, NTRK2, PCSK1, SIM1, CARTPT, ENPP1, PPARG, PPARGC1B, PYY, SDC3, UCP1, ADIPOQ, NAMPT, CFD, RETN, PPARGC1A, CCK, NPY, SLC2A4, ADD1, SREBF1, PTPN1, IRS-1, GHRL, BDNF, NEGR1, SH2B1, GIPR, TMEM18, FTO, SLC22A1*). Briefly, the samples were enzymatically fragmented and molecularly barcoded and passed through the stages of library generation, target enrichment, sample indexing and amplification. The concentration of each library was determined by using Qiaseq Library Quant Assay Kit (ref. no. 333314, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each library was diluted to 4 nM, and pooled in equimolar ratio. The final pool was denatured with freshly prepared 0.2 N NaOH and then

diluted to 20 pM and sequenced as 251x2 bp paired-end chemistry according to the sequencer manufacturer's instructions (MiSeq, Illumina, San Diego, CA) (11).

Sequencing Data Analysis

Demultiplexed FASTQ files were processed individually using Qiagen Bioinformatics solutions. Secondary analysis was performed by using Qiagen, QCI Analyze Universal 1.5.0. Tertiary analysis and interpretation were performed using Qiagen Clinical Insight Interpret (all programs from Quiagen, Hilden, Germany).

Sanger Sequencing

Detected variants were also analysed and confirmed by Sanger sequencing according to the manufacturer's protocols. The amplicons were analyzed by direct sequencing with ABI 3500 (Life Technologies, Waltham, Massachusetts, USA). Analysis of sequence results was performed by Mutation Surveyor Programme (SoftGenetics, USA).

Data Analysis

Mutations and/or polymorphisms were screened for using next-generation sequencing. All the genes that were investigated have various roles in energy homeostasis, such as energy intake, energy expenditure, adipose tissue functions and glucose metabolism. Genetic variant pathogenicity was examined using the following standard *in silico* analyses; MutationTaster, PolyPhen-2, CADD, Stratum and I-Mutation-2.0: prediction. Novel mutations detected were verified by Sanger sequencing (12,13,14).

Results

A total of 105 patients meeting the inclusion criteria were included in the study. Table 1 shows the key clinical and genetic characteristics of the children carrying the obesity-related gene variations. We described six novel mutations in five candidate genes in seven out of the 105 patients, and previously described mutations in *MC4R* detected in four patients. The novel variations detected were two in *SIM1*, one in *POMC*, one in *PCSK*, one in *LEPR* and two in *MC4R* (Table 1). Family members of these affected children were also genetically screened for the same pathogenic variants. Family pedigrees of the children carrying the novel variations are shown in Figure 1.

Genetic Results

Table 1 shows the key clinical characteristics of the patients carrying novel mutations. We identified six novel mutations potentially contributing to the severe obesity in these subjects. In Patient 1, a novel homozygous *SIM1* variant (p.W306C, c.918 G>T in exon 8) was detected. He was two years and three months of age at the time of the study, his BMI-SDS was 5.6, and obesity onset age was one and a half years. His birth weight was 2900 g. He had no other endocrinological or developmental abnormalities. His parents were second degree relatives. His father and mother were also obese and both were heterozygous for the same variation.

The second patient (Patient 2) carrying a novel heterozygous *SIM1* variant (p.Q36X, c.106 G>T in exon 1), was five years old, her birth weight was 2300 g, her BMI-SDS was 4.7 and she had severe obesity from two years of age. Her growth velocity and developmental history were normal and she had no additional endocrinological or developmental abnormalities. There was no consanguinity in her family. Her father was obese and heterozygous for the same variants.

A novel heterozygous *POMC* variant (p.Y160H, c.478 T>C in exon 3) was detected in a male patient who was 14 years old (Patient 3). He had no other abnormalities except severe obesity and hyperphagia. There was no consanguinity in his family, but his obese mother was heterozygous for the same variation.

In Patient 4, a novel heterozygous *PCSK1* variant (p.W130G fs Ter8, c.388delT) was detected. He was two years and four months old and had no endocrinological abnormalities. His parents were not obese and had no genetic variation detected on our panel.

Two siblings in the same family (Patients 5 and 6) were homozygous for a novel *MC4R* variant (p.D126E, c.378C>A in exon 1). They had severe obesity, intractable hyperphagia and accelerated growth which is typical for *MC4R* deficiency. Their parents were severely obese and close relatives, and they were both heterozygous for the same variantion.

The last female patient (Patient 7) was 14 years old, severely obese and heterozygous for a novel *LEPR* variant (c.12A>C, p.Q4H in exon 3) (Figure 2). Her obese father was heterozygous for the same variant.

In addition to these novel variants, previously described mutations in *MC4R* were found in four patients (p.R165W, c.493C>T in exon 1 in three of four, and p.V166I, c.496G>A in exon 1 in one) (15,16).

Discussion

In this study, variants in 41 genes which are known to be involved in causing obesity in patients with non-syndromic early onset severe obesity were investigated. Two novel

Table 1. Clinical and genetic characteristics of the patients with obesity-related gene variations

Patient no	Birth weight (gr)	Obesity onset (years)	Current age (years), gender, ethnicity	BMI-SDS	Height SDS	Mutant gene	Zygosity/ variation/ protein change	Functional prediction	Clinical findings	Consanguinity	Parents' BMI (father/ mother)	Parents' zygosity (father/ mother)
1	2900	1.5	2.5, M, Kurdish	5.6	1.8	*SIM1* Exon 8	Homozygous c.918G > T p.W306C	Disease causing	Hyperphagia	Yes	36/29	Heterozygous/ heterozygous
2	2300	2	5, F, Turkish	4.7	0.3	*SIM1* Exon 1	Heterozygous c.106G > T PQ36X	Disease causing	Hyperphagia	No	35/28	Heterozygous/ wild
3	4000	2	14, M, Turkish	3.2	0.4	*POMC* Exon 3	Heterozygous c.478T > C p.Y160H	Disease causing	Hyperphagia, hyperlipidemia	No	27/47	Wild/ heterozygous
4	2600	1	2.4, M, Turkish	3.6	-0.8	*PCSK1* Exon 3	Heterozygous c.388delT p.W130G fsTer8	Disease causing	Hyperphagia, IR	No	21/32	Wild/wild
5	3250	1	6, M, Turkish	6.5	2.4	*MC4R* Exon 1	Homozygous c.378C > A p.D126E	Disease causing	Hyperphagia, IR, hyperlipidemia, hepatosteatosis	Yes	38/37	Heterozygous/ heterozygous
6	2250	1	9, M *Brother of patient 5*	3.9	3.2	*MC4R* Exon 1	Homozygous c.378C > A p.D126E	Disease causing	Hyperphagia, IR, hyperlipidemia, hepatosteatosis	-	-	-
7	3200	2	16, F, Turkish	3.4	1.8	*LEPR* Exon 3	Heterozygous c.12A > C p.Q4H	Disease causing	Hyperphagia, IR, hyperlipidemia	No	33/26	Heterozygous/ wild
8	3100	2.5	11, F, Turkish	3.1	2	*MC4R* Exon 1	Heterozygous c.493C > T p.R165W	Previously described (R)	Hyperphagia, hyperlipidemia, hepatosteatosis	No	35/26	Heterozygous/ wild
9	3200	1	4, M, Turkish	3.2	2.2	*MC4R* Exon 1	Heterozygous c.496G > A p.V166I	Previously described (R)	Hyperphagia, hyperlipidemia, hepatosteatosis	No	28/33	Wild/ heterozygous
10	3000	3,5	7.5, F, Syrian	3.5	1.9	*MC4R* Exon 1	Heterozygous c.493C > T p.R165W	Previously described (R)	Hyperphagia, IR, hyperlipidemia, hepatosteatosis	Yes	32/25	Heterozygous/ wild
11	4100	2	9, M, Turkish	3.6	2.1	*MC4R* Exon 1	Heterozygous c.493C > T p.R165W	Previously described (R)	Hyperphagia, IR, hyperlipidemia	Yes	43/26	Heterozygous/ wild

IR: insulin resistance [defined as HOMA-IR > 3.5; HOMA-IR = fasting glucose (mmol/L) x fasting insulin mIU/mL/22.5].

BMI-SDS: body mass index-standard deviation score, M: male, F: female

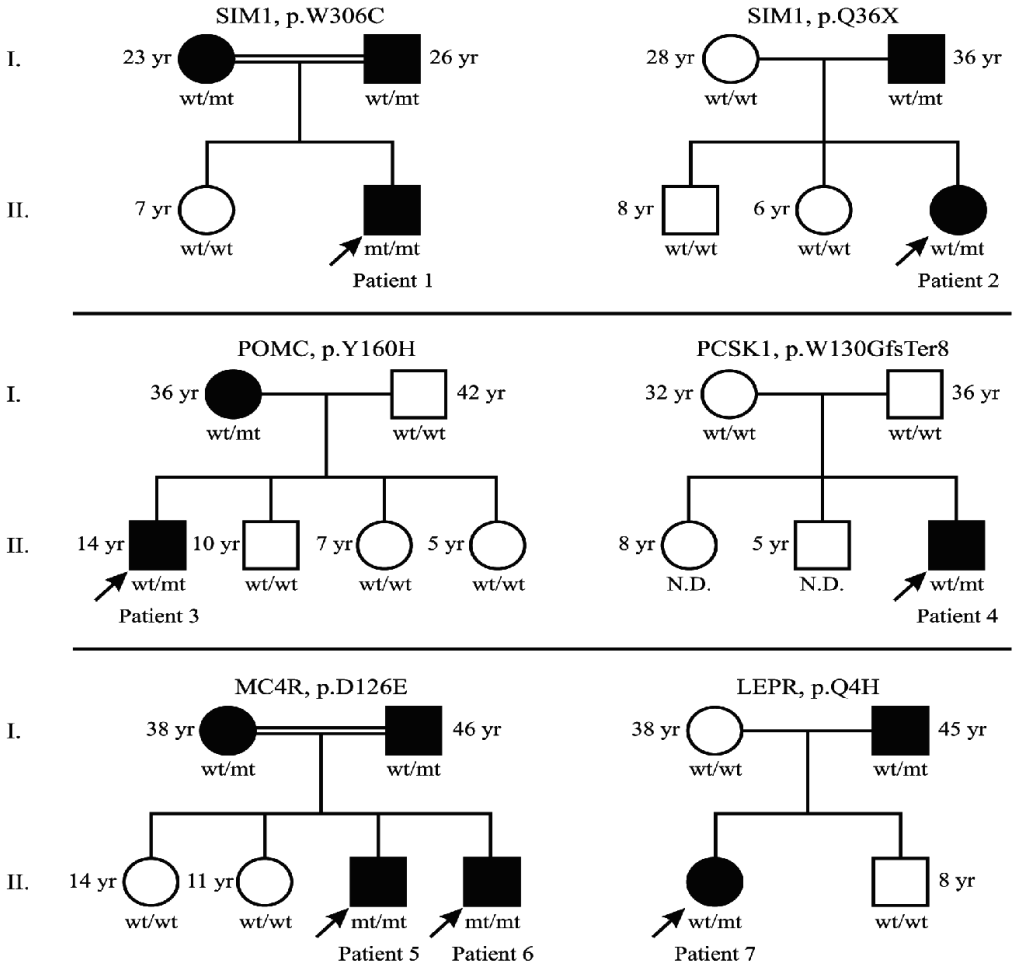

Figure 1. Pedigrees of the families bearing novel variants in obesity related genes. Arrows indicate probands in each family. Genotypes were defined as wild type (wt) or mutant (mt) for corresponding variations

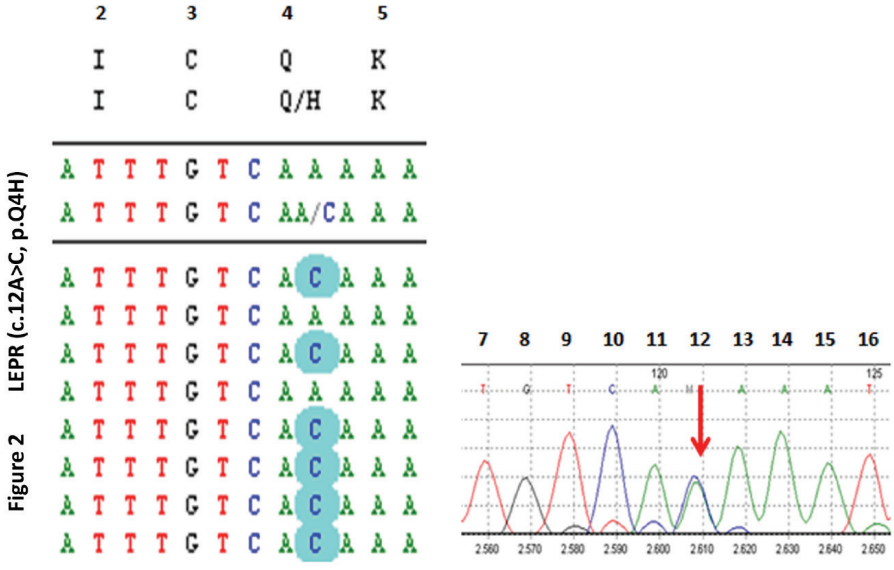

Figure 2. DNA sequencing by the next-generation sequencing (NGS) method revealed a novel heterozygous c.12A > C, p.Q4H mutation in *LEPR*. Related mutations are highlighted in NGS sequences and designated by red arrows in Sanger sequences

SIM1 variants in two unrelated patients, a novel *POMC* variant, a novel *PCSK 1* variant, two siblings with the same *MC4R* variant and a novel *LEPR* variant were identified in our cohort.

Single-minded-1 gene *(SIM1*) is located on chromosome 6q16.3-q21 and consists of 11 exons spanning 75kb. *SIM1* encodes a hypothalamic transcription factor in the basic helix loop helix/Per Arnt Sim (bHLH-PAS) family. Its main function has been described as the formation of the paraventricular nucleus of the hypothalamus which is critical for food intake regulation. *SIM1* also plays an important role in the regulation of energy homeostasis by interacting with the melanocortin signalling pathway and loss-of-function variants in this gene are one of the few known causes of monogenic obesity in both humans and mice (17,18). Recently, it has been reported that chromosomal abnormalities such as translocation between chromosome 1p22.1 and 6q16.2, deletion of the 6q16.2 region and heterozygous point mutations in the *SIM1* region are responsible for early-onset severe obesity in humans (19,20,21). In these reports, patients had increased fat mass with increased body fat percentage in addition to hyperphagia, increased linear growth, learning disabilities and Prader-Willi-like phenotype. Experimentally, it has been observed that homozygous *Sim1* knockout mice *(Sim1 -/-*) do not survive due to lack of the hypothalamic neurons which produce multiple neuropeptides including oxytocin, vasopressin, corticotropin-releasing hormone, thyrotropin-releasing hormone and somatostatin. However, heterozygous mice (*Sim1 +/-)* develop partial failure of hypothalamic neurons resulting in hyperphagia and obesity similar to *mc4r-mutant mice* (22). In our study group, we described one patient with a homozygous missense *SIM1* variant (p.W306C, c.918 G>T in exon 8) and another patient with heterozygous nonsense *SIM1* variant (p.Q36X, c.106 G>T in exon 1). The homozygous patient had severe obesity due to hyperphagia from eighteen months of age and his obese parents were also heterozygous for the same *SIM1* variant. This p.W306C variant is located in the PAS domain, which has a critical role in *SIM1* activity (23). Stratum and I-Mutant 2.0 prediction analysis suggest that the Gibbs free energy (delta delta G, DDG) value of this mutant protein would be -1.7 and CADD score was 35, indicating a decrease in the stability of the mutant protein structure**.** Therefore, this variant is likely to be pathogenic because of changes in the protein structure and redox status leading to reduced SIM1 activity. Previously, pathogenic variants have been described in this region (23,24,25), and it appears that this new variant located in the same region is also pathogenic. In addition, and contrary to what might be expected, identification of accelerated growth at his physical examination and the resemblance of his phenotype to the *MC4R* variants led us to hypothesize that this *SIM1* variant might induce considerable functional loss in MC4R activation. Needless to say, functional studies would be required to confirm this.

The mother of Patient 2, in whom a novel heterozygous nonsense *SIM1* variants (p.Q36X, c.106 G>T in exon 1, CADD score: 37) was identified, did not carry the same variant, whereas his obese father was haploinsufficient for p.Q36X. This new variant located in the bHLH domain of *SIM1* is predicted to play a significant role in DNA dimerization and binding, so it is likely to be pathogenic according to Polyphen-2 and CADD analysis. In addition to the critical location of this variant, its pathogenity is enhanced because it also produces a premature stop codon resulting in a truncated protein. Previously, a loss-of-function, heterozygous *SIM1* variant (T46R) was described in the same region (24,25). Although most of the heterozygous *SIM1* variants that cause obesity have been described as causing growth retardation and a Prader-Willi-like syndrome in addition to the accompanying obesity (24,25), developmental and intellectual capacity was normal in our patient.

Proopiomelanocortin (POMC) is produced by the POMC/CART (cocaine and amphetamine-related transcript) neurons in the hypothalamus, and is the precursor of adrenocorticotropic hormone (ACTH), beta-endorphin, beta-lipotropin (beta-LPH), corticotropin-like intermediate peptide (CLIP) and α-, β- and γ-melanocyte-stimulating hormones (MSH), some of which regulate melanin synthesis, adrenal functions and inhibit food intake through interaction with the MC4R signalling pathway (26,27,28). Homozygous loss-of-function mutations in *POMC* have been reported to be very rare and a cause of severe obesity, ACTH deficiency and hypopigmentation in mice and humans (29,30). It is suggested that the MC4R signalling pathway is affected secondary to the impairment of interaction with MC4R and α-MSH in heterozygous missense *POMC* variants without complete POMC deficiency, and subsequently severe obesity develops in humans (29,30,31). In this study, a novel heterozygous *POMC* variant (p.Y160H) was described in a patient with early-onset, severe obesity whose obese mother was also affected by the same variant. This variant was located in the CLIP region of the ACTH domain of *POMC*. The DDG value of this mutant protein was -1.62 kj/mol, predicted by Stratum and I-mutant 2.0 analysis, and CADD score was 25.8 leading to a decrease in the stability of the mutant protein. PolyPhen-2 analysis predicted that this novel variant is likely to be pathogenic. Although the

function of CLIP is not fully understood in humans, it is considered that variants affecting this region may confer the phenotype through an altered MC4R signalling pathway.

The proprotein convertase subtilisin/kexin type 1 gene *(PCSK1*) encodes the prohormone convertase enzyme (PC1/3) and is abundantly expressed in the hypothalamus (32). PC1/3 deficiency is described as an autosomal recessive disorder. Although heterozygous PC1/3 deficiency is associated with obesity, homozygote loss-of-function mutations usually present with early onset severe obesity and hyperphagia in addition to malabsorptive diarrhea in the neonatal period, central diabetes insipidus, reactive hypoglycemia and hypoadrenalism (33,34,35).

However, the described phenotype may be variable depending on which parts of the *PCSK1* gene structure have been affected. In Patient 4, a novel heterozygous frameshift *PCSK1* variant (p.W130G fsTer8, C388delT) was found. However, the same variant was not present in his parents. This novel variant is located in a catalytic domain of *PCSK1* and leads to a frameshift mutation and deletion followed by stop-codon that is predicted to produce a non-functional truncated protein. Its CADD score was 36. It has been described that pathogenic variants within the same domain reduce the PCSK1 activity (34,35). Therefore, it seems highly likely that this novel variant would be pathogenic.

MC4R is the receptor for α-MSH and plays a key role in controlling energy homeostasis, food intake and satiety. *MC4R* mutations are the most common genetic cause of monogenic obesity and also contribute in polygenic forms. Loss-of-function *MC4R* mutations are associated with early-onset severe obesity due to hyperphagia, hyperinsulinemia and increased linear growth. Currently more than 150 variants have been identified, and the prevalence of pathogenic *MC4R* variants reported in various obese populations is highly variable, ranging from 0.5% to 6% (1,36,37,38). We found a novel homozygous *MC4R* variant, D126E, in exon 1 in two siblings. This mutation is located on the helical transmembrane domain/putative ligand binding site (NCBI-search tool). Its DDG value was -1.33 kj/mol predicted by Stratus and I-Mutation 2 prediction, and CADD score was 24.5, suggesting a possible decrease in the function of the mutant protein. This variant may lead to a decrease in the binding capacity of MC4R to α-MSH, as previously described in the pathogenic variants, I137T, R165W and G98R located in the same region of *MC4R* (39,40,41,42). Thus this novel variant, D126E, is likely to be pathogenic. Our affected siblings were extremely obese, and they had increased height velocity for age. Their parents were heterozygous for the same variant and they were also severely obese. Additionally, we found two different previously described mutations in *MC4R* in four patients (p.R165W, c.493C>T in exon 1 in three, and p.V166I, c.496G>A in exon 1 in one) (Table 1).

Leptin and *LEPR* mutations are associated with early onset severe obesity, severe hyperphagia and some neuroendocrine abnormalities, such as hypogonadotropic hypogonadism, impaired growth hormone secretion and hypothalamic hypothyroidism (43,44). Patient 7 had a novel heterozygous *LEPR* mutation (p.Q4H, c.12A>C in exon 3, Figure 2). She was severely obese and had no endocrinopathy. The heterozygous *LEPR* variant detected in this patient is located in the signal peptide and may destroy protein synthesis and/or processing (sorting and location). Its DDG was -1.13 kj/mol, signifying a decrease in protein stability and CADD score was 10. Deletions causing dysfunction in the signal peptide located in the extracellular domain of *LEPR* have been reported (44). Although it is hard to speculate about this variant without performing an analysis to confirm abnormal protein processing, the patient's phenotype and heterozygosity of the obese father for the same variant led us to suppose that this novel variant is most likely pathogenic. However, definitive functional analysis should be performed to confirm pathogenicity.

In the literature, there are a few similar studies detecting obesity-related genes with a targeted DNA custom panel. In a recent report by Foucan et al (45), 59 obesity-related genes were screened by next-generation sequencing in 25 obese children in Guadeloupe Island and five mutations in *MC4R, SIM1, SH2B1* and *NTRK2* genes were described. The prevalence of monogenic obesity in this cohort was 10% which is similar to the present study.

Study Limitations

There were some limitations associated with our study. We could not conduct a functional analysis of the mutant genes. However, we believe that the relationship between the genotype and phenotype of the patients and the assessment of possible functional losses that would result from the novel mutations provide compelling evidence of the effect of these novel mutations.

Conclusion

We identified six different novel variants within five obesity-related genes (*SIM1, POMC, PCSK1, MC4R* and *LEPR*) in seven out of 105 children with early-onset severe obesity in a Turkish population. Additionally, we found previously known mutations in the *MC4R* gene in four patients, thus monogenic obesity prevalence was 10.4% in our cohort. In order to understand whether these novel variants are

specific to the Turkish community in which consanguineous marriages are common, further broad-based genetic screening studies are needed.

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Ethics

Ethics Committee Approval: The study protocol was approved by the regional Ethical Committees (Malatya Clinical Research Ethics Committee, 21.01.2018, no: 2018-20).

Informed Consent: Informed consent was obtained from the parents of all children before their participation.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Ayşehan Akıncı, Design: Ayşehan Akıncı, Doğa Türkkahraman, Data Collection or Processing: Ayşehan Akıncı, Doğa Türkkahraman, İbrahim Tekedereli, Leyla Özer, Bahri Evren, İbrahim Şahin, Tarkan Kalkan, Yusuf Çürek, Emine Çamtosun, Esra Döğer, Aysun Bideci, Ayla Güven, Erdal Eren, Özlem Sangün, Atilla Çayır, Pelin Bilir, Ayça Törel Ergür, Oya Ercan, Analysis or Interpretation: Ayşehan Akıncı, İbrahim Tekedereli, Doğa Türkkahraman, Leyla Özer, Tarkan Kalkan, Literature Search: Ayşehan Akıncı, Writing: Ayşehan Akıncı.

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Glucose Metabolism Evaluated by Glycated Hemoglobin and Insulin Sensitivity Indices in Children Treated with Recombinant Human Growth Hormone

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What is already known on this topic?

Growth hormone (GH) has an anti-insulin effect. Children treated with recombinant human GH (rhGH) may develop abnormalities in glucose metabolism and present a higher incidence of type-2 diabetes mellitus. This applies particularly to subjects with predisposing conditions such as obesity or positive family history.

What this study adds?

In this study, conventional use of rhGH, in a large GH deficient pediatric population, resulted in increased hemoglobin A1c and worsened insulin sensitivity after one year of therapy. However, at the subsequent follow-up, these indices had not deteriorated further and were not associated with significant changes in glucose metabolism. This therapy proved to be safe, even in subjects considered at risk for glucose metabolism alteration.

Abstract

Objective: To evaluate glucose metabolism and insulin sensitivity in children with idiopathic growth hormone (GH) deficiency, treated with recombinant human GH (rhGH), and to identify possible risk factors for the development of glucose abnormalities in this population.
Methods: We retrospectively collected data from 101 patients (60 males, median age 10.4 years, 77 prepubertal), with confirmed GH deficiency, enrolled before starting rhGH and followed up during the first three years of treatment. Glucose metabolism was evaluated annually by oral glucose tolerance test (OGTT) and glycated hemoglobin A1c (HbA1c). OGTT was used to calculate insulin sensitivity (HOMA-S) and insulin resistance (HOMA-IR), defined as HOMA-IR >3.
Results: RhGH was effective in improving growth and dosages significantly reduced after the first year of therapy. No patient developed diabetes mellitus. After one year of therapy, a significant increase in HbA1c ($p=0.0042$) and insulin levels (fasting $p<0.0001$, 60 min $p=0.0018$, 120 min $p=0.0003$) was observed, with a higher prevalence of IR ($p<0.05$). These indices did not alter further during the follow-up and were not related to GH dose or to family history of diabetes. A significant correlation was found only for IR indices and pubertal status, weight and age ($p<0.05$).
Conclusion: In this retrospective study on a large GH deficient pediatric population, conventional use of replacement therapy resulted in an increase in HbA1c and IR after one year of therapy, regardless of rhGH dosage. These alterations did not worsen significantly in the following two years and were not associated with overt diabetes.
Keywords: Glucose metabolism, growth hormone treatment, children, insulin sensitivity, glycated hemoglobin

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Introduction

Growth hormone (GH) exerts a variety of different metabolic actions, including playing a role in glucose homeostasis (1). It contributes to maintaining normoglycemia and is considered an insulin antagonist, especially during fasting, via stimulation of hepatic gluconeogenesis and suppression of insulin-mediated glucose uptake in peripheral tissues (2).

The benefits of human recombinant GH (rhGH) replacement therapy in improving height in children with GH deficiency (GHD) are well recognized (3). RhGH therapy can also improve body composition, lipid profile and bone mineral density (4). As regards carbohydrate metabolism, observational studies have reported an increased incidence of type 2 diabetes in GH-treated children (5,6). Although the incidence of type 2 diabetes is low (one case for every 3000 person-years of treatment), monitoring glucose levels before and, periodically, during treatment, has been recommended, especially in subjects with pre-existing risk factors such as obesity, positive family history of type 2 diabetes and pretreatment insulin resistance (IR) (5,6).

In terms of rhGH effects on insulin sensitivity, GH treatment leads to a compensatory increase in insulin secretion before the appearance of overt glucose abnormalities (1). Thus, decreased insulin sensitivity may be detected even without changes in glucose tolerance (7).

In recent years, a variety of different parameters and indices have been used to study the influence of GH treatment on glucose and insulin homeostasis (8). Biomarkers such as glycated hemoglobin A1c (HbA1c) and indices of glucose tolerance are now widely employed in the diagnosis and monitoring of patients with glucose abnormalities, but only one report explored their potential application in the field of rhGH therapy over a one year follow-up period (9).

The aim of our study was to evaluate the influence of GH replacement therapy on glucose metabolism and insulin sensitivity in a cohort of idiopathic GHD children over a three year follow-up period. The secondary aim was to identify risk factors that could predict the development of impaired glucose metabolism in this population.

Methods

Study Design

We retrospectively collected information on all the children consecutively diagnosed with isolated GHD at the Institute for Maternal and Child Health-IRCCS "Burlo Garofolo" (Trieste, Italy) between March 1st, 2007 and December 31st, 2013. The diagnosis of GHD was established based on the clinical, auxological and biochemical criteria set by AIFA (Agenzia Italiana del Farmaco, Italian Medicines Agency) at the time of first evaluation (10). Auxological and laboratory evaluations were collected before starting rhGH (baseline), and after one, two and three years of treatment. All patients were regularly followed-up every six months.

Height and body mass index (BMI) were expressed as standard deviation scores (SDS) based on the Italian reference growth charts (11) using Growth Calculator 3 Software (Weboriented.it. Growth Calculator 3). Pubertal status was assessed with Tanner staging.

Parents provided informed consent to obtain and store blood samples for research purposes, in accordance with the Declaration of Helsinki of 1975. The study was approved by the Institutional Review Committee of IRCCS "Burlo Garofolo" (approval number: RC 32/18 Line 2).

Growth Hormone Treatment

Biosynthetic rhGH (Genotropin®, Humatrope®, Norditropin®, NutropinAq®, Omnitrope®, Saizen®, or Zomacton®) was administered once daily at bedtime, for a total of six or seven injections per week. Initial subcutaneous dose was 25-35 mcg/kg/day, which was gradually modified during the follow-up based on growth velocity and insulin-like growth factor-1 (IGF-1) concentration.

Monitoring of Glucose Metabolism

Before starting GH treatment and every year at follow-up, monitoring of glucose metabolism was carried out on each patient, after an overnight fast: fasting glucose, fasting insulin and HbA1c were determined and an oral glucose tolerance test (OGTT) was performed (glucose load of 1.75 g/kg of body weight up to a maximum of 75 g). Blood samples for glycaemia and insulinemia were collected after 60 and 120 minutes (Glu60, Glu120 and Ins60, Ins120, respectively).

Altered glucose metabolism was defined according to the American Diabetes Association criteria for prediabetes (12) and included impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or impaired HbA1c (39-47 mmol/mol, using IFCC method). Diabetes was diagnosed if fasting glucose was ≥126 mg/dL, or Glu120 was ≥200 mg/dL, or HbA1c was ≥48 mmol/mol. In the absence of unequivocal hyperglycemia, results were confirmed by repeat testing (12).

Hyperinsulinemia was diagnosed if fasting insulin was ≥15 μU/mL in prepubertal and ≥20 μU/mL in pubertal children or with Ins60 ≥150 μU/mL or Ins120 ≥75 μU/mL (13). We assessed basal insulin secretion by Homeostasis Model Assessment for β-cell function index (HOMA-β) and

insulin sensitivity (HOMA-S) using the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/. HOMA Calculator). IR was evaluated by Homeostasis Model Assessment Insulin Resistance index (HOMA-IR), applying the Matthews formula [fasting insulin (μU/mL) x fasting glucose (mg/dL)/405] (14). A diagnosis of IR was made if the HOMA-IR value was >3, in accordance with literature (15,16).

Hormone and Biochemical Assays

All biochemical data were measured in our laboratory using standard methods. Glycemia was measured via a hexokinase enzymatic reaction by Cobas 501/502 (Roche Diagnostics, Indianapolis, IN, USA). Insulinemia was measured using an electrochemiluminescence immunoassay by Elecsys immunoanalizer and Cobas e (Roche Diagnostics, Indianapolis, IN, USA). HbA1c was assessed using turbidimetric inhibition immunoassay by Cobas Integra 400 Tina-quant Hemoglobin A1c Gen.2 (Roche Diagnostics, Indianapolis, IN, USA). Serum GH was assessed with a two-site chemiluminescent immunometric assay on the IMMULITE 2000 analyzer (Siemens Healthcare Diagnostics, United Kingdom, UK) with a sensitivity of 0.01 ng/mL. Serum total IGF-I was assayed using a solid-phase, enzyme-labeled chemiluminescent immunometric assay by IMMULITE 2000 (Siemens Healthcare Diagnostics, United Kingdom, UK) with a sensitivity of 20 ng/mL.

Statistical Analysis

All statistical analyses were conducted with Stata/IC 14.2 (StataCorp LLC, College Station, USA). Data were described as frequencies and percentages or as medians and interquartile ranges, as appropriate. The Wilcoxon sign-rank test for paired samples was employed to compare repeated measures taken at two different points in time. Spearman correlations were used to compare the ranks of two continuous variables. The Mann-Whitney rank-sum test was carried out to compare unrepeated measures between two groups. The McNemar test was used to compare proportions for paired nominal data. Bivariate and multivariate logistic regressions were carried out to study associations between dichotomous outcomes and one or more independent variables. A p value <0.05 was considered statistically significant.

Results

Patient Characteristics

We studied 101 GHD-children (60 males). All children failed two GH stimulation tests, with GH peaks being below 10 ng/mL [first peak median (range) 6.20 (4.51-7.74); second peak median 6.41 (3.89-7.90)]. At baseline, 77 children (76.3%) were prepubertal (Tanner stage 1). Median (range) age at start of GH treatment was 10.4 (7.7-12.5) years.

The clinical and biochemical features of the population at baseline and after one, two and three years of therapy are shown in Table 1.

Growth

At baseline, GHD children displayed short stature and low IGF-1 concentrations, as expected. A significant and

Table 1. Clinical and biochemical features at baseline and at completion of first, second and third year of growth hormone treatment

	Baseline n = 101	1st year n = 101	2nd year n = 98	3rd year n = 73
rhGH dose (μg/kg/day)	28.6 (24.7-30.6)	25.6 (22.0-30.0)**	25.8 (20.6-30.0)†	25.40 (21.50-32.24)†
Height (SDS)	-2.26 (-2.65 to -1.96)	-1.72 (-2.13 to -1.39)**	-1.42 (-2.01 to -0.94)**††	-1.16 (-1.73 to -0.81)**††
Weight (SDS)	-1.77 (-2.41 to -1.10)	-1.47 (-2.15 to -0.78)**	-1.23 (-1.83 to -0.54)**††	-1.04 (-1.64 to -0.24)**††
BMI (SDS)	-0.50 (-1.45 to 0.17)	-0.57 (-1.68 to 0.25)	-0.70 (-1.40 to -0.20)	-0.42 (-1.11 to 0.29)*
IGF-1 (ng/mL)	117 (74-167)	329 (174-469)**	367 (214-573)**††	421 (227-556)††
Fasting glucose (mg/dL)	82 (76-85)	82 (76-88)	81 (76-88)	82 (76-88)
Insulin secretion indices				
Fasting insulin (μU/mL)	4.6 (2.4-7.0)	7.9 (4.9-13.6)**	9.6 (6.4-12.9)††	9.0 (6.2-12.5)††
HOMA-B	100.0 (79.4-124.5)	128.0 (93.7-160.5)**	142.3 (108.3-168.5)††	135.1 (108.3-167.5)††
Insulin sensitivity indexes				
HOMA-IR	0.76 (0.53-1.02)	1.16 (0.72-1.72)**	1.23 (0.88-1.68)††	1.17 (0.82-1.61)††
HOMA-S	131.5 (97.7-189.1)	86.1 (58.2-136.3)**	81.5 (59.5-113.8)††	85.7 (63.9-122.6)††

Data reported are medians and interquartile ranges. p-values are between baseline and 1st year, between 1st and 2nd year and between 2nd and 3rd year (Wilcoxon sign-rank test for paired samples, *$p<0.05$, **$p<0.001$ versus previous year; †$p<0.05$, ††$p<0.001$ versus baseline).

rhGH: recombinant human growth hormone, SDS: standard deviation scores, BMI: body mass index, IGF-1: insulin-like growth factor-1

consistent increase in height SDS and IGF-1, together with an increase in body weight SDS, was noticed over the study period ($p < 0.0001$, see Table 1). BMI SDS did not change significantly until the second year of treatment, and subsequently increased during the third year ($p = 0.0133$).

The dose of rhGH significantly decreased after the first year of treatment ($p < 0.0001$) and remained stable in subsequent years. No correlations were found between the dose of rhGH during treatment and any of the other variables examined: age; height; puberty; and BMI.

Evaluation of Glucose Metabolism

No patient developed diabetes mellitus during the study period.

HbA1c significantly increased after one year of treatment, from 25.5 ± 11.9 mmol/mol to 30.9 ± 9.9 mmol/mol ($p = 0.0042$) and thereafter remained stable over the following years (second year 32.6 ± 10.6 mmol/mol, third year 34.7 ± 6.5 mmol/mol, Figure 1). Compared to baseline values, glycated hemoglobin was significantly increased during all three years of follow-up. The increase occurred mostly in the first year, continuing in the subsequent years but not so rapidly and without statistical significance.

OGTT did not detect significant increases in glucose concentrations over the years, while a significant increase in insulin levels was found after the first year of treatment versus baseline, in fasting insulin ($p < 0.0001$), and in the 60 ($p = 0.0018$) and 120 minutes ($p = 0.0003$) samples. Insulin concentrations were significantly correlated with age, BMI, IGF-1 and pubertal status, at baseline and in the follow-up period (Table 2). Along with an increase in insulin secretion, a significant increase in HOMA-B was observed (Table 1). No further significant increases were observed in the following years (Figure 2).

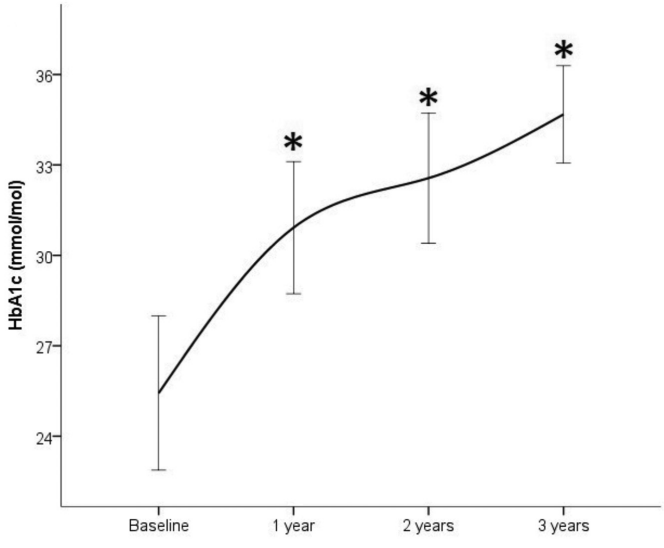

Figure 1. Glycated hemoglobin A1c during the three years of follow-up. *$p < 0.05$ vs baseline

HbA1c: hemoglobin A1c

Before the therapy was started, alterations in glucose metabolism were detected in six (5.9%) patients, four presenting IGT and two presenting impaired HbA1c. In these patients, glucose metabolism normalized during the follow-up (only for one patient impaired HbA1c was confirmed after one year, but normalized in subsequent follow-ups). During the study period, IFG was present in five (5.0%) patients and 12 (11.9%) developed IGT. Glucose and HbA1c alterations were confirmed only occasionally in these patients during the follow-up period (Figure 3). These cases were managed with dietary and lifestyle advice, without stopping the treatment. In the 29 subjects with a positive family history of type 2 diabetes, the risk of developing glucose metabolism alterations was not increased when compared with the rest of the cohort.

In a multivariate logistic regression model that considered age, gender, BMI and pubertal status, none of the model variables was significantly associated with IFG, IGT or HbA1c.

Insulin Resistance

A significant increase in HOMA-IR and decrease in HOMA-S were observed between baseline and the first year of treatment (Table 1). Prevalence of IR (altered HOMA-IR) increased from baseline to first year (from 0% to 6.9%, $p = 0.045$), with a non-significant decrease in the second (1.2%) and third (4.6%) years.

Univariate analysis revealed that IGF-1 concentrations were significantly ($p < 0.01$) and positively correlated with HOMA-IR and inversely correlated with HOMA-S. Weight and age were significantly ($p < 0.01$) correlated with these indices (positively with HOMA-IR and inversely with HOMA-S). No correlation was found with BMI. As expected, HOMA-IR was significantly lower and HOMA-S significantly higher than baseline in pubertal children, after the first year.

Discussion

Data from the main registries on children treated with rhGH (5,6) suggest that this therapy may accelerate the onset of type 2 diabetes mellitus in predisposed patients, with a prevalence of 0.36% of abnormal glucose metabolism and a six-fold increase in the incidence of type 2 diabetes. Several other studies have investigated the effects of rhGH therapy on glucose metabolism in children (17,18,19,20,21,22). Nevertheless, as highlighted in a recent systematic review

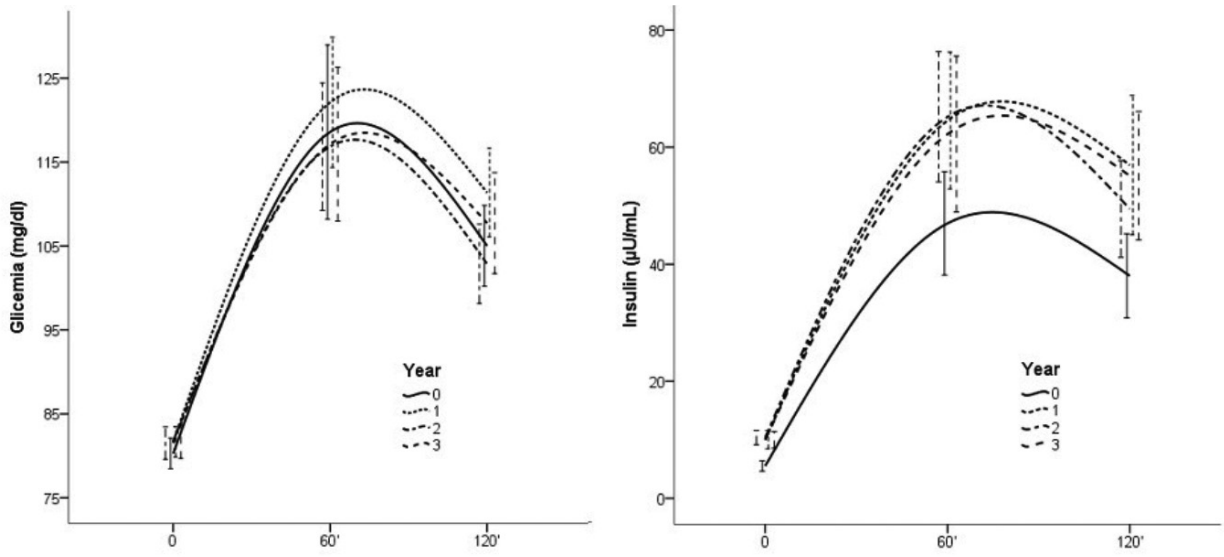

Figure 2. Time response of glucose and insulin levels during oral glucose tolerance test, at baseline and in the three years of follow-up. A significant increase was found only in insulin levels after the first year (see text for p values)

Table 2. Bivariate analysis of the relation between insulinemia (fasting, at 60 minutes and at 120 minutes during oral glucose tolerance test) and relevant variables over the study period

		Age	IGF-1	BMI SDS	Puberty	Peak of GH
Fasting insulin	Baseline	p = 0.4607	p = 0.5446	p = 0.3623	**p = 0.0001**	p = -0.0480
		p < 0.0001	**p < 0.0001**	**p = 0.0003**		p = 0.6480
	1st year	p = 0.4846	p = 0.6352	p = 0.3275	**p < 0.0001**	p = -0.0594
		p < 0.0001	**p < 0.0001**	**p = 0.0039**		p = 0.6078
	2nd year	p = 0.4816	p = 0.7366	p = 0.3629	**p = 0.0040**	p = -0.1230
		p < 0.0001	**p < 0.0001**	**p = 0.0006**		p = 0.2592
	3rd year	p = 0.4271	p = 0.6446	p = 0.2374	p = 0.1568	p = -0.6819
		p = 0.0003	**p < 0.0001**	p = 0.0513		p = 0.0514
Insulin 60 min	Baseline	p = 0.3907	p = 0.2378	p = 0.3305	**p = 0.0232**	p = -0.3396
		p = 0.0011	p = 0.0628	**p = 0.0063**		**p = 0.0049**
	1st year	p = 0.3367	p = 0.4401	p = 0.3270	p = 0.1743	p = -0.1950
		p = 0.0061	**p = 0.0003**	**p = 0.0083**		p = 0.1226
	2nd year	p = 0.2922	p = 0.5365	p = 0.2976	p = 0.3004	p = -0.1715
		p = 0.0156	**p < 0.0001**	**p = 0.0137**		p = 0.1653
	3rd year	p = 0.4381	p = 0.3592	p = 0.0465	p = 0.1057	p = -0.2200
		p = 0.0007	**p = 0.0071**	p = 0.7310		p = 0.1066
Insulin 120 min	Baseline	p = 0.4684	p = 0.4655	p = 0.3166	**p = 0.0007**	p = -0.3484
		p = 0.0001	**p = 0.0001**	**p = 0.0091**		**p = 0.0039**
	1st year	p = 0.4610	p = 0.5398	p = 0.3821	**p = 0.0012**	p = -0.1702
		p = 0.0001	**p < 0.0001**	**p = 0.0018**		p = 0.1788
	2nd year	p = 0.2981	p = 0.5813	p = 0.2502	**p = 0.0017**	p = -0.0890
		p = 0.0136	**p < 0.0001**	**p = 0.0396**		p = 0.4737
	3rd year	p = 0.4271	p = 0.6446	p = 0.2374	p = 0.1508	p = -0.0514
		p = 0.0003	**p < 0.0001**	p = 0.0513		p = 0.6819

Significant p-values in bold. p-values are Spearman's rank correlation coefficients, and are associated with their p-values. p-values for "puberty" are the result of Mann-Whitney rank-sum tests.

GH: growth hormone, SDS: standard deviation scores, BMI: body mass index, IGF-1: insulin-like growth factor-1

(8), only in relatively few studies glucose metabolism abnormalities were the main outcome. The use of different methods to study glucose metabolism and the heterogeneity of the populations evaluated precluded the possibility of obtaining strong evidence of possible glycemic alterations caused by rhGH. The two largest case-control studies reported no significant increase in metabolic disorders, but presented conflicting results with low global clinical significance on the effects of rhGH on insulin sensitivity markers (19,22).

In this study, which included a large cohort of GHD children treated with conventional doses of rhGH for three years, therapy was well tolerated, without major changes in glucose metabolism occurring. No children developed overt diabetes mellitus. In line with previous data (9,19,23), we found an increase in HbA1c and insulin levels, HOMA-IR and HOMA-B values, with a concomitant decrease in HOMA-S. The significant increase in HbA1c, insulin concentrations and IR indices after the first year of treatment, compared to baseline, persisted in subsequent years of follow-up but did not significantly increase from one year to the next. Albeit non statistically significant and of little or none clinical impact, values were not 'stable' at three years, showing a slight but persistent increase. The lack of data after three years do not allow to define if this alteration is persistent or self limiting in a longer period. Larger studies with longer follow may help to better understand this issue.

The observed increase did not translate into significant alterations in either basal or OGTT-measured glucose metabolism: glucose abnormalities were only mild and transitory, and unrelated to rhGH doses, BMI or positive family history. This does not necessarily mean that GH administration does not increase glucose production by stimulating insulin secretion. As reported by Baronio et al (21), the enhanced insulin secretion observed in children with GHD might be not due to IR, but rather to a positive influence of GH treatment on β-cell secretory capacity. In our study, rhGH doses were maintained in the recommended range for isolated GHD (25-35 mcg/kg/day) (24) for the entire follow-up period. Even if the dosage was higher during the first year, when a significant impairment in HbA1c and in insulin sensitivity indices was observed, no significant correlation was found. Our data cannot answer the question of whether, for higher doses of GH, the effect of therapy in inducing IR and diabetes might be more significant.

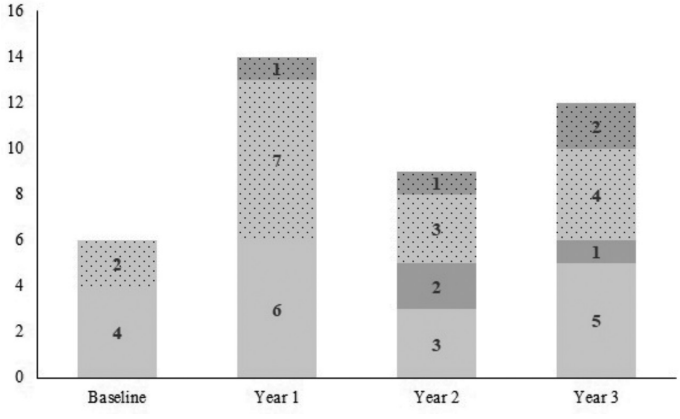

Figure 3. Number of growth hormone deficient children with glucose metabolism alterations before starting growth hormone therapy and in the follow-up. Dotted area: impaired hemoglobin A1c. Non-dotted area: impaired fasting glucose or impaired glucose tolerance. Light grey area: newly diagnosed glucose metabolism alterations. Dark grey area: alterations confirmed from the previous year

Remarkably, 6% of patients presented with pretreatment alterations in glucose metabolism, but none of these patients developed diabetes, nor confirmed persistent alterations during treatment. This is in line with the results of Radetti et al (19), who observed a normalization of glucose tolerance in children presenting with IGT before starting rhGH treatment. We speculate that the increased linear growth and the likely improvements in lean body mass composition induced by GH replacement, may have ameliorated glucose metabolism in these patients.

Few studies have tried to identify predictive factors relating to glucose metabolism alterations in children treated with rhGH. The two largest studies (5,6) postulated that the most relevant predictors for the development of IR and diabetes are individual predisposition and presence of pre-existing metabolic risk factors such as obesity, family history of diabetes, pretreatment IR, previous cranial irradiation and glucocorticoid treatment. In the present study, the development of abnormal glucose metabolism, defined as IFG, IGT or impaired HbA1c, was not predicted by any of these factors. No correlation was found between rhGH dosage, positive family history for diabetes, BMI and presence of IFG, IGT or impaired HbA1c, although this conclusion is limited by the small number of detected cases.

Study Limitations

The major limitation of this study is its retrospective nature. In addition, the gold standard method for the detection of insulin sensitivity, i.e. the euglycemic hyperinsulinemic clamp, was not used. Markers for the assessment of insulin secretion included fasting insulin and HOMA-B, while HOMA-IR was used as a surrogate estimate of insulin sensitivity. The fact that glucose metabolism was not re-evaluated after rhGH discontinuation is a further limitation of the study. Furthermore, a longer follow-up period would have been more informative in patients who needed to

maintain therapy after the first three years. Therefore, the risk factors for the persistence of glucose abnormalities cannot be adequately analysed.

Conclusion

In conclusion rhGH replacement therapy at recommended dosages may be considered safe in terms of metabolic effects. A significant increase in HbA1c and IR after one year of therapy was observed. These alterations persisted, but did not worsen significantly in the following two years and did not bear to overt diabetes in any case. Glucose abnormalities were infrequent and, in the majority of cases, not confirmed in the subsequent follow-up, even in the presence of pretreatment metabolic impairment. Therefore, pre-existing glucose metabolism alterations should not represent a limitation to starting rhGH therapy and new onset alterations during treatment should be appropriately managed by intervening on predisposing factors, rather than by modifying rhGH dosage.

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Ethics

Ethics Committee Approval: The study was approved by the Institutional Review Committee of IRCCS "Burlo Garofolo" (approval number: RC 32/18 Line 2).

Informed Consent: Parents provided informed consent for blood samples to be obtained and stored for research purposes, in accordance with the Declaration of Helsinki of 1975.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Maria Chiara Pellegrin, Elena Faleschini, Claudio Germani, Gianluca Tornese, Concept: Claudio Germani, Egidio Barbi, Gianluca Tornese, Design: Elena Faleschini, Claudio Germani, Egidio Barbi, Gianluca Tornese, Data Collection and Processing: Maria Chiara Pellegrin, Daria Michelon, Elena Faleschini, Claudio Germani, Analysis and Interpretation: Maria Chiara Pellegrin, Egidio Barbi, Gianluca Tornese, Literature Search: Maria Chiara Pellegrin, Daria Michelon, Claudio Germani, Writing: Maria Chiara Pellegrin, Daria Michelon, Elena Faleschini, Claudio Germani, Egidio Barbi, Gianluca Tornese.

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Impact of Socioeconomic Characteristics on Metabolic Control in Children with Type 1 Diabetes in a Developing Country

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What is already known on this topic?

Several predictors of type 1 diabetes mellitus outcomes in children have been studied, which include socioeconomic factors. A number of these social factors have been shown to negatively impact metabolic control including low socioeconomic status, single-parent family and inadequate parental supervision. There is a dearth of evidence on the impact of this type of factor in developing countries.

What this study adds?

Our study aimed to identify socioeconomic factors affecting metabolic control in children and adolescents with type 1 diabetes in a developing country. Identifying patients with high risk of poor metabolic control including those with low socioeconomic status and lower parental education level, will help in implementing early effective strategies for diabetes care and will result in a better metabolic control.

Abstract

Objective: Adequate glycemic control in children with type 1 diabetes reduces the risk of future complications. Identifying factors affecting haemoglobin A1c (HbA1c) is crucial to management of metabolic control. We aimed to identify possible socioeconomic predictors of poor metabolic control this patient group in Jordan, a developing country with limited resources.

Methods: Medical charts of children with type 1 diabetes attending the pediatric endocrine clinics in two major diabetes centers were reviewed. HbA1c ≥7.5% (58 mmol/mol) was considered to reflect poor metabolic control. Logistic regression analysis was performed to identify predictors of poor glycemic control. The association between socioeconomic characteristics and metabolic control was evaluated using multiple correspondence analysis (MCA).

Results: Two hundred and fifty-nine children were enrolled in the study. One fifth of the patients (20.5%) achieved HbA1c <7.5%. Patients with dietary non-compliance [odds ratio (OR): 3.533, confidence interval (CI): 1.803 - 6.926; p<0.001], and those who were overweight (OR: 3.869, CI: 1.218 - 12.294; p=0.022) were more likely to have poor metabolic control. Children whose mothers had a bachelor's degree or higher were less likely to have poor metabolic control compared to children whose mothers had only elementary education (OR: 0.241, CI: 0.079 - 0.734; p=0.012). MCA revealed an association between low socioeconomic status and poor metabolic control. Children with deceased mothers had significantly higher HbA1c of 10.6±1.86% compared to an average of 8.7±1.45% for the rest of participants (p=0.005).

Conclusion: Low socioeconomic status, lower levels of maternal education and maternal death were associated with poor metabolic control. Identifying children with these risk factors might play an important role in optimizing metabolic control and provide better diabetes care.

Keywords: Type 1 diabetes, HbA1c, metabolic control, socioeconomic status, Jordan

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Introduction

Type 1 diabetes is associated with microvascular and macrovascular complications, including diabetic retinopathy and nephropathy (1,2). The Diabetes Control and Complications Trial and the Epidemiology of Diabetes Interventions and Complications study showed that the progression of microvascular complications can be reduced by strict glycemic control (3). Adequate glycemic control, especially in the first five years of diabetes, slows the development of microvascular complications (4). These findings support the importance of maintaining a low haemoglobin A1c (HbA1c) at <7.5%, equivalent to 58 mmol/mol, which is recommended by the International Society for Pediatric and Adolescent Diabetes in children and adolescents (5). Achieving adequate metabolic control has proven to be a challenge worldwide. Therefore identifying possible predictors of metabolic control will be of benefit in adopting appropriate strategies employed to optimize outcomes.

Socioeconomic characteristics of patients are associated with glycemic control. Low socioeconomic class, single-parent family structure and lower parental supervision have been reported as predictors of poor metabolic control (6). Socioeconomic characteristics are variable among different communities and so it is crucial to assess these factors in diabetic children in each individual population, both in developed and developing countries, and to examine the association between those socioeconomic characteristics and metabolic control.

The aim of this study was to identify socioeconomic determinants of metabolic control in children with type 1 diabetes in a Jordanian population, which would help in developing appropriate strategies for diabetes care and education. In addition, it would facilitate the identification of high-risk patients who may require a more personalized management strategy.

Methods

Data were collected by medical chart review of patients seen at pediatric endocrine clinics of two institutions: Jordan University Hospital and the National Centre for Diabetes, Endocrinology and Genetics, from February 2012 through December 2017. The University of Jordan Research Ethics Board approval was obtained (no: 51/2014-2015). Informed consent was not required as it is a chart review, retrospective study. Patients were eligible for the study if they had type 1 diabetes, age <18 years, and had at least one year of follow up.

Diagnosis of type 1 diabetes in our cohort was mainly based on the clinical picture. Variable antibodies were positive in variable percentages, with glutamic acid decarboxylase antibodies showing the highest propotion, followed by insulin autoantibodies and insulinoma antigen 2 antibodies. Antibody status supported the diagnosis of type 1 diabetes, but diagnosis was mainly based on clinical presentation with exclusion of patients with type 2 diabetes, maturity onset diabetes of the young and neonatal diabetes.

Body mass index (BMI) was used to categorize patients into two groups; normal and overweight, using the Centre for Disease Control and Prevention growth charts (7). Children with BMI values <85th percentile were categorized as normal, and those with BMI values ≥85th percentile were categorized as overweight.

Glycemic control was reflected by mean HbA1c values measured over the last year of follow up. Children with a HbA1c value of <7.5% (58 mmol/mol) were considered to be in metabolic control; while those with HbA1c ≥7.5% not in control. Dietary compliance was assessed by counting carbohydrates or determining portions.

When assessing the effect of place of residence, the participants were categorized into four groups depending on the distance from both centers, since they are adjacent to each other.

The socioeconomic status was expressed in terms of: 1) paternal and maternal level of education categorized into three groups - illiterate or elementary school, high school and bachelor's degree or higher (Master's degree and PhD); 2) paternal and maternal occupation was categorized into three groups - professional job, manual job and unemployed; and 3) total family monthly income was categorized into three groups: less than 400 Jordanian Dinars (JDs); which is the low income category, 400-800 JDs, and more than 800 JDs.

Statistical Analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences Statistics for Windows, version 23 (IBM Corp., Armonk, NY, USA).

Characteristics of the patients in the two metabolic groups, in control and not in control, were compared using Pearson chi-square and Fisher's exact tests. Comparison of continuous variables among groups was conducted using One-Way ANOVA and Scheffe post-hoc analysis were used to identify the groups that were significantly different from each other.

Possible predictors of poor metabolic control were analyzed using multiple logistic regression (forward method). Metabolic control, expressed as HbA1c values categorized as a dichotomous nominal variable, was considered the dependent variable.

When logistic regression analysis was conducted, children with deceased mothers and/or fathers (11 cases) were excluded from regression analysis. We removed those cases to avoid large odds ratio (more than 6 digits) with inapplicable confidence intervals, a condition known as complete separation.

Multiple correspondence analysis (MCA) was conducted to analyze the association between socioeconomic characteristics and metabolic control. MCA allows researchers to analyze the pattern of relationships of several categorical dependent variables and detect underlying structures. P values less than 0.05 were considered statistically significant.

Results

A total of two hundred and fifty-nine children with a mean ± standard deviation (SD) age of 11.14 ± 3.61 years were enrolled in the study. One hundred and forty participants (54.1%) were males and 119 (45.9%) were females. Mean ± SD HbA1c of the whole patient group was 8.77% ± 1.48% with 53 participants (20.5%) having an HbA1c level less than 7.5%, while HbA1c was ≥7.5% in two hundred and six children (79.5%).

The main characteristics of participants in the two metabolic groups are shown in Table 1. There were no significant differences between the two metabolic control groups concerning gender, age at diagnosis, type of insulin regimen and whether the child lived with both parents or with either one of them. Children who achieved their target HbA1c tended to have less than five siblings, normal BMI and adequate dietary compliance.

The socioeconomic characteristics are shown in Table 2. Among those, both maternal and paternal educational levels were significantly different between the two metabolic groups. Participants in the metabolically controlled group had a higher percentage of parents with a bachelor's degree or higher. There were no significant differences between the two groups in terms of monthly income, parental job, parental age and parental medical condition.

The percentage of patients who experienced at least one episode of diabetic ketoacidosis, hospitalization and/or emergency room (ER) visits due to hyperglycemia in the metabolically-controlled group was significantly lower than in the metabolically-uncontrolled group: 0% *vs* 9.2% p = 0.022; 3.8% *vs* 17.5%, p = 0.012; and 0% *vs* 9.2%, p = 0.022, respectively. However, hospitalization and ER visits due to hypoglycemia were not statistically different between the two groups: 0% *vs* 2.9%, p = 0.209; and 1.9% *vs* 3.4%, p = 0.571, respectively.

The association of the participants' socioeconomic characteristics with metabolic control was investigated by conducting logistic regression analysis using the forward method (Table 3).

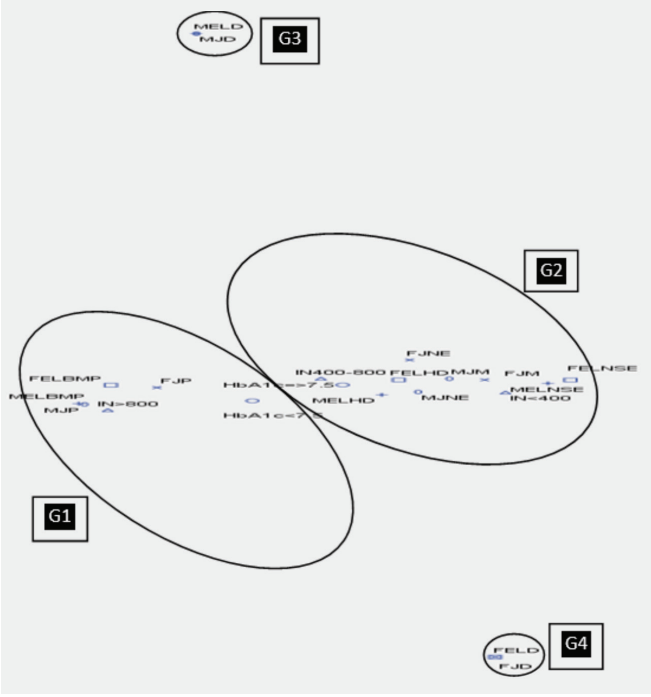
Poor metabolic control was associated with dietary non-compliance, being overweight, and low maternal educational level. Participants who were overweight were three and a half times more likely to have HbA1c ≥7.5% than those with normal weight. Patients with dietary non-compliance were almost four times more likely to experience poor metabolic control than those who were compliant. Participants whose mothers had a bachelor's degree or higher were less likely to have poor metabolic control than those whose mothers were illiterate or had only elementary education [odds ratio (OR): 0.241, confidence interval (CI): 0.079 - 0.734; p = 0.012].

MCA was conducted to explore patterns between socioeconomic status and metabolic control. The MCA model shown in Figure 1 explained 80% of total variability in the model and revealed four groups of patients. Group number 1 consisted of patients who were metabolically controlled, had a high family income, and both parents had professional jobs from the highest education level group. Group number 2 consisted of patients with poor metabolic control, low to intermediate monthly family income, and both parents had manual jobs or were unemployed with an education level lower than bachelor's degree. Group number 3 (G3) consisted of patients whose mothers were deceased. Similarly, Group number 4 (G4) consisted of patients whose fathers were deceased.

Further analysis of the G3 showed that they had a significantly higher HbA1c of 10.6 ± 1.86% compared to an average of 8.7 ± 1.45% for the rest of participants (p = 0.005). However, children whose fathers were dead also had a higher HbA1c that was statistically non-significant, 9.3 ± 2.16% compared to 8.8 ± 1.46% for the rest of the children (p = 0.523).

Discussion

The percentage of children who achieved target HbA1c (20.5%) was similar to that reported in studies reported from developed countries (8,9). Gender in our study was not associated with metabolic control, while evidence from



0 MJP = Mother's job - professional	+ MELNSE = Mother's education level - no school or elementary school
0 MJM = Mother's job - manual	+ MHELHD = Mother's education level - high school or diploma
0 MJNE = Mother's job - not employed	+ MELBMP = Mother's education level - bachelor's degree or higher
0 MJD = Mother's job - dead	+ MELD = Mother's education level - dead
X FJP = Father's job - professional	□ FELNSE = Father's education level - no school or elementary school
X FJM = Father's job - manual	□ FHELHD = Father's education level - high school or diploma
X FJNE = Father's job - not employed	□ FELBMP = Father's education level - bachelor's degree or higher
X FJD = Father's job - dead	□ FELD = Father's education level - dead
O HbA1c <7.5	Δ IN <400 = Monthly income <400 JDs
O HbA1c ≥7.5	Δ IN 400-800 = Monthly income 400-800 JDs
	Δ IN >800 = Monthly income >800 JDs

G1	Group 1 with HbA1c <7.5, family income >800 JDs, both parents had professional jobs and bachelor's degree or higher educational level
G2	Group 2 with HbA1c ≥7.5, family income <800 JDs, both parents had manual jobs or unemployed with educational level of diploma or less
G3	Group 3 with deceased mothers
G4	Group 4 with deceased fathers

HbA1c: haemoglobin A1c, JDs: Jordanian Dinars, G1: group number 1, G2: group number 2, G3: group number 3, G4: group number 4

Figure 1. Association between socioeconomic factors and metabolic control in multiple correspondence analysis

studies concerning gender is conflicting (10,11,12). Longer duration of diabetes was found to be associated with poor metabolic control, similar to previous studies (13).

In our study, the MCA model revealed an association between low socioeconomic status and poor metabolic control, a finding which was also demonstrated in several previous studies (8,14,15,16). This effect even persists through adulthood where, in adults, low socioeconomic status was reported to increase mortality risk in patients with childhood-onset type 1 diabetes (17).

Poor metabolic control was associated with number of siblings exceeding four, which may be attributed to reduced attention and care that was provided to the patient by parents trying to manage a large number of children, a finding in agreement with previous reports (18).

The incidence of hypoglycemia was not significantly different in the controlled and the uncontrolled metabolic groups, while the rate of hyperglycemia was significantly higher in the metabolically uncontrolled group, again similar to previous reports (19). These results show that fear of hypoglycemia should not prevent families from achieving metabolic control for their children (20).

Monthly income was not significant in predicting metabolic control. This is in contrast to results from other studies that found a negative linear association between income and metabolic control (16,21,22). This association may be attributed to the fact that lower-income households rarely contact a primary care provider when facing a diabetes-related problem (23). However, in our study this lack of association between income and metabolic control can be explained by the fact that most of the subjects were covered by insurance and had access to the same level of diabetes care provided by specialized pediatric endocrinologists, regardless of family income. Distance between patient's residence and the two adjacent diabetes centers, was not significantly related to metabolic control, probably due to the fact that 50% of our cohort lived in the same city and 86% of patients lived within 70 kilometers. In addition, both personal and public transport was relatively easily accessible to the families (11).

One of the socioeconomic characteristics predicting metabolic control in our study was parental educational level. The higher the maternal and paternal education the better the metabolic control. Paternal educational level in studies such as those from Italy and Saudi Arabia (8,24), was reported to have no effect on metabolic control. In our study the caregiver responsible for most of the diabetes care was the patient's mother, even in the families where the patient lived with both parents (92% of our

Table 1. Characteristics of participants in the two metabolic control groups (n = 259)

	HbA1c		
Participant's characteristics	**< 7.5% (n = 53)**	**≥7.5% (n = 206)**	**p value**
Female gender n (%)	25 (47.2%)	94 (45.6%)	0.841
Age at last visit (years)			0.053
1-5	5 (9.4%)	13 (6.3%)	
> 5-10	22 (41.5%)	62 (30.1%)	
> 10-15	24 (45.3%)	95 (46.1%)	
≥15	2 (3.8%)	36 (17.5%)	
Latest insulin regimen			0.745
Multiple dose injection	34 (64.2%)	140 (68.0%)	
Triple dose injection	19 (35.8%)	65 (31.6%)	
Pump	0 (0%)	1 (0.5%)	
Number of siblings			0.036
≤4	43 (81.1%)	135 (65.5%)	
> 4	10 (18.9%)	71 (34.5%)	
Duration of diabetes (years)			0.001
≤5	52 (98.1%)	16 (79.1%)	
> 5	1 (1.9%)	43 (20.9%)	
Count carbohydrates			
Yes	30 (56.6%)	57 (28.2%)	< 0.001
Living arrangements			0.703
Lives with both parents	50 (94.3%)	189 (91.7%)	
Lives with the mother	1 (1.9%)	9 (4.4%)	
Lives with the father	2 (3.8%)	8 (3.9%)	
Distance from the two institutions (kilometers)			0.765
Amman (city where the two centers are situated)	33 (62.3%)	125 (60.7%)	
Less than 70 kilometers from Amman	14 (26.4%)	51 (24.8%)	
71-185 kilometers from Amman	6 (11.3%)	26 (12.6%)	
More than 185 kilometers from Amman	0 (0%)	4 (1.9%)	
Type of insurance			
Ministry of Health and University of Jordan (90% coverage)	17 (32.1%)	41 (19.9%)	0.088
Royal court (100% coverage)	35 (66.0%)	146 (79.6%)	
Private (no coverage)	1 (1.9%)	1 (0.5%)	

HbA1c: haemoglobin A1c

cohort). This is understandable since mothers are usually the primary caregivers coordinating efforts and medical recommendations provided by the members of the medical team. Improving mother's knowledge and targeting those with lower educational level may improve glycemic control in this subgroup of children. Furhthermore, children whose mothers are dead; were found to have significant poor metabolic control since they are deprived of direct maternal care and supervision. It is extremely important to give those children special attention to optimize their diabetes care as much as possible with the help of their caregivers.

Study Limitations

Our study was a retrospective study which mainly relied on information present in the medical records. One of the limitations of our study is that pubertal status was not collected from the medical charts. Another important limitation is the fact that the study involved patients registered in two centers in the capital city. A more comprehensive study involving different geographical areas with possible different socioeconomic determinants of metabolic control might have been more helpful in explaining the health inequalities that possibly exist in a society, despite the presence of the same policy of medical

Table 2. Parental characteristics of participants in the two metabolic control groups (n = 248, 11 children with deceased father and/or mother were excluded)

	HbA1c		
	< 7.5% (n = 52)	≥7.5% (n = 196)	p value
Consanguineous parents (yes)	8 (15.4%)	24 (12.2%)	0.548
Paternal age			0.210
< 50 years	42 (80.8%)	137 (69.9%)	
≥50 years	10 (19.2%)	59 (30.1%)	
Paternal medical condition			0.356
No medical problem	48 (90.6%)	177 (85.9%)	
At least one medical condition	4 (7.7%)	24 (12.2%)	
Paternal occupation			0.272
Professional	30 (57.7%)	89 (54.4%)	
Manual	19 (36.5%)	89 (45.4%)	
Non-employed	3 (5.8%)	18 (9.2%)	
Paternal education level			0.029
No school or elementary school	2 (3.8%)	33 (16.8%)	
High school or diploma	15 (28.8%)	63 (32.1%)	
Bachelor's degree or higher	35 (67.3%)	100 (51%)	
Maternal age			0.111
< 45 years	40 (76.9%)	128 (65.3%)	
≥45 years	12 (23.1%)	68 (34.7%)	
Maternal medical condition			0.658
No medical problem	48 (92.3%)	177 (90.3%)	
At least one medical condition	4 (7.7%)	19 (9.7%)	
Maternal occupation			0.960
Professional	15 (28.8%)	54 (27.6%)	
Manual	1 (1.9%)	3 (1.5%)	
Non-employed	36 (69.2%)	139 (70.9%)	
Maternal education level			0.046
No school or elementary school	5 (9.6%)	43 (21.9%)	
High school, or diploma	26 (50.0%)	102 (52.0%)	
Bachelor's degree or higher	21 (40.4%)	51 (26.0%)	
Family monthly income in JDs			0.199
< 400	8 (15.4%)	48 (24.5%)	
400-800	36 (69.2%)	109 (55.6%)	
> 800	8 (15.4%)	39 (19.9%)	

JDs: Jordanian Dinars

care. It is important to evaluate the effect of socioeconomic factors in both developed and developing countries to explain the universal challenge of achieving adequate metabolic control despite all the recent advancement in the field of diabetes care.

Conclusion

Early metabolic control is essential in preventing future complications of type 1 diabetes. Identifying predictors of poor metabolic control might help in improving clinical care provided for patients.

Some predictors of poor glycemic control are modifiable, such as dietary non-compliance and being overweight, which can be controlled to improve glycemic control. Other predictors such as low maternal education level are non-modifiable, but these factors help in identifying those children and adolescents with type 1 diabetes at high risk

Table 3. Logistic regression analysis of possible predictors of poor metabolic control (n = 248; 11 children with deceased father and/or mother were excluded)

	Simple logistic regression				Multiple logistic regression			
	B¥	OR$^{\Delta}$	CI (95%)	p value	B¥	OR$^{\upsilon}$	CI (95%)	p value
Gender (female)	-0.107	0.898	0.487-1.657	0.731				
Age at last visit (years)				0.117				
1-5$^{\infty}$								
> 5-10	0.094	1.099	0.350-3.453	0.872				
> 10-15	0.344	1.410	0.457-4.348	0.550				
≥15	1.907	6.731	1.159-39.085	0.034				
Father's job				0.227				
Professional$^{\infty}$								
Manual	0.457	1.579	0.828-3.011	0.165				
Not employed	0.704	2.022	0.557-7.350	0.285				
Paternal education level				0.092				
No school or elementary school$^{\infty}$								
High school, or diploma	-1.596	0.203	0.046-0.896	0.035				
Bachelor's degree or higher	-1.658	0.190	0.042-0.862	0.031				
Mother's job				0.961				
Professional$^{\infty}$								
Manual	-0.182	0.833	0.081-8.602	0.878				
Not employed	0.070	1.073	0.544-2.116	0.840				
Maternal education level				0.054				0.043
No school or elementary school$^{\infty}$								
High school, or diploma	-0.785	0.456	0.164-1.267	0.132	-1.014	0.363	0.124-1.064	0.065
Bachelor's degree or higher	-1.264	0.282	0.098-0.812	0.019	-1.423	0.241	0.079-0.734	0.012
Count carbohydrate portions								
Yes$^{\infty}$								
No	1.248	3.485	1.849-6.568	< 0.001	1.262	3.533	1.803-6.926	< 0.001
Body mass index								
Normal$^{\infty}$								
Overweight	1.027	2.792	0.947-8.230	0.063	1.353	3.869	1.218-12.294	0.022

$^{\infty}$Reference group, ¥Regression coefficient, $^{\Delta}$Unadjusted odds ratio, $^{\upsilon}$Adjusted odd ratio, OR: odds ratio, CI: confidence interval

of poor metabolic control. This group of children needs individualized care plans to ensure that target HbA1c levels are achieved. Children, whose mothers are dead, probably require special attention since they are at high risk of poor glycemic control. Engaging their caregivers and providing comprehensive education concerning the care plans of diabetic children is of great importance. These children might also need early, intensive and more frequent education and training on personal insulin requirements and administration since they lack maternal guidance and care.

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Ethics

Ethics Committee Approval: The University of Jordan Research Ethics Board approval was obtained (no: 51/2014-2015).

Informed Consent: Informed consent was not required as it is a chart review retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Abeer Alassaf, Rasha Odeh, Kamel Ajlouni, Design: Abeer Alassaf, Rasha Odeh, Data Collection or Processing: Abeer Alassaf, Rasha Odeh, Sarah Ibrahim, Analysis or Interpretation: Abeer Alassaf, Rasha Odeh, Sarah Ibrahim,

Lubna Gharaibeh, Literature Search: Abeer Alassaf, Lubna Gharaibeh, Writing: Abeer Alassaf, Rasha Odeh, Lubna Gharaibeh, Kamel Ajlouni.

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ORIGINAL ARTICLE

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Accuracy of Tri-ponderal Mass Index and Body Mass Index in Estimating Insulin Resistance, Hyperlipidemia, Impaired Liver Enzymes or Thyroid Hormone Function and Vitamin D Levels in Children and Adolescents

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What is already known on this topic?

Recently, the tri-ponderal mass index (TMI) has been reported as an alternative to body mass index (BMI). TMI has been reported to be nearly stable throughout adolescence and that it may estimate body fat levels more accurately than BMI, especially in adolescents.

What this study adds?

This study documented the usability of the proposed TMI values in Turkish children and investigated the relationship between TMI and some biochemical parameters. To the best of our knowledge, this is the first study to investigate the power of TMI as a predicter of liver enzyme concentrations. This is also the first application of TMI in Turkish children.

Abstract

Objective: Tri-ponderal mass index (TMI) has been reported to estimate body fat more accurately than body mass index (BMI). This study aimed to compare the efficacy of TMI and BMI in predicting insulin resistance (IR), hyperlipidemia, impaired liver enzymes or thyroid hormone function and vitamin D concentration.

Methods: One hundred and forty-three overweight or obese children, based on BMI-standard deviation (SD) scoring (BMI-SDS) were studied retrospectively. TMI thresholds for overweight status were 16.0 kg/m^3 for boys and 16.8 kg/m^3 for girls and 18.8 kg/m^3 for boys and 19.7 kg/m^3 for girls for obese status.

Results: Twenty-two overweight and eight obese children by BMI-SDS were classified as normal by TMI. Of the overweight children 22 (22.7%) had IR and IR was detected in 2 of 8 obese children with normal TMI. There was no increase in liver enzymes in any of the children with normal TMI. Forty-four obese children were overweight according to TMI and IR was detected in 40.9%. Thyroid stimulating hormone levels were significantly higher in BMI-based obese children. Vitamin D levels were similar in all groups of both classifications.

Conclusion: When TMI was used there may be a risk of overlooking IR. However, if it is assumed that liver enzymes are elevated as a result of visceral adiposity, TMI can be used as an auxiliary parameter to show visceral effects of adiposity. Normal TMI may indicate that visceral organ functions have not deteriorated yet. More studies are needed to evaluate TMI as a clinical tool.

Keywords: Body mass index, hyperlipidemia, impaired liver enzymes, insulin resistance, tri-ponderal mass index

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Introduction

Childhood obesity is a major health problem of worldwide concern (1,2,3). During the past 20 years, the proportion of obese children and adolescents has significantly increased in most countries (1,2,3). Obesity in adolescents is a major risk factor for adulthood obesity (3). Childhood obesity is also strongly linked to comorbidities such as hypertension, hyperlipidemia, impaired glucose metabolism and type 2 diabetes, obstructive sleep apnea, non-alcoholic fatty liver disease and metabolic syndrome in childhood or later in life (3). Body mass index (BMI) is commonly used to diagnose obesity in children and adolescents. Recently, the tri-ponderal mass index (TMI) has been reported to be more stable throughout childhood and adolescence and to estimate body fat levels more accurately than BMI, especially in adolescents which is supported by y dual-energy X-ray absorptiometry (4,5). In line with the increasing interest on TMI, recent studies of TMI in both obese (4,5,6,7,8,9) and non-obese children (10) have been published. The aim of this study was to compare the efficacy of BMI and the recently proposed TMI in forecasting insulin resistance (IR), hyperlipidemia, impaired liver enzymes, thyroid hormone function and vitamin D concentrations.

Methods

Participants

In this retrospective study, a medical chart review was performed to collect data from the Pediatric Endocrinology Outpatient Clinics of the Near East University, Nicosia, Northern Cyprus. The medical records of all children and adolescents seen between January 2016 and December 2017 with a diagnosis of obesity were investigated. Initial data selection sought children and adolescents aged six to18 years with obesity or overweight according to their BMI-standard deviation (SD) score (BMI-SDS) at their first visit to the endocrine clinic and followed at the endocrine clinic for at least one year. Children with a BMI-SDS between +1.0 and +2.0 were accepted as overweight and those with a BMI-SDS greater than or equal to +2.0 as obese. Patients excluded from the study included those with syndromatic obesity, endocrine disorders associated with obesity such as hypothyroidism, Cushing's syndrome, hypothalamic obesity and postcranial surgery or with non-endocrine chronic illness which require medications that might impact body weight (systemic steroids, psychiatric medications) and patients with missing data.

Clinical and Biochemical Parameters

Routine clinical follow-up of patients at every clinic visit (usually every 4-6 months) during the time period of the study, included measurement of weight (with underwear, using a standard Seca digital weight scale), height (using a commercial Harpenden-Holtain stadiometer) and evaluation of pubertal stage according to the criteria of Marshall and Tanner. BMI and TMI were calculated as weight in kilograms divided by height in meters squared (kg/m^2) and as weight divided by height cubed (kg/m^3), respectively. The SDS of height, weight, and BMI were calculated according to the data of Neyzi et al (11) for Turkish children and adolescents. Considering that TMI is more stable in children and adolescents (4,5), established TMI thresholds used in the study. TMI thresholds used in the study to diagnose overweight status were 16.0 kg/m^3 for boys and 16.8 kg/m^3 for girls and were 18.8 kg/m^3 for boys and 19.7 kg/m^3 for girls to diagnose obese status (4). Fasting blood glucose, insulin, homeostasis model assessment-IR (HOMA-IR), high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol, triglycerides (TG), total cholesterol (TC), liver function enzymes, thyroid hormones and 25-hydroxyvitamin D [$25(OH)D_3$] were evaluated. HOMA-IR was used to evaluate IR using the formula: HOMA-IR = [insulin (mU/l) × glucose (mmol/l)]/22.5 (3). The HOMA-IR thresholds of Turkish children were used to define IR as follows: 2.22 for prepubertal girls; 2.67 for prepubertal boys; 3.82 pubertal girls; and 5.22 for pubertal boys (12). TC ≥200 mg/dL and TG ≥150 mg/dL (≥1.69 mmol/L) were accepted as high (13). Thresholds for liver enzymes were accepted according to laboratory references [serum glutamic oxaloacetic transaminase (SGOT): 5-34 U/L, serum glutamic-pyruvic transaminase (SGPT): SGPT: 0-55 U/L]. Instead of a sex-specific cut-off for HDL, a single cut-off was used which was <1.03 mmol/L or <40 mg/dL as proposed by the International Diabetes Federation consensus definition of metabolic syndrome in children and adolescents (13). Vitamin D status was classified as sufficiency (>50 nmol/l or >20 ng/mL), insufficiency (30-50 nmol/l or 12-20 ng/mL) and deficiency (<30 nmol/l or <12 ng/mL), based on the consensus statement of the Endocrine Society (14). Abdominal ultrasound (USG) was performed to detect non-alcoholic fatty liver disease in patients with IR and/or elevated liver enzymes.

Statistical Analysis

The Statistical Package for Social Sciences Software (SPSS 21, Chicago, IL, USA) was used for data analysis. Skewness and Kurtosis Z-values and Shapiro-Wilk's test were used to test the distribution of data. All dependent variables are not normally distributed for each category of an independent variable (Shapiro-Wilk's test $p<0.05$ and Skewness and Kurtosis Z-values were not between -1.96 to +1.96). Thus, non-parametric methods was used in data analysis. The

Kruskal-Wallis H test was used to determine if there were statistically significant differences between more than two groups of an independent variable on a continuous or ordinal dependent variable whereas Mann-Whitney U test was used to determine if there were statistically significant differences between two groups. Finally, a chi-square test was used for testing relationships between categorical variables from a single population. All continuous variables were expressed as median, maximum and minimum values. Statistical significance was assumed when $p < 0.05$.

Results

A total of 143 patients were enrolled in the study. Of the total cohort, 58% (n = 83) were female and 42% (n = 60) were male. The mean ± SD age of the patients was 11.1 ± 2.9 (range 6.3-17.6) years. Based on BMI-SDS, the overweight group consisted of 37 patients (25.9%) and the obese group of 106 patients (74.1%), respectively. When the study sample was classified based on TMI thresholds, three groups were identified. These were normal 21% (n = 30), overweight 41.3% (n = 59) and obese 37.8% (n = 54). Twenty-two overweight and eight obese children were classified as normal by TMI. No patient classified as overweight by BMI-SDS was classified as obese by TMI. Forty-four obese children were classified as overweight according to TMI. There were 54 (37.7%) patients who were classified as obese, based on both BMI-SDS and TMI (Table 1).

The median values of fasting blood glucose, insulin, HOMA-IR, TC, HDL, LDL, TG, SGOT, SGPT, 25(OH)D_3, thyroid stimulating hormone (TSH) and free thyroxine (fT4) are presented in Table 2. The median values of serum TG, SGOT and SGPT differed within the groups according to TMI classification. The serum levels of TG, SGOT and SGPT in patients with normal TMI were significantly lower than those of both obese and overweight patients (Table 2). Median values of fasting blood glucose and TSH concentratons significantly differed between the overweight and obese patients based on BMI-SDS classification (Table 2). TMI classification did not effect the median values of thyroid hormones. However, all patients with normal TMI had normal TSH values. According to the BMI classification, all patients with elevated TSH were obese (Table 3). Serum 25(OH)D_3 levels were similar in all groups according to both classification (Table 2, 3).

The incidence of IR in the total study group was 37.1% according to HOMA-IR thresholds for Turkish children. Based on BMI-SDS, eight (21.6%) of the overweight patients and 45 (42.5%) of the obese patients had IR. Based on TMI, seven (23.3%) of the normal, 21 (35.5%) of the overweight, 25 (46.3%) of the obese patients had IR (Table 3). The frequency of IR was significantly higher in obese children than in overweight when BMI was used to classify the study group (Table 3). Moreover, when we classified the study group according to BMI, another parameter that was significantly different between the obese and overweight groups was the frequency of low HDL levels. However only the frequency of elevated SGOT differed, although not significantly, within the groups when classified according to TMI (p = 0.054) (Table 3).

22.7% of overweight children with normal TMI had IR, 9.1% high TC, 50% had LDL > 100 mg/dL and 4.5% had low HDL and high TG. Two of eight obese children (BMI-SDS > + 2) with normal TMI had IR and low HDL. There was no increase in liver enzyme levels in any child with normal TMI (Table 4). Forty-four obese children, who were overweight according to TMI, had IR 40.9%, low HDL 34.1% and at least one elevated liver enzyme was present in 11.4%. Isolated IR was detected in 46.3% of 54 patients who were obese according to the both BMI-SDS and TMI (Table 4).

In all insulin resistant cases (n = 53), hepatosteatosis was observed in 15 (28.3%) patients (n = 6, 40% female; n = 9, 60% male) and at least one elevated liver enzyme

Table 1. Distribution of groups according to body mass index and tri-ponderal mass index

		TMI			n (% in all study group)
		Normal n (% TMI in BMI subgroups: % BMI in TMI subgroups)	OW n (% TMI in BMI subgroups: % BMI in TMI subgroups)	OB n (% TMI in BMI subgroups: % BMI in TMI subgroups)	Total
BMI	OW	22 (59.5%:73.3%)	15 (40.5%:25.4%)	0 (0%:0%)	37 (25.9%)
	OB	8 (7.5%:26.6%)	44 (41.5%:74.6%)	54 (50.9%:100%)	106 (74.1%)
n (% in all study group)	Total	30 (20.9%)	59 (41.3%)	54 (37.7%)	143 (100%)

BMI: body mass index, OB: obese, OW: overweight, TMI: tri-ponderal mass index

was detected in seven (13.2%) patients of whom seven also had elevated SGOT and five had elevated SGPT. None of these seven had normal TMI, whereas two of them had overweight TMI value and the remaining five were obese by TMI (Table 5). All seven had at least Grade 2 hepatosteatosis on abdominal USG. Conversely, only eight (17.4%) of the patients who had IR without an increase in liver enzymes (n = 46) had hepatosteatosis on abdominal USG (Grade 1 hepatosteatosis n = 6; Grade 2 hepatosteatosis n = 2). None of these patients had Grade 3 stetatosis. Remarkably, none of these eight patient had normal TMI value (Table 5).

Discussion

This study investigated the usability of the proposed TMI values in Turkish children. The relationships between TMI and some biochemical parameters were also presented. This study compares the utility of TMI and BMI in forecasting IR, hyperlipidemia, impaired liver enzymes or thyroid hormone functions and 25(OH)D_3 level. The current study is the first in Turkish children and to the best of our knowledge is the first to investigate the use of TMI in predicting abnormalities in liver enzymes.

The use of BMI as a surrogate of adiposity is especially problematic in the pediatric population, because the relative contributions of fat mass and lean body mass to body weight vary by age, sex, pubertal status, and population ancestry. Annual increases in BMI from midchildhood onward are largely because of increases in lean body mass rather than to increases in fat mass and differences in BMI percentiles indicate differences in fat mass only for high percentiles of BMI (15). If the goal is to define overweight status in children and adolescents based on percentiles of body fat or visceral adiposity, BMI-SDS may be overdiagnosing adolescents as overweight (4). Thus, the debate on overdiagnosis of overweight using BMI has been highlighted recently (4,5,15,16). This overdiagnosis may increase health care-related costs and also cause stress in both families and patients (4). Thus, if we use TMI, the number of children who are diagnosed as overweight or obese is likely to decrease. This is important because adolescents may be more sensitive than adults to being classified as overweight (4). Indeed, in our study, 22 overweight and eight obese children were classified as normal when we used TMI and there were no patients that TMI classified as obese while BMI-SDS classified as overweight. However, when we try to prevent overdiagnosis of overweight with BMI, the patient

Table 2. The median, minimum and maximum values of variables in the study sample and in groups classified by tri-ponderal mass index and body mass index-standard deviation score

	TMI group				Total group	BMI group		
	Normal	OW	OB	p		OW	OB	p
	Median (minimum:maximum)				Median (minimum:maximum)			
BG (mg/dL)	93 (84:107)	92 (65:112)	91 (74:117)	0.17	92 (65:117)	95 (82:112)	91 (65:117)	* < 0.01
Insulin (uU/mL)	12.3 (3.8:34.3)	14 (5.4:39.8)	16.4 (2:71.7)	0.13	115.2 (2:71.7)	12.8 (3.8:34.3)	15.8 (2:71.7)	0.18
HOMA-IR	3 (0.9:8.4)	3.4 (1.2:8.3)	3.8 (0.4:15)	0.28	3.5 (0.4:15)	3 (0.9:8.4)	3.5 (0.4:15)	0.29
TC (mg/dL)	164.5 (114:224)	158 (117:231)	164.5 (92:252)	0.94	163 (92:252)	164.5 (114:226)	163 (92:252)	0.90
HDL (mg/dL)	45 (35:72)	46 (23:72)	46 (26:78)	0.74	45 (23:78)	48.5 (35:72)	45 (23:78)	0.10
LDL (mg/dL)	101 (60:155)	91 (51:157)	94.5 (48:169)	0.17	93 (48:169)	96.5 (61:155)	92 (48:169)	0.73
TG (mg/dL)	64.5 (31:169)	88.5 (41:382)	87.5 (33:214)	*0.04	85 (31:382)	72.5 (31:166)	87.5 (33:382)	0.09
SGOT (U/L)	19.5 (11:28)	23 (11:98)	23 (13:96)	* < 0.01	22 (11:98)	22 (11:38)	22 (12:98)	0.43
SGPT (U/L)	16 (10:40)	21 (5:194)	23 (12:148)	* < 0.01	21 (5:194)	20 (10:59)	21 (5:194)	0.07
Vit D_3 (ng/mL)	21.2 (10.3:42)	19.2 (6.2:67)	20.9 (7.4:39.7)	0.45	20.6 (6.2:67)	22.8 (10:67)	19.4 (6.2:44)	0.27
TSH (uIU/mL)	2 (0.9:4)	2.1 (0.4:9.8)	2.7 (0.7:9)	0.21	2.3 (0.4:9.8)	1.9 (0.9:4)	2.6 (0.4:9.8)	0.03*
fT4 (ng/dL)	1 (0.7:1.4)	1 (0.8:1.7)	1 (0.9:1.7)	0.91	1 (0.7:1.7)	1 (0.7:1.4)	1 (0.8:1.7)	0.99

Kruskal-Wallis H test.

*Statistically significant.

BG: fasting blood glucose, BMI: body mass index, fT4: free thyroxine, HDL: high density lipoprotein, HOMA-IR: homeostasis model assessment-insulin resistance, LDL: low density lipoprotein, OB: obese, OW: overweight, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic-pyruvic transaminase, TMI: tri-ponderal mass index, TSH: thyroid stimulating hormone, Vit D_3: 25-hydroxyvitamin D, TG: triglycerides, TC: total cholesterol

at risk should not be overlooked. So, both approaches may have some risks and undesirable consequences.

Both blood glucose and TSH were significantly different by BMI classification compared to TMI classification. Although, mean values of insulin and HOMA-IR were not significantly different between the BMI overweight and BMI obese groups, the frequency of IR according to the cut off values based on age and puberty, pointed to significant differencee in these two groups. Higher fasting glucose levels in obese patients than in those who are overweight may be expected, consistent with an increasing frequency of IR. Thus BMI may reflect IR and associated higher glucose values better than TMI. In contrast, using TMI classification, mean values of serum TG, SGOT and SGPT in patients with normal TMI, were significantly lower than those of both obese and overweight patients classified by BMI. Moreover, in our study, none of the insulin resistant cases with elevated liver enzymes or none of the insulin resistant cases with USG-proven hepatosteatosis had normal TMI. No patient with a normal classification by TMI had elevated liver enzymes. Although BMI has been reported as a good predictor of elevated SGPT

Table 3. Frequency of pathologic biochemical parameters according to body mass index and body mass index groups

		TMI group (n)				BMI group (n)				
		Normal	OW	OB	p	Total	OW	OB	Total	p
IR	(+)	7	21	25		53	8	45	53	
	(-)	23	38	29	0.18	90	29	61	90	*0.024
	Total	30	59	54		143	37	106	143	
High total cholesterol (>200 mg/dL)	(+)	2	9	7		18	4	14	18	
	(-)	28	50	47	0.47	125	33	92	125	0.78
	Total	30	59	54		143	37	106	143	
Low HDL (<40 mg/dL)	(+)	3	18	14		35	4	31	35	
	(-)	27	41	40	0.1	108	33	75	108	*0.02
	Total	30	59	54		143	37	106	143	
High LDL (>100 mg/dL)	(+)	14	21	23		58	16	42	58	
	(-)	16	38	31	0.86	85	21	64	85	0.85
	Total	30	59	54		135	37	106	143	
High triglyceride (>150 mg/dL)	(+)	2	7	9		18	2	16	18	
	(-)	28	52	45	0.42	125	35	90	12	0.14
	Total	30	59	54		143	37	106	143	
High SGOT	(+)	0	5	9		14	1	13	14	
	(-)	30	54	45	0.054	129	36	93	129	0.1
	Total	30	59	54		143	37	106	143	
High SGPT	(+)	0	4	5		9	1	8	9	
	(-)	30	55	49	0.26	134	36	98	134	0.32
	Total	30	59	54		143	37	106	143	
$25(OH)D_3$	N	18	26	25	0.65	69	22	47	69	0.32
	MD	9	24	20		53	11	42	53	
	SD	3	9	9		21	4	17	21	
	Total	30	59	54		143	37	106	143	
High TSH	(+)	0	3	4	0.26	7	0	7	7	0.09
	(-)	30	56	50		136	37	99	136	
	Total	30	59	54		143	37	106	143	

Chi-square test.

*Statistically significant.

BMI: body mass index, High LDL: low density lipoprotein >100 mg/dL, High TC: total cholesterol >200 g/dL, High TG: triglyceride >150 mg/dL, IR: insulin resistance according to homeostasis model assessment-IR thresholds of Turkish children, LE: liver enzyme, Low HDL: high density lipoprotein <40 mg/dL, MD: mild deficiency, SD: severe deficiency, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic-pyruvic transaminase, OW: overweight, TMI: tri-ponderal mass index, TSH: thyrotrophin-stimulating hormone, $25(OH)D_3$: 25-hydroxyvitamin D

in adolescents (17,18), the accuracy of the TMI in detecting impaired liver enzymes seems to be better than BMI. It has been reported that TMI may estimate body fat percentage more accurately than BMI (4,5). Accordingly, all of the study findings that present the correlation between TMI and liver enzymes or hepatosteatosis can be considered to support this recent information of estimation body fat percentage. The gold standard dual-energy X-ray absorptiometry is not always practical for screening body fat percentages, especially in children, so a simple calculation of TMI may be a practical approach to the assessment of increase in body fat. Moreover, TMI offers certain cut off values and does not need age-specific percentiles like as BMI, and thus provides ease of use (4,5). This may be particularly helpful in identifying a child with a higher risk of visceral adiposity and may also be helpful in referring these at-risk patients to the pediatric endocrine clinic. Conversely, some criticism also exists regarding TMI usage (6,7). It has been reported that fat distribution is more important than body fat percentage in determining adult obesity-related outcomes, such as type 2 diabetes. TMI does not account for fat distribution without distinguishing fat mass from muscle mass (6). Moreover, in contrast to studies which support TMI, BMI-SDS, BMI and waist circumference have also been reported as the most relevant anthropometric markers to predict metabolic risk in youth and these markers have been reported as superior to TMI (7).

Table 4. Comparison of variables between groups according to the group combinations of body mass index and tri-ponderal mass index

		Group combinations, n(%)				
		TMI normal and BMI OW	TMI normal and BMI OB	TMI OW and BMI OW	TMI OW and BMI OB	TMI OB and BMI OB
IR	(+)	5 (22.7%)	2 (25%)	3 (20%)	18 (40.9%)	25 (46.3%)
	(-)	17 (72.7%)	6 (75%)	12 (80%)	26 (59.1%)	29 (53.7%)
High TC	(+)	2 (9.1%)	0	2 (13.3%)	7 (15.9%)	7 (13%)
	(-)	20 (90.9%)	8 (100%)	13 (86.7%)	37 (84.1%)	47 (87%)
Low HDL	(+)	1 (4.5%)	2 (25%)	3 (20%)	15 (34.1%)	14 (25.9%)
	(-)	21 (95.5%)	6 (75%)	12 (80%)	29 (65.9%)	40 (74.1%)
High LDL	(+)	11 (50%)	3 (37.5%)	5 (33.3%)	16 (36.4%)	23 (42.6%)
	(-)	11 (50%)	5 (62.5%)	10 (66.6%)	28 (63.6%)	31 (57.4%)
High TG	(+)	1 (4.5%)	1 (12.5%)	1 (6.7%)	6 (13.6%)	9 (16.7%)
	(-)	21 (95.5%)	7 (87.5%)	14 (93.7%)	38 (86.4%)	45 (83.3%)
High SGOT	(+)	0 (0%)	0 (0%)	1 (6.7%)	4 (9.1%)	9 (16.7%)
	(-)	22 (100%)	8 (100%)	14 (93.7%)	40 (90.9%)	45 (83.3%)
High SGPT	(+)	0 (0%)	0 (0%)	1 (6.7%)	3 (6.8%)	5 (9.3%)
	(-)	22 (100%)	8 (100%)	14 (93.7%)	41 (93.2%)	49 (90.7%)
At least 1 high LE	(+)	0 (0%)	0 (0%)	1 (6.7%)	5 (11.4%)	9 (16.7%)
	(-)	22 (100%)	8 (100%)	14 (93.7%)	39 (88.6%)	45 (83.3%)
Total		22	8	15	44	54

BMI: body mass index, High LDL: Low density lipoprotein >100 mg/dL, High TC: Total cholesterol >200 mg/dL, High TG: triglyceride >150 mg/dL, IR: insulin resistance according to homeostasis model assessment-IR thresholds of Turkish children, LE: liver enzyme, Low HDL: high density lipoprotein <40 mg/dL, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic-pyruvic transaminase, OB: obese, OW: overweight, TMI: tri-ponderal mass index

Table 5. Frequency of high liver enzymes and hepatosteatosis in insulin resistant cases

		TMI group									
		Normal		OW			Obese				Total
HS Grade		0	1	0	1	2	0	1	2	3	
At least 1 LE high (n)	Yes	0	0	0	0	2	0	0	3	2	7
	No	7	0	17	1	1	14	5	1	0	46
Total IR (+) (n)		7		21			25				53

HS: hepatosteatosis, IR: insulin resistance according to homeostasis model assessment-IR thresholds of Turkish children, OW: overweight, TMI: tri-ponderal mass index, LE: liver enzyme

In our study, USG-proven hepatosteatosis was found to be slightly more predominant in males. Although the frequency of IR in our group of patients was 37.1%, at least one high liver enzyme was present in seven and USG-proven hepatosteatosis was detected only in 15 insulin resistant cases respectively. This means that laboratory-demonstrated IR may not always correlate with visceral adiposity which causes organ damage or dysfunction. Moreover, the frequency of IR was close to 50% in patients who were evaluated as obese in both classifications. From this, it can be assumed that there may be other factors contributing to the formation of both IR and visceral adiposity, other than weight gain versus height, calculated by either method. The reasons for this difference may reside in genetic or ethnic differences, to variable socioeconomic status, environment or and to interactions among these variables (4). However, this study highlighted that these multifactorial effects on IR may be more correlated with BMI whereas TMI may be more sensitive to detect multifactorial causes leading to visceral adiposity.

The current study also tried to demostrate the relations between serum lipid levels and weight versus height by both BMI and TMI. Only the frequency of low HDL levels was shown to be correlated with BMI, while the mean TG concentrations were significantly different when using the TMI classification. However, many studies report an association of BMI and lipid levels in children (19,20,21,22). Although a statistically significant association between LDL concentration and BMI has been determined in a population-based, cross-sectional study (19) or BMI has been reported to correctly identify 77% of the total dyslipidemic disorders in obese children (22), our study did not show any relation between both BMI or TMI and LDL concentrations or total dyslipidemic disorders. However, the initial categorization of overweight or obesity was BMI based. It would be of interest to increase the sample size and the include children with normal BMI in future studies of BMI and TMI and lipid abnormalities in young people.

In addition, this study is the first study to test the relation between vitamin D concentrations and thyroid hormones using the TMI classification. However, in the current study, no correlations between vitamin D concentration and either BMI or TMI were found. Subclinical hypothyroidism is defined as elevated TSH levels with normal total thyroxine (T4) or fT4 (23) concentrations. Subclinical hypothyroidism is known to be common in obesity (23). According to BMI values, all patients with elevated TSH levels were obese. However, all patients with normal TMI had normal TSH values. This may indicate that normal TMI can also mean thyroid gland functions have not yet been impaired. This issue remains to be studied and discussed.

Study Limitations

The nature of this study required us to rely on data from medical records. Retrospective design and small sample size were the main limitations of our study. In this retrospective study, waist circumference data could not be evaluated from records. In addition, we did not include blood pressure measurements due to the possibility of obtaining unreliable results under suboptimal conditions, where the white coat hypertension effect could not be ruled out (optimal conditions would include seated subjects with a 5-min rest and use of an appropriately-sized cuff). Also, abdominal USG results were limited to patients with IR, due to financial constraints.

Conclusion

In conclusion, classification by TMI may risk overlooking IR. However, if it is assumed that liver enzymes are elevated as a finding of visceral adiposity, TMI can be used as an auxiliary parameter to show the visceral effects of adiposity. Normal TMI may indicate that visceral organ functions have not deteriorated or visceral organ damage has not yet begun. Thus, TMI can provide different benefits in clinical practice. On the other hand, BMI should be continued to be used because there is a huge body of work on its utility until there are more studies based on TMI. We recommend that BMI and TMI may have different advantages and it would be more appropriate to use them together in clinical practice. Age-specific TMI cut-offs for screening high adiposity; and to compare TMI-based and BMI-based references for indicating visceral adiposity in Turkish children and adolescents are also needed. Overall, there is a need for more and larger prospective studies of the utility of TMI. These would eventually lead to the establishment of both national and international standards for TMI.

Ethics

Ethics Committee Approval and Informed Consent: Since the study was performed retrospectively, ethics committee approval and patient informed consent form were not needed.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Neşe Akcan, Rüveyde Bundak, Concept: Neşe Akcan, Rüveyde Bundak, Design: Neşe Akcan, Rüveyde Bundak, Data Collection or Processing:

Neşe Akcan, Rüveyde Bundak, Analysis or Interpretation: Neşe Akcan, Rüveyde Bundak, Literature Search: Neşe Akcan, Rüveyde Bundak, Writing: Neşe Akcan, Rüveyde Bundak.

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Serum Neuron-specific Enolase and S100 Calcium-binding Protein B in Pediatric Diabetic Ketoacidosis

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What is already known on this topic?

Cerebral edema is the most serious and devastating event in diabetic children during episodes of diabetic ketoacidosis (DKA). However, there are only three studies which have evaluated brain injury markers in children with DKA. These studies report increased plasma levels of neuron-specific enolase (NSE) and S100 calcium-binding protein B (S100B) in patients with DKA.

What this study adds?

In contrast to previous reports this study analyzed simultaneous measurement of both NSE and S100 protein B and compared these with children with type 1 diabetes mellitus without DKA and a healthy control group. Serum NSE is elevated in DKA and the increase in NSE concentrations correlate directly with severity of acidosis in DKA. NSE is also significantly elevated in childen with diabetes but without DKA when compared to healthy controls. Thus NSE may be a useful marker of neuronal injury.

Abstract

Objective: Neuron-specific enolase (NSE) and S100 calcium-binding protein B (S100B) are markers of different neurological disorders. The aim was to investigate the relationship between NSE and S100B serum concentrations and the severity of diabetic ketoacidosis (DKA) in diabetic children.

Methods: Eighty children with DKA, 40 with type 1 diabetes mellitus (T1DM) without DKA and 40 healthy controls were enrolled. Severity of DKA was assessed according to blood pH and bicarbonate concentration. Serum NSE and S100B were measured in all participants. In the DKA group serum NSE and S100B were measured at three time points, at admission and at 12 hours and 24 hours after starting treatment.

Results: Children with DKA showed significantly higher serum levels of NSE at all time points compared to children with T1DM without DKA and controls ($p < 0.01$), while serum S100B concentrations did not differ between the three cohorts. Children with T1DM but without DKA also had significantly higher serum levels of NSE ($p < 0.01$) compared to healthy controls. Patients with low Glasgow Coma Scale score (GCSS) and those with moderate and severe DKA had significantly higher levels of NSE at all time points ($p < 0.01$ for each) compared to patients with normal GCSS and those with mild DKA. No significant differences were found in serum S100B levels according to the severity of DKA and GCS ($p > 0.05$). Younger age, lower GCSS, higher glucose and HbA1c, lower pH and lower serum bicarbonate were the risk factors associated with elevated NSE.

Conclusion: Serum NSE is elevated in all patients with type 1 DM and, in patients with DKA, correlates with severity of DKA. However, serum S100B concentration did not differ between T1DM with or without DKA and healthy controls.

Keywords: Neuron-specific enolase, ketoacidosis, brain injury, S100B, type 1 diabetes mellitus, children

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Introduction

Type 1 diabetes mellitus (T1DM) is the most common chronic metabolic disorder in childhood. Twenty-five to forty percent of children with T1DM present with diabetic ketoacidosis (DKA) at the time of diagnosis (1). DKA is the leading cause of morbidity and mortality in children with T1DM. DKA is characterized by a triad of severe hyperglycemia, metabolic acidosis and hyperketonemia (2).

Cerebral edema is the most serious and devastating event during episodes of DKA (3). Fifty four percent of pediatric DKA patients show neurologic injury, which may manifest as headache, dizziness, depressed mood and/or muscle weakness, without overt cerebral edema (2,4). Ghetti et al (2) and others reported that overall cognitive function is affected by even one episode of DKA (5,6). To date, most data on pediatric DKA related to brain injury consist of data derived from animal studies, small observational studies and case reports (7,8). It is known that many risk factors, such as duration and severity of DKA before treatment, intravenous mannitol or hypertonic saline or overhydration, are involved in the development of cerebral edema (9,10,11). Most pediatric patients with DKA suffer an acute neurologic decompensation several hours after the start of treatment, indicating a potential relationship with treatment strategies. The identification of an early marker that can be easily measured in the blood and either precedes or coincides with the clinical decompensation of diabetic children would be of value as an indicator of adjustment of management strategy to minimize neurologic injury (12,13). Recent studies have shown that the estimation of neuronal derived proteins in biologic fluids [serum and cerebrospinal fluid (CSF)] can be used for the evaluation neurologic injury. Hamed et al (14) investigated the utility of the proteins, neuron-specific enolase (NSE), myelin basic protein, S100 calcium-binding protein B (S100B) and glial fibrillary acidic protein (GFAP) in their study. These brain injury biomarkers have been studied in traumatic brain injury, but studies assessing the value of these biomarkers in pediatric DKA are very limited (15,16,17).

In this present study, the aim was to investigate serum concentrations of NSE and S100B and their relationship to different clinical, radiological and laboratory variables in children with DKA and in children with T1DM without DKA and compare these to non-diabetic healthy age-matched controls.

Methods

This is a cross-sectional case-control study. The patients were recruited from the pediatric outpatient clinic and pediatric intensive care unit of Al Hada and Taif military hospitals, Saudi Arabia. The study included 40 apparently healthy children of matched age and sex who visited the general pediatric outpatient clinic for purposes of immunization and/or routine health monitoring as controls.

Diagnosis of DKA was based on hyperglycemia (>200 mg/dL equivalent to 11.1 mmol/L) and metabolic acidosis (serum pH <7.3, bicarbonate <15 mEq/L equivalent to 15 mmol/L), with evidence of increased ketoacidosis in blood (measurable serum or urine ketones, increased anion gap) (18). The diagnosis of diabetes was confirmed according to the World Health Organization diagnostic criteria (19). Children were subdivided into two groups. Group 1 included children with newly diagnosed T1DM with DKA as a first presentation and group 2 included children with a known diagnosis of T1DM and who developed DKA as a complication. Cerebral edema was diagnosed clinically when patients developed sudden changes in their mental/clinical state, such as a severe headache, recurrent vomiting, seizures, hypertension, inappropriate slowing of the heart rate and/or signs of increased intracranial pressure. Subclinical cerebral edema was defined as minor changes in mental status, with or without being given mannitol therapy, but not developing into overt cerebral edema (3).

Exclusion criteria were:

- Children less than one year of age.

- A pre-existing medical condition other than T1DM, such as a neurologic or neurodevelopmental abnormality documented by brain computed tomography or magnetic resonance imaging (MRI).

- History of recent head trauma.

- Other known complications of type T1DM (e.g., neuropathy, retinopathy and/or nephropathy).

- Hypoglycemic attacks.

- Administration of insulin or intravenous fluids before enrollment.

- T1DM with a hyperosmolar hyperglycemic state.

The CONSORT flow diagram of DKA patients is shown in Figure 1.

The study was conducted during the time period from July 2015 to March 2018. Informed consent was taken from all participants in the study. A Local Ethical Committee in Al Hada and Taif military hospitals approved the study (approval number: 53131370). The study protocol conforms to the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments.

All patients were subjected to:

- Complete history taking which included age, gender, age of onset, duration of illness, dose of insulin, insulin regimen, compliance with treatment and history of any episodes of DKA.

- A thorough clinical examination including mental state, assessment of conscious level using Glasgow Coma Scale (GCS) for age and assessment of cranial nerve function (20).

- Laboratory investigations included complete blood count, random blood sugar, serum electrolytes (sodium, potassium, calcium), blood urea, serum creatinine, hemoglobin A1c (HbA1c), osmolality, analysis of arterial blood gases (pH, PO_2, PCO_2, and HCO_3) and analysis of urine for detection of ketone bodies.

DKA ranges from mild to severe and will influence the treatment and disposition of the patient. DKA classification in this study was based on two variables; pH and HCO_3. Pediatric DKA may be classified as severe (arterial pH < 7.1 and HCO_3 < 5), moderate (pH < 7.2 and HCO_3 < 10) or mild DKA (pH < 7.3 and HCO_3 < 15) (21). According to the GCS score, patients with DKA were divided into (1) patients with GCS score = 15 and (2) patients with GCS score < 15. All patients with DKA were treated according to the standard guidelines (22). None of our patients received any sedation.

Children with T1DM without DKA were divided into two groups based on metabolic control: (1) patients with good control, with HbA1c values of < 7.5; and (2) Patients with poor control with HbA1c values > 9.0% (23). There were no children in this group with HbA1c values between 7.5-8.9%. In patients with DKA, a 4 mL venous blood sample

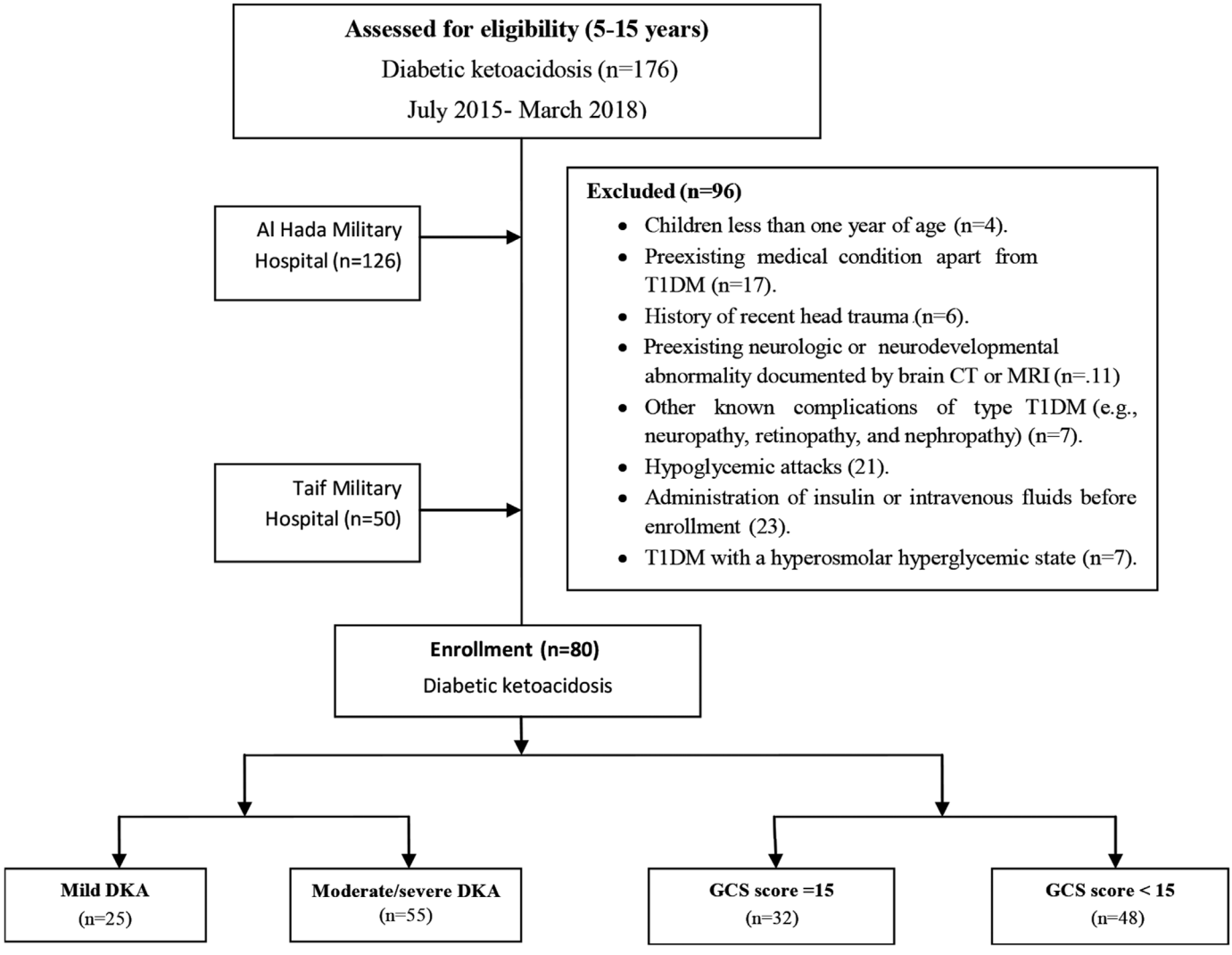

Figure 1. CONSORT flow diagram of patients with diabetic ketoacidosis

T1DM: type 1 diabetes mellitus, MRI: magnetic resonance imaging, GCS: Glasgow Coma Scale, DKA: diabetic ketoacidosis

was taken at the time of admission for the assay of NSE and S100B concentrations, before the initial saline bolus. Subsequent samples were taken at 12 hours and 24 hours after the start of treatment.

- In the healthy controls and in T1DM patients without DKA, only baseline blood samples were taken.

All blood samples were centrifuged and stored at -80 °C until the time of assay.

NSE and S100B were tested individually by enzyme-linked immunosorbent assays (ELISA) (EMD Millipore, Merck, Germany), according to the instructions of the manufacturer. For the NSE and S100B tests serum volumes of 220 µL and 60 µL were used with sensitivities of 0.19 ng/mL and 50 pg/mL, respectively. The detection limits of the assays were 0-100 ng/mL and 0.25-25 pg/mL for NSE and S100B, respectively (24).

NSE measurements are compromised by even slight hemolysis, as it is abundant in red blood cells. Therefore hemolysis was avoided as far as possible during the procedure. Established pre-analytical precautions were followed to ensure minimal hemolysis and thus greater accuracy of returned results. In those samples which were hemolyzed, a correction was applied and is described briefly hereafter. Toman et al (25) derived an equation to correct for hemolysis in samples by measuring baseline NSE in samples and then intentionally hemolyzing them and remeasuring. The individualized hemolysis correction equation is:

NSE (corrected) = NSE (measured) - [Hb (serum)] [NSE (RBCs/Hb)] + 0.0844 [Hb (serum)] + 1.1.

This was shown to correct 95% of the intentionally hemolyzed samples to within ±5 ng/mL of corresponding baseline NSE concentrations, compared to only 74% of samples using a generalized formula.

This equation was used in the study in an attempt to reduce the sample rejection rates, which approached 11% in our institution.

- Brain imaging: Brain MRI was performed in all patients with DKA, after stabilization and hemodynamic stability, to demonstrate any brain injury.

Statistical Analysis

Data analysis was carried out using the SPSS software package, version 10.0 (IBM Inc., Chicago, IL, USA). Homogeneity of the data was assessed with the Kolmogorov-Smirnov test. All data sets were normally distributed. Therefore data were presented as means ± standard deviation. ANOVA test was used to test the significance of means. The correlation between different variables was assessed using Pearson correlation test. The determination of risk factors that were significantly associated with increased levels of NSE was performed using multiple logistic regression analysis. The odds ratios and significance at 95% confidence intervals were calculated.

The relationship of NSE with GCS, pH and HCO_3 was re-evaluated by partial correlation after controlling for age, duration of diabetes and metabolic control (well controlled/poor controlled) using Spearman's rho test. $P < 0.05$ was considered significant.

Results

A total of 176 patients with DKA were admitted to the pediatric endocrinology clinic during the study period. Of these patients, 96 were excluded for a number of reasons (see Figure 1). The study was thus conducted with 80 children with DKA and 40 children with T1DM without DKA. The mean age of children with DKA was 10.4 ± 3.6 years and 68.75% of patients were males. The 40 children with T1DM without DKA were 26 (65%) males. Their ages ranged from 4 to15 years with a mean age of 10.7 ± 3.2 years. Demographic and clinical characteristics of patients are shown in (Table 1).

The clinical manifestations of DKA among our patients were rapid acidotic breathing, acetone breath, repeated vomiting, polyuria, polydipsia, enuresis and acute abdomen. Decreased level of consciousness was reported in 60% of children with DKA at the time of admission, indicated by a GCS score < 15, suggesting the presence of cerebral edema. Blood pH and serum concentrations of bicarbonate, corrected sodium, urea nitrogen and creatinine were all consistent with the diagnosis of DKA. Mild and moderate DKA was present in 31.25% while severe DKA was reported in 37.5% (Table 1). The proportion of the samples with evidence of hemolysis was 11%, but none exceeded 2%.

Children with DKA showed significantly higher serum concentrations of NSE at the three time points: admission (13.9 ± 2.8 ng/mL); 12 hours (27.8 ± 2.3 ng/mL); and 24 hours (36.7 ± 5.6 ng/mL) after starting treatment compared to children with T1DM without DKA (10.2 ± 2.2 ng/mL, $p < 0.01$) and to healthy controls (5.17 ± 1.5 ng/mL, $p < 0.01$). When NSE concentrations in children with T1DM without DKA were compared with those of healthy controls there were significantly higher concentrations in the T1DM patients (10.2 ± 2.2 ng/mL versus 5.17 ± 1.5 ng/mL, $p < 0.01$, respectively).

No significant difference were found between the studied groups in terms of S100B concentrations ($p > 0.05$). Neither did the serum S100B change significantly at the three time points measured in the DKA group: at admission (53.2 ± 6.7 pg/mL); at 12 hours (52.4 ± 7.2 pg/mL); and 24 hours (50.6 ± 7.7 pg/mL) after starting treatment ($p > 0.05$) (Table 2).

Patients with low GCS score had significantly higher concentrations of NSE at admission (16.7 ± 7.4 ng/mL), 12 hours (30.9 ± 4.8 ng/mL) and 24 hours (22.7 ± 7.1 ng/mL) compared to patients with normal GCS score which were 6.42 ± 2.9 ng/mL, 5.18 ± 2.5 ng/mL and 7.17 ± 0.6 ng/mL at the same time points respectively ($p < 0.01$) and in comparison with T1DM patients without DKA whose mean NSE concentration was 10.2 ± 2.2 ng/mL ($p < 0.01$; see Table 3).

Patients with duration of T1DM ≥5 years had significantly higher mean concentrations of NSE than those with shorter duration, both for patients with DKA (11.17 ± 3.2 ng/mL versus 7.96 ± 2.7 ng/mL, respectively; $p = 0.038$) and for

Table 1. Clinical, demographic and laboratory data of the studied patients

	Children with DKA (n = 80)	Children with T1DM without DKA (n = 40)	p
Age range	5-15	4-15	
Mean ± SD	10.4 ± 3.6	10.7 ± 3.2	0.32
Sex			
Male, n (%)	55 (68.75)	26 (65%)	0.36
Female, n (%)	25 (31.25)	14 (35%)	0.48
DKA with newly diagnosed T1DM, n (%)	60 (75)	-	
DKA with established diagnosis of T1DM, n (%)	20 (25)	-	
Duration of illness, [a]years	5.7 ± 2.4	4.8 ± 2.9	0.23
Dose of insulin , [a]IU	0.92 ± 0.28	0.88 ± 0.31	0.07
[a]Compliance with therapy			
Good	32 (40)	23 (57.5)	
Poor	48 (60)	17 (42.5)	
Random blood sugar, [a]mg/dL	428.40 ± 136.80	202.60 ± 58.7	0.04
HbA1c (%)	9.3 (2.5)	6.4 (2.3)	0.02
History of episodes of DKA[a], n (%)	11 (44)	-	0.02
Patients according to duration of illness			
< 5 years	26 (32.5)	11 (27.5)	
≥5 years	54 (67.5)	29 (72.5)	
GCS score at time of admission, n (%)		-	
GCS score = 15	32 (40)		
GCS score < 15	48 (60)		
Blood urea nitrogen, mg/dL	22.5 ± 17.5	-	
Serum creatinine, mg/dL	1.04 ± 0.5		
Corrected serum sodium, mEq/L	142.3 ± 7.4		
Serum osmolarity, mmol/L	312.8 ± 30.2		
pH	7.06 ± 0.12	-	
Bicarbonate, mEq/L	8.3 ± 2.8		
Patients according to metabolic control			
Good control		18 (45)	
Poor control		22 (55)	
Patients according to the severity of DKA, n (%)		-	
Mild	25 (31.25)		
Moderate	25 (31.25)		
Severe	30 (37.5)		

DKA: diabetic ketoacidosis, T1DM: type 1 diabetes mellitus, GCS: Glasgow Coma Scale, SD: standard deviation, NSE: neuron-specific enolase

[a]Results for children with established diagnosis T1DM

Table 2. Neuron-specific enolase and S100 calcium-binding protein B at 3-time points among studied groups

	Children with DKA (n = 80)	Children with T1DM without DKA (n = 40)	Controls	p value
NSE (ng/mL)				
Baseline	13.9 ± 2.8	10.2 ± 2.2	5.17 ± 1.5	0.003
p value	***0.0001**	0.001†		
12 h after start of treatment	27.8 ± 2.3			0.0001
p value	***0.0001**			
24 h after start of treatment	36.7 ± 5.6			0.0001
p value	***0.0001**			
S100B (pg/mL)				
Baseline	53.2 ± 6.7	51.6 ± 6.8	48.9 ± 7.3	0.638
p value	*0.542	0.648†		
12 h after start of treatment	52.4 ± 7.2			0.275
p value	*0.842			
24 h after start of treatment	50.6 ± 7.7			0.374
p value	*0.482			

DKA: diabetic ketoacidosis, T1DM: type 1 diabetes mellitus, NSE: neuron-specific enolase, S100B: S100 calcium-binding protein B

*p value: patients with DKA *vs* controls

†value: patients with T1DM without DKA *vs* controls

Table 3. Serum levels of neuron-specific enolase (ng/mL) by Glasgow Coma Scale, duration of illness and metabolic control

	Children with DKA			Children with T1DM without DKA	Controls
	At admission	At 12 hour after start of treatment	At 24 hour after start of treatment		
GCS score < 15	16.7 ± 7.4	30.9 ± 4.8	22.7 ± 7.1	10.2 ± 2.2	5.25 ± 1.2
P1 value	0.0001	0.0001	0.0001		
P2 value	0.001	-	0.361		
P3 value	0.001	0.361	-		
P4 value	0.0001	0.0001	0.0001		
P value				0.001	
GCS score = 15	6.42 ± 2.9	5.18 ± 2.5	7.17 ± 0.6		
P1 value	0.216	0.223	0.035		
P2 value	0.038	-	0.028		
P3 value	0.058	0.026	-		
P4 value	0.412	0.436	0.026		
P5 value	0.0001	0.0001	0.0001		
Patients by duration of illness					
< 5 years	7.96 ± 2.7			6.23 ± 2.3	
≥5 years	11.17 ± 3.2			10.88 ± 3.2	
***p value**	0.038			0.042	
Patients by metabolic control					
Good control				6.37 ± 2.4	
Poor control				12.36 ± 3.3	
****p value**				0.032	

NSE: neuron-specific enolase, T1DM: type 1 diabetes mellitus, DKA: diabetic ketoacidosis, GCS: Glasgow Coma Scale, P1 *vs* controls, P2 *vs* 12 h after starting treatment; P3, *vs* 24 h after starting treatment; P4, *vs* diabetic children without DKA, P5: levels with low GCS scores *vs* those with normal GCS scores, P: diabetic children without DKA *vs* controls

*p: DKA and diabetic children without DKA with duration of illness ≥5 year *vs* those with duration of illness < 5 year

**p: diabetic children without DKA with poor metabolic control *vs* those with good metabolic control

patients without DKA (10.88 ± 3.2 ng/mL versus 6.23 ± 2.3 ng/mL, respectively; p = 0.042).

When metabolic control was investigated it was found that serum concentrations of NSE were significantly higher among diabetic children with poor metabolic control without DKA than in those with good control (12.36 ± 3.3 ng/mL versus 6.37 ± 2.4 ng/mL; p = 0.032).

Patients with severe DKA had significantly higher mean concentrations of NSE at the each time point compared to patients with moderate DKA (19.6 ± 8.4 ng/mL versus 17.3 ± 7.8 ng/mL; 37.7 ± 5.3 ng/mL versus 33.7 ± 5.3 ng/mL and 28.3 ± 9.3 ng/mL versus 25.3 ± 7.2 ng/mL at admission, 12 and 24 hours, respectively), and compared to patients with mild DKA (19.6 ± 8.4 ng/mL versus 9.12 ± 3.2 ng/mL, 37.7 ± 5.3 ng/mL versus 6.22 ± 2.9 ng/mL and 28.3 ± 9.3 ng/mL versus 9.13 ± 0.9 ng/mL, respectively; p < 0.01). The mean NSE concentration was not significantly different from the control group at the 12th hour in the mild DKA group (6.22 ± 2.9 ng/mL versus 5.25 ± 1.2 ng/mL, p > 0.05; see Table 4).

There were no differences in mean concentration of S100B between patients with low GCS scores compared with those with normal GCS scores at any of the time points examined (see Table 5). Neither were there differences between the low GCS score group and T1DM patients without DKA at any time point (Table 5). The serum concentration of S100B did not differ between the patients with T1DM with and without DKA when compared for duration of illness (Table 5).

When patients were compared by severity of DKA no significant differences were found in mean concentrations of S100B at any of the time points examined (see Table 6). No significant correlation was found between mean concentration of S100B and any of the patient laboratory or demographic data, regardless of the time point examined.

Serum concentrations of NSE at 24 hours after starting treatment for DKA showed significant negative correlation with age (p = 0.0001), GCS score (p = 0.0001), pH (p = 0.02), and bicarbonate concentration (p = 0.04) (see Table 7). However, there was significant positive correlation between mean NSE concentration at 24 hours after starting treatment for DKA and baseline NSE concentration (p = 0.0001), duration of illness (p = 0.03), random blood sugar concentration (p = 0.0001) and HbA1c (p = 0.001) (Table 7).

Multiple regression analysis was used to assess the relationship between mean concentration of NSE at 24 hours after starting treatment and a range of risk factors.

Table 4. Serum levels of neuron-specific enolase (ng/mL) of the subjects by severity of diabetic ketoacidosis

	Children with DKA (ng/mL)			Children with T1DM without DKA ng/mL	Controls ng/mL
	At admission	At 12 hours after start of treatment	At 24 hours after start of treatment		
Severe DKA	19.6 ± 8.4	37.7 ± 5.3	28.3 ± 9.2	10.2 ± 2.2	5.25 ± 1.2
P1 value	0.0001	0.0001	0.0001		
P2 value	0.001	-	0.361		
P3 value	0.001	0.361	-		
P4 value	0.0001	0.0001	0.0001		
p value				0.001	
Moderate DKA	17.3 ± 7.8	33.7 ± 5.3	25.3 ± 7.2	10.2 ± 2.2	5.25 ± 1.2
P1 value	0.0001	0.0001	0.001		
P2 value	0.001	-	0.361		
P3 value	0.001	0.361	-		
P4 value	0.0001	0.0001	0.0001		
p value				0.001	
Mild DKA	9.12 ± 3.2	6.22 ± 2.9	9.13 ± 0.9	10.2 ± 2.2	5.25 ± 1.2
P1 value	0.006	0.276	0.007		
P2 value	0.010	-	0.010		
P3 value	0.073	0.010	-		
P4 value	0.412	0.076	0.246		
P5 value	0.0001	0.0001	0.0001		

NSE: neuron-specific enolase, T1DM: type 1 diabetes mellitus, DKA: diabetic ketoacidosis, GCS: Glasgow Coma Scale, P1, *vs* controls, P2, *vs* 12 h after starting treatment, P3, *vs* 24 h after starting treatment, P4, *vs* diabetic children without DKA, P5, levels for those with moderate/severe DKA *vs* those with mild DKA, P, diabetic children without DKA *vs* controls

Table 5. Serum levels of S100 calcium-binding protein B (pg/mL) of the subjects by Glasgow Coma Scale and duration of illness

Patients with DKA by GCS score	Children with DKA			Children with T1DM without DKA	Controls
	At admission	At 12 hours after start of treatment	At 24 hours after start of treatment		
GCS score < 15	53.7 ± 5.4	44.9 ± 4.9	42.7 ± 8.4	51.6 ± 6.8	48.9 ± 7.3
P1 value	0.435	0.274	0.245		
P2 value	0.275	-	0.347		
P3 value	0.712	0.136	-		
P4 value	0.745	0.312	0.318		
P value				0.347	
GCS score = 15	51.2 ± 4.6	45.6 ± 4.5	47.8 ± 5.6	51.6 ± 6.8	48.9 ± 7.3
P1 value	0.318	0.534	0.095		
P2 value	0.227	-	0.124		
P3 value	0.164	0.415	-		
P4 value	0.234	0.346	0.527		
P5 value	0.123	0.112	0.078		
GCS values by duration of illness					
< 5 years	74.56 ± 3.8			73.85 ± 4.1	
≥5 years	73.19 ± 4.2			73.34 ± 3.3	
***p value**	0.673			0.436	

S100B: S100 calcium-binding protein B, T1DM: type 1 diabetes mellitus, DKA: diabetic ketoacidosis, GCS: Glasgow Coma Scale, P1, *vs* controls, P2, *vs* 12 h after starting treatment, P3, *vs* 24 h after starting treatment, P4, *vs* diabetic children without DKA, P5, levels with low GCS scores *vs* those with normal GCS scores, P, diabetic children without DKA *vs* controls.

*p: DKA and diabetic children without DKA with duration of illness ≥5 years *vs* those with duration of illness < 5 years

Table 6. Serum levels of S100 calcium-binding protein B (pg/mL) by severity of diabetic ketoacidosis

S100B levels	Children with DKA			Children with T1DM without DKA	Controls
	At admission	At 12 hours after start of treatment	At 24 hours after start of treatment		
Severe DKA	50.9 ± 7.4	48.2 ± 6.1	46.3 ± 6.5	51.6 ± 6.8	48.9 ± 7.3
P1 value	0.163	0.242	0.073		
P2 value	0.094	-	0.421		
P3 value	0.312	0.311	-		
P4 value	0.524	0.187	0.217		
p value				0.634	
Moderate DKA	50.1 ± 6.2	46.2 ± 6.1	43.3 ± 6.3	51.6 ± 6.8	48.9 ± 7.3
P1 value	0.163	0.213	0.083		
P2 value	0.064	-	0.421		
P3 value	0.314	0.312	-		
P4 value	0.528	0.147	0.317		
p value				0.512	
Mild DKA	47.1 ± 3.7	46.2 ± 4.9	46.1 ± 5.2	51.6 ± 6.8	48.9 ± 7.3
P1 value	0.412	0.254	0.079		
P2 value	0.247	-	0.731		
P3 value	0.156	0.311	-		
P4 value	0.567	0.677	0.185		
P5 value	0.981	0.541	0.541		

S100B: S100 calcium-binding protein B, T1DM: type 1 diabetes mellitus, DKA: diabetic ketoacidosis, GCS: Glasgow Coma Scale, P1, *vs* controls, P2, *vs* 12 h after starting treatment, P3, *vs* 24 h after starting treatment, P4, *vs* diabetic children without DKA, P5, levels for those with moderate/severe DKA *vs* those with mild DKA.

p: diabetic children without DKA *vs* controls

Significant association was found with age (p = 0.001), GCS score (p = 0.007), random blood sugar concentration (p = 0.008), HbA1c (p = 0.03), blood pH (p = 0.04) and blood bicarbonate (p = 0.003) (see Table 8).

The serum NSE concentration in DKA was further assessed, controlling for covariables that may potentially influence the NSE level, which included age, duration of diabetes and metabolic control status. After adjustment, the serum NSE concentration was still independently associated with GCS, pH and bicarbonate.

MRI of the brain showed no significant abnormalities in any of our patients.

Table 7. Correlations between the serum levels of neuron-specific enolase and clinical, demographic and laboratory variables among diabetic children with diabetic ketoacidosis

Variable	Serum NSE levels at 24 hour time point after starting treatment	
	r	p
Serum levels of NSE at admission	0.762	0.0001
Age	-0.721	0.0001
Duration of illness	0.436	0.03
Random blood sugar	0.813	0.0001
HbA1c	0.782	0.001
GCS score	-0.717	0.0001
pH	-0.426	0.02
Bicarbonate	-0.296	0.04

NSE: neuron-specific enolase, GCS: Glasgow Coma Scale, HbA1c: hemoglobin A1c

Table 8. Multiple regression analysis of risk factors associated with increased serum levels of neuron-specific enolase in diabetic children with diabetic ketoacidosis

Variable	Adjusted odds ratio* (95% confidence interval)	p value
Age	1.7 (2.13-3.86)	0.001
GCS score	2.62 (2.98-4.64)	0.007
Random blood sugar	1.5 (2.11-2.26)	0.008
HbA1c (%)	1.19 (1.06-1.27)	0.03
pH	1.13 (1.89-2.38)	0.04
Bicarbonate	3.42 (3.96-6.87)	0.003

NSE: neuron-specific enolase, HbA1c: hemoglobin A1c, GCS: Glasgow Coma Scale

*Odds ratios means the adjusted odds for the following changes in variables: increase in age by 1 year, decrease in GCS score by 1, increase in random blood sugar by 100 mg/dL, decrease in HbA1c by 1%, decrease in pH by 0.1 and increase in bicarbonate concentration by 1 mEq/L

Discussion

Serum concentrations of NSE and S100B, two markers of neuronal damage, were measured, immediately before and over the course of the first 24 hours of treatment for DKA in pediatric T1DM patients in this study. In addition the relationship between the severity of DKA and markers of brain injury were investigated.

NSE, a soluble protein of 45 kDa, is a glycolytic enzyme present almost exclusively in neurons and neuroendocrine cells, although it is also found in platelets and erythrocytes. Erythrocyte-derived NSE is important when using NSE as a clinical marker of neuronal injury as mild hemolysis of only 2% may increase serum concentrations of NSE five-fold (26). In our subjects the proportion of samples with hemolysis was 11%, but none exceeded the 2% limit. In addition, the correction factor of Toman et al (25) was applied in these samples in order to correct, as accurately as possible, the final NSE serum concentrations prior to comparative analysis.

When neuronal membranes are injured, NSE and S100B will diffuse to the extracellular fluid compartment and to the CSF. Therefore, estimation of these markers in CSF may be a clinically attractive method of assessment, as it may be more sensitive than serum measurement and will largely negate the complication of erythrocyte derived NSE. However, there are additional clinical risks and ethical issues which hinder performing lumbar puncture and CSF collection. Measurement of serum NSE and S100B has been used as evidence of alterations in the blood-brain-barrier (BBB) in certain instances of DKA. Hence, interpretation of results using only serum levels was possible (27).

In the recent literature, there are only three studies which have evaluated brain injury markers in children with DKA. Hamed et al (17) compared serum NSE levels among DKA patients without documented cerebral edema with normal and abnormal GCS scores (GCS < 15 and GCS = 15) and healthy controls. The results showed that DKA patients with GCS < 15 had significantly higher serum NSE concentrations than both the DKA patients with GCS = 15 and healthy controls, while the difference between the DKA group with GCS = 15 and healthy controls was not significant. These results showed that serum NSE was elevated in DKA and also that it correlated with hyperglycemia, ketosis and acidosis (17). Interestingly children with T1DM without DKA also had significantly higher serum concentrations of NSE compared to healthy controls. The mean NSE concentration did not differ significantly in the mild DKA group at 12 hours after starting treatment when compared to the control group. One explanation for this would be that the severity of

acidosis was responsible for the significant increase in NSE.

We did not observe a significant elevation in the level of S100B in the DKA group. This finding can be attributed to a mis-match between the methodology of ELISA kit and the biological characteristics of S100B (28). Unfortunately, ELISA assays take 4-6 hours to run and generally present high inter- and intra-coefficients of variation resulting in a worse functional sensitivity of the assay (29). Coupled with the half-life of S100B being in the range of 60 to 120 minutes in patients with brain injury, the measured net amount of S100B in serum samples will inevitably be less than the original concentration due to the rate of degradation when using ELISA methodology (30).

Çatlı et al (31) (2018) studied NSE, S100B and GFAP levels in 29 patients with DKA, 30 with T1DM and 35 healthy children. They found S100B was significantly higher in the DKA group than the healthy control and T1DM groups, while GFAP and NSE levels were not different from controls and T1DM patients. No significant differences were found in GFAP, NSE and S100B levels according to the severity of DKA, diabetes duration and GCS.

Kaya et al (16) (2015) investigated the pre-treatment and post-treatment oxidant capacity, antioxidant capacity and S100B levels in cases of DKA. They hypothesised that long-term exposure to high blood glucose concentrations leads to an increase in the oxidative stress in patients with DKA that led to an increase in S100B concentration, which implies neuronal damage.

In our study significantly increased serum concentrations of NSE were found in diabetic patients without DKA and without detectable CNS disorders, neuropathies, and retinopathy. In addition, serum concentrations of NSE were significantly higher in diabetic children without DKA but with poor metabolic control than those who showed good control. Hyperglycemia-induced pericyte loss and oxidative stress contribute to BBB disruption (32). These neuroanatomical changes observed in experimental models of diabetes may accurately reflect what is occurring in the clinical setting (33). It was reported that cognitive dysfunction in T2DM appears to be due to permanent brain damage with significant elevation in NSE level and correlated with the level of glycemic control (34).

Gonder-Frederick et al (35) (2009) reported a disturbance in the cognitive functions of school-aged children with T1DM due to repeated attacks of hyperglycemia. A previous study, conducted by Antenor-Dorsey et al (36), observed changes in brain imaging in the form of increased diffusiveness in the superior parietal lobule and hippocampus attributed to repeated attacks of hyperglycemia, associated with ketosis with or without academia. Experimental and human studies have indicated that chronic hyperglycemia associated with DM resulted in a brain injury which particularly affected memory and learning abilities. The mechanisms underlying brain injury in experimental models include: disruption of BBB; alteration of insulin transporter and decrease in insulin receptors, which are expressed in discrete neuronal populations in the CNS; reduction in the uptake of glucose into the neurons; impairment of energy metabolism; and impairment of the capacity of the brain to generate the connections vital to memory and learning (37). Other investigators reported raised concentrations of NSE in diabetic patients with and without overt neurologic complications (38). It is well-known that T1DM has long-term complications affecting cognitive functions (39). An understanding of the nature and onset of the neurological insults associated with diabetic children is essential to prevent or mitigate these complications.

Among diabetic patients, high blood glucose has been associated with elevated concentrations of serum NSE. In addition to central nervous system disorders, hyperglycemia-induced pericyte loss contributes to disruption of BBB (32).

Another important finding of our study was the elevation in serum concentrations of NSE during DKA and its correction after starting treatment. Our findings support the hypothesis that during the critical time-period where acute complications of DKA have been reported, the levels of NSE remain high (17). In our study, we reported significantly higher serum concentrations of NSE in patients with GCS score < 15 compared to patients with normal GCS score at each time point. We also found significantly higher serum concentrations of NSE in patients with moderate to severe DKA compared to patients with mild DKA at the same three time points. It was notable that the concentrations of NSE remained high and were continuing to rise at 24 hours post start of treatment, coinciding with the initial recovery of clinical manifestations of DKA. The persistence of higher concentrations of NSE after improvement of the manifestations of DKA suggests that neuronal injury may recover partially but not completely. Alternatively, the healing process in neurological tissues is know to be relatively slow and so persistent NSE elevation after the first 24 hours of DKA might be expected. It was notable that T1DM children without overt DKA also had significantly higher concentrations of NSE ($p < 0.01$) than non-diabetic children, which also supports the hypothesis that neuronal injury in T1DM may be permanent. An alternative hypothesis would be that neuronal injury may begin early at the cellular level in the context of T1DM without DKA and may be associated with cognitive impairment. Repeated episodes

of DKA may carry the risk of progressive neuronal injury. Also, we observed some patients with normal GCS score had a significant elevation in the serum concentrations of NSE after improvement of their condition. This suggests neuronal injury may occur in the absence of brain edema in children with DKA. Our data were consistent with previous studies that reported evidence of neuronal injury without brain edema. Wootton-Gorges et al (40) reported a progressive decrease in N-acetyl aspartate/creatine ratio as evidence of permanent brain injury in a teenager with T1DM and repeated episodes of DKA without clinically apparent cerebral edema.

S100B is a relatively small protein, 9-14 kDa, synthesized largely by glial cells although a small proportion is synthesized by neurons, Schwann cells and in non-neuronal peripheral sources, including cardiomyocytes, alveolar cells, chondrocytes and adipocytes (41).

In our study, S100B protein did not show significant differences between the DKA, T1DM, and healthy control groups although this may have been due to methodological problems, as described earlier.

In research studies, statistical power is generally calculated with two main objectives. Firstly, it can be calculated before data collection to decide the sample size needed for the current study based on information from previous studies. Secondly, it can also be calculated after data analysis. When the result turns out to be non-significant, statistical power can be calculated to verify whether the non-significance result is due to lack of relationship between the groups or due to the lack of statistical power (42). The power of our study was calculated for the comparison of NSE and S100B between children with DKA, children with T1DM without DKA and controls using G power software version 3.1.2. 9. We took into consideration the mean values of NSE and S100B in the studied groups and the Alpha level was kept at 0.05. The power calculated was 1.0 and 0.845 for NSE and S100B respectively. We can confirm that the non-significant result of the S100B analysis between the three groups is robust, as no difference was detected although there was sufficient power (0.845) to detect any difference, if present.

There are a few controversial studies showing that S100B can be used as a marker for cerebral edema in pediatric DKA (15,16,43). Experimental studies reported low levels of S100B in the DKA group. This finding was attributed to glial cell dysfunction and not glial cell loss and that S100B is not a reliable marker of neuronal injury (15). We did not detect any significant increase or decrease in S100B in children with DKA over a short term follow-up (12 hr-24 hr) to predict neuronal injury. We did not find any correlation between S100B concentrations and other laboratory and demographic factors at the time points studied. It was reported that S100B levels were not raised in subclinical cerebral edema in children with DKA (31). However, Kaya et al (16) found significantly higher S100B concentrations in children with DKA but without accompanying cerebral edema than controls, but did not find a significant difference in S100B concentrations before and after initiation of therapy.

In our study, we found the risk factors and early predictors of higher serum concentrations of NSE in children with DKA were younger age, lower GCS score, higher degrees of hyperglycemia, longer duration of illness and more severe acidosis and ketosis. Previous studies have shown that a range of factors are suitable predictors of higher levels of NSE during DKA, but none of these variables has been singled out as the most important determining factor (17,44,45). Regarding clinical risk factors, the degree of acidosis and younger age appeared to be the greatest risk factors for alterations in cerebral structure. However, the degree of acidosis was the most important determining factor of an impaired level of consciousness in children with DKA without cerebral edema (46). Different biomarkers reflecting inflammation including tumor necrosis factor-alpha and interleukin-6 and cerebral dysfunction and/or possible injury (S100B, GFAP), as well as genetic markers of brain injury risk in children with DKA, were studied by Nett et al (47) They demonstrated the potential importance of these markers in the pathophysiology of CNS dysfunction and/or possible injury in DKA.

Under normal conditions with an intact BBB, brain-derived proteins of different molecular weights (such as S100B and NSE) do not cross the BBB (24,46,47). With the disruption of the BBB, blood levels of these proteins can be used as a marker for brain injury (48). During the treatment of DKA, it was observed that the whole brain and regional BBB permeability increased in most patients (33). Although the mechanisms underlying the increase in BBB permeability is still unclear, it is suggested that DKA can disrupt the tight endothelial junctions through inflammatory and immunologic responses (49). Furthermore, many factors such as matrix metalloproteinase activity, hyperglycemia and insulin administration are associated with increased permeability of the BBB (33).

Study Limitations

We did not measure NSE in the CSF of our patients for as our scientific committee did not approve CSF sampling for the study although CSF measurements would likely be more sensitive to CNS damage. We did not repeat MRI to

detect subclinical cerebral edema at the time of diagnosis and during clinical follow-up. Also, neurocognitive function, which is a good marker of brain dysfunction, was not assessed. This is explained by the fact that our study focused on investigating serum concentrations of NSE and S100B in children with DKA and its relationship with different clinical and laboratory variables. The study only examined serum concentrations of NSE and S100B over a period of 24 hours. Longer follow-up would have clarified the progression of serum NSE concentrations as they returned to normal. Lastly, we did not undertake a sample size calculation before conducting our study.

Conclusions

Serum NSE was found at a significantly higher concentration in T1DM children, with or without DKA, than non-diabetic children. This might suggest a degree of neurologic dysfunction, even in the absence of DKA. In our study, cerebral edema was absent in brain imaging in children with DKA. Elevated NSE concentrations in patients with abnormal GCS and the positive correlation between NSE and severity of acidosis suggest that NSE might be a reliable marker of neuronal injury. However, S100B did not show a simultaneous increase with NSE. This can be attributed to a methodological error with the ELISA kit. To clarify subclinical brain injury related to pediatric DKA, further longer term and larger studies are recommended to assess neurocognitive functions.

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Ethics

Ethics Committee Approval: The Local Ethical Committee for research study at Al Hada and Taif military hospitals, Taif, Saudi Arabia approved the study (approval number: 53131370).

Informed Consent: Taken from each participant.

Peer-review: Externally and internally peer-reviewed.

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Clinical and Biochemical Phenotype of Adolescent Males with Gynecomastia

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What is already known on this topic?

The imbalance between estrogen and androgen activity is considered to be the main cause of this gynecomastia. Nonetheless, studies that actually present data on the estrogen to testosterone (E2/TTE) ratio in gynecomastia patients are scarce.

What this study adds?

This study describes the relationship between alterations in sex hormones and the evolution of gynecomastia. Altered E2/TTE ratio might be responsible for part of cases described previously as idiopathic. This study highlights the importance of checking the E2/TTE ratio in gynecomastia patients.

Abstract

Objective: Gynecomastia is defined as a benign proliferation of male breast glandular tissue. Its prevalence during puberty varies between 50-60% and is also common in neonatal and elderly males. It develops mainly due to the disequilibrium between estrogen and androgen activity in breast tissue, where estradiol (E2) binds to estrogen receptors and stimulates ductal and glandular cells. The aim of this work was to investigate the relationship between sex hormone alterations and the natural history of gynecomastia.

Methods: Participants in this study were young males referred to an outpatient clinic, between January 2011 and February 2016, with breast enlargement. Thyroid function, liver function, hormone concentrations and tumor markers were measured and anthropometric assessment was conducted.

Results: Subjects comprised 93 males, aged 9 to 18 (mean ± standard deviation age 13.8 ± 2.6) years. In 63 of 93 (67.7%) the gynecomastia was confirmed and 28 were followed-up for a median period of three months. None of the boys showed any reduction in breast size during follow-up. There was no correlation between body mass index Z-score and breast size. Breast enlargement progressed in nine boys (32.1%). A positive correlation between estrogen to testosterone (E2/TTE) ratio and Tanner B stage ($r = 0.47$; $p = 0.034$) was observed.

Conclusion: The E2/TTE ratio may be a helpful tool in diagnosing gynecomastia. Altered E2/TTE ratio might be responsible for a proportion of cases described previously as idiopathic. Additionally, weight loss does not imply reduction of breast size in boys. Nonetheless it should be the first step in the management of prolonged gynecomastia.

Keywords: Gynecomastia, puberty, estradiol, testosterone, ratio

Introduction

Gynecomastia is defined as a benign, unilateral or bilateral proliferation of male breast glandular tissue. It is the most common breast alteration in males and has a trimodal age distribution, occurring in neonatal, pubertal, and elderly males. Gynecomastia is observed in 50% to 60% of boys during their puberty, usually bilaterally. It may be asymmetric in size (1,2). Physiologically gynecomastia has been reported to resolve within six months to two years after onset. Persistance indicates presence of a pathology which requires further evaluation (3,4,5,6).

The imbalance between estrogen and androgen activity is considered to be responsible for gynecomastia (7,8). Gynecomastia occurs in response to increased estrogen production and/or activity or because of decreased

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production and/or activity of testosterone (TTE) (9). Increased prevalence of gynecomastia raises the question of a factor which is associated with the pathophysiologic mechanism of gynecomastia.

A rapid increase in estradiol (E2), occurring before and delaying a similar increase in TTE, causes an elevated E2/TTE ratio at the onset of puberty. E2 binds to estrogen receptors in the breast tissue and stimulates ductal and glandular cell proliferation, leading to gynecomastia. Opposing this effect, TTE exerts a generalized inhibitory action on growth and differentiation, perhaps through a specific anti-estrogenic action (10).

Aromatase, which converts androstenedione and TTE to estrone and E2, respectively, is the most important factor in establishing equilibrium (11). Overexpression and increased activity of aromatase is a key factor in development of gynecomastia. This upregulation contributes to excessive local production of estrogen, decreased estrogen degradation and changes in the levels or activity of estrogen or androgen receptors (12,13).

Gynecomastia is strongly associated with obesity (14,15). Aromatization takes place in the adipose tissue and it is the main source of E2 in men. Subsequently, higher production and activity of aromatase are the key factors leading to gynecomastia in obese men (6,16). Furthermore, the increased body weight contributes to breast tissue proliferation by increased leptin level (12). Due to an increasing prevalence of adolescent obesity, it is essential to identify patients with gynecomastia among all boys presenting with breast enlargement.

Testicular tumors, as well as adrenal tumors, may secrete estrogen, causing disruption in the E2/TTE ratio (17,18). In addition, all forms of male hypogonadism lead to TTE deficiency, disrupting sex hormone homeostasis (4).

In addition, puberty is the period of the fastest linear growth in children, as remonstrated by peak height velocity (PHV) and at that time, insulin-like growth factor-1 (IGF-1) and growth hormone (GH) reach maximum levels. Both GH and IGF-1 are responsible for linear growth and also stimulate breast tissue proliferation through their respective receptors located in breast tissue (19). Occurrence of PHV and gynecomastia in a similar period of a young boy's life may suggest that there is a relationship between them (20,21).

There are also other causes of gynecomastia that should not be disregarded, such as drug-induced gynecomastia, systemic illness and familial disorders. Common drug contributors include antipsychotics, antiretrovirals and prostate cancer therapies with long-term use (22,23). Gynecomastia is also a common sign of chronic liver disease and human immunodeficiency virus infection (24,25).

In some cases, the etiology remains uncertain and a pathomechanism responsible for gynecomastia cannot always easily be determined. A medical history of all young boys who present with gynecomastia should be carefully reviewed and each patient should be subjected to a thorough physical examination.

Methods

All study subjects presented to the Upper Silesian Child Health Centre in Katowice. 93 male patients, ages ranged from 9 to 18 years with a mean ± standard deviation (SD) age of 13.8 ± 2.6 years at presentation, were referred to our endocrine outpatient clinic because of breast enlargement and were examined between January 2011 and February 2016. Of these, 11 were excluded due to steatomastia and 19 due to a reduction in breast size at the time of consultation. Sixty-three boys were diagnosed with gynecomastia and enrolled in the study group. Follow-up visits every 3-6 months were planned. Of these 63 subjects, two had a family history of gynecomastia and three had delayed puberty. There were also four cases of hyperprolactinemia and one patient had an additionally history of galactorrhoea concurrent with a normal prolactin (PRL) level. Thus the pathological gynecomastia group consisted of 11 patients and were compared to the rest, described as pubertal gynecomastia (n = 52). None of the patients had a history of primary hypogonadism, drug-induced gynecomastia, a human chorionic gonadotropin (hCG)-secreting tumor or elevated aminotransferase concentrations.

The patients were divided into two groups with respect to Tanner breast development criteria. Thus, 42 of the 63 boys (66.7%) were classified as Tanner stage 2 (B2) of breast development and 21 boys (33.3%) as > B2.

Clinical Phenotype

Anthropometric measurements included weight and height measurements. Body mass index (BMI) was calculated using the standard formula of weight (kg) divided by height (m) squared. Weight was measured using a scale with a precision of 100 g. Height was measured by a stadiometer sensitive to 0.1 cm. Height SD score (hSDS) was calculated from population standards for healthy children using the following formula: hSDS = child's height-height for 50 pc/0.5 * (height 50 pc-height 3 pc). Short stature was defined as hSDS below -2.0 SD.

Given a child's age, sex, BMI, and the appropriate reference standard, the BMI Z-score was calculated using The Pediatric

Z-score Calculator. The tool is available at the website of The Children's Hospital of Philadelphia, Research Institute (http://stokes.chop.edu/web/zscore/) and can be used for subjects aged between two and 20 years. A BMI Z-score over +2.0 SD was classified as obesity, between +2.0 and +1.0 SD as overweight, between -1.0 and -2.0 as underweight and under -2.0 SD as significant weight deficiency (26). The boys' sexual maturity stages were assessed using the Tanner scale (27).

Biochemical Phenotype

E2, TTE, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and PRL blood concentrations were measured using a chemiluminescent immunoassay by Immulite 2000 kit (Diagnostic Products Corp., Los Angeles, CA, USA). Serum free thyroxine (fT4) and thyroid stimulating hormone (TSH) concentrations were measured with a chemiluminescent immunometric assay (Immulite 2000 Free T4 Siemens Healthcare Diagnostics, Immulite 2000 Third Generation TSH, Diagnostic Products Corp., Los Angeles, CA, USA). Based on standardized E2 and TTE results (see the Statistical Analysis section below), the E2/TTE ratio was calculated. To exclude other causes of breast enlargement, such as cirrhosis and testicular tumors, alpha-fetoprotein (AFP), hCG, alanine transaminase (ALT) and aspartate transaminase (AST) were also measured, in accordance with International Federation of Clinical Chemistry (Beckman Coulter, USA).

Statistical Analysis

All statistical analyses were performed using a Statistica 13 PL software (StatSoft Inc., Tulsa, Oklahoma, USA). A p value of <0.05 was considered significant. Shapiro-Wilk test was utilized to verify the normality of E2 and TTE distribution. In order to calculate the E2/TTE ratio, raw results were compared by using Standard Score. The analysis was stratified by gynecomastia status. The comparisons between two parametric values were made by using Student's T-test or Mann-Whitney U test for non-parametric distributions. The correlation between quantitative values was analyzed by using Pearson's correlation and Spearman's rank correlation coefficient for ordinal variables. All results were reported as mean ± SD.

Ethics

All procedures in our study were performed in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration. Due to its retrospective design and non-experimental nature, a formal consent or a formal approval by a bioethics committee were not required.

Results

Clinical Results

The mean ± SD age of the patients was 13.8 ± 2.6 years at the time of the first consultation. At the initial assessment, seven (11.1%) boys were obese, 24 (38.1%) were overweight, 29 (46.0%) had a normal weight and three (4.8%) boys had weight deficiency. Mean ± SD BMI was 22.9 ± 4.3, mean ± SD BMI Z-score was 0.83 ± 1.0 and the mean ± SD height SDS was 0.5 ± 1.3. Among all 63 patients, only 28 (44.4%) turned up to scheduled visits in the endocrinology outpatient clinic and the longest follow-up lasted seven visits extending to 38 months. Fourteen (50%) boys were advised to lose weight and eight (28.6%) children followed the recommendation. Two of 14 (14.3%) of them achieved normal BMI Z-score, but reduction in size of breast during the observation period was not noted. There was no correlation between BMI Z-score and breast size ($p>0.05$). Breast size at Tanner B stage of 16/28 (57.1%) boys did not change. Breast enlargement progressed in 12/28 (42.9%) of the boys.

Gynecomastia was bilateral in 46/63 (73.0%) of the subjects. The median B Tanner stage at presentation was B2 (n = 42; 67.0%). B3 stage was reported in 15 (23.8%) cases and six (9.5%) patients had B4 stage. Tanner stage 4 for pubic hair appeared most often (n = 13, 20.6%), and mean ± SD testicular volume was 12.2 ± 5.5 mL when gynecomastia was observed for the first time.

The clinical characteristics of the patients with gynecomastia, divided into two groups (B2 or >B2), are shown in Table 1. There were no statistically significant clinical differences between early and more advanced stage of the disease.

We were able to identify the cause of gynecomastia in 11 cases (17.4%) which were classified as the pathological

Table 1. Clinical characteristics of adolescent boys with gynecomastia stratified by Tanner B stage

	B2 stage (n = 42)	>B2 stage (n = 21)	p value
Age (years)	13.6 ± 2.5	14.1 ± 2.2	0.25
Height (cm)	165.5 ± 13.7	168.1 ± 10.2	0.28
Weight (kg)	62.6 ± 17.1	66.3 ± 14.0	0.24
hSDS	0.46 ± 1.2	0.68 ± 1.4	0.54
BMI	22.4 ± 4.0	23.3 ± 4.0	0.43
BMI Z-score	0.72 ± 1.0	1.06 ± 0.9	0.20
Tanner G stage	3 (2-4)	3 (2-4)	0.85
Tanner P stage	3 (2-4)	3 (3-4)	0.30

Data are presented as mean ± standard deviation (SD) or median (interquartile range). hSDS: height SD score, BMI Z-score: body mass index SD score, G: genitals; P: pubic hair (components of Tanner scale)

gynecomastia group (n = 11). In this group the mean ± SD age was 14.9 ± 3.0 years, mean ± SD hSDS was 0.3 ± 1.0 and mean ± SD BMI Z-score was 0.6 ± 0.8 at first presentation. These results did not differ statistically from the pubertal gynecomastia group.

Hormonal Results

Hormonal results of all patients are presented in Table 2. E2 concentration was elevated in six (12.5%) boys. Of these, one was obese and two were overweight. There were five (10.9%) patients with TTE results below the reference interval and two of them were also overweight. None of the patients had elevated E2 and decreased TTE simultaneously Figure 1 displays a flow chart of patients included in E2/TTE ratio evaluation. A statistically significant positive correlation between E2/TTE ratio and Tanner B stage was found ($r = 0.47$; $p = 0.034$, see Figure 2). E2/TTE ratio did not correlate with BMI Z-score. The mean basal LH and FSH concentrations were in the pubertal ranges. TSH concentrations were elevated in six (12.8%) boys, although all of them had normal fT4 concentration. Among these, one boy was obese and four were overweight. Four (11.1%) boys had hyperprolactinemia (617.2; 604.6; 567.4 and 381.6 mIU/L) and one patient had a history of galactorrhoea concurrent with normal PRL level. ALT, AST, hCG and AFP of all patients were within normal ranges.

The comparison of B2 and >B2 groups revealed that patients with a breast stage >2 Tanner B tended to have higher E2/TTE ratios (0.8 ± 1.8 versus -0.3 ± 1.5; $p = 0.057$; Figure 3, 4). There were no other differences between groups and all results are presented in Table 3.

Table 2. Hormone levels in all boys with pubertal gynecomastia

Hormone	n	Level, mean ± SD
E2 (pmol/L)	48	25.03 ± 18.27
TTE (ng/L)	42	258.56 ± 215.89
E2/TTE ratio (SD)	42	0.34 ± 1.73
TSH (µU/mL)	47	2.92 ± 1.56
LH (mIU/mL)	37	3.34 ± 1.89
FSH (mIU/mL)	23	4.73 ± 5.44
PRL (mIU/L)	36	150.29 ± 169.40
ALT (IU/L)	24	18.36 ± 8.05
AST (IU/L)	23	23.03 ± 5.73
hCG (mIU/mL)	31	0.68 ± 0.71
AFP (IU/mL)	32	1.63 ± 0.89

E2: estradiol, TTE: testosterone, PRL: prolactine, ALT: alanine transaminase, AST: aspartate transaminase, hCG: human chorionic gonadotropin, AFP: alpha-fetoprotein alanine transaminase, SD: standard deviation, TSH: thyroid stimulating hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone

Additionally, we investigated the clinical profile of six boys (14.3% out of 42 boys who had calculated E2/TTE ratio) whose E2/TTE ratio was over +1 SD (3.4 ± 1.1). These boys were in the middle of puberty (14.7 ± 2.1 years of age) with a BMI Z-score of 1.0 ± 0.6 and a hSDS value of -0.1 ± 1.5. All were at Tanner stage B3 (n = 2) or B4 (n = 4). They had TTE concentrations within the reference interval (242.3 ± 97.7 ng/dL). Mean ± SD E2 concentration among this group was 40.3 ± 7.6 pg/mL and three boys had elevated E2 level (45.9 ± 4.5 pg/mL) while three had normal E2 levels (34.2 ± 4.9 pg/mL).

Finally, we analysed hormonal results in the pathological gynecomastia group, which were available for nine of 11 (81.8%) patients. Median (range) values for E2 were 23.8 (50.5) pmol/l and for TTE were 209.8 (825.4) ng/L ng/L. Mean E2/TTE ratio was 0.2 ± 1.8. Due to the small number of subjects with hormonal results in this group, comparison with the pubertal gynecomastia group would be unreliable and was not undertaken.

Discussion

Gynecomastia may be a cause of psychosocial discomfort, stress and worsening of self-image in a boy. It is important

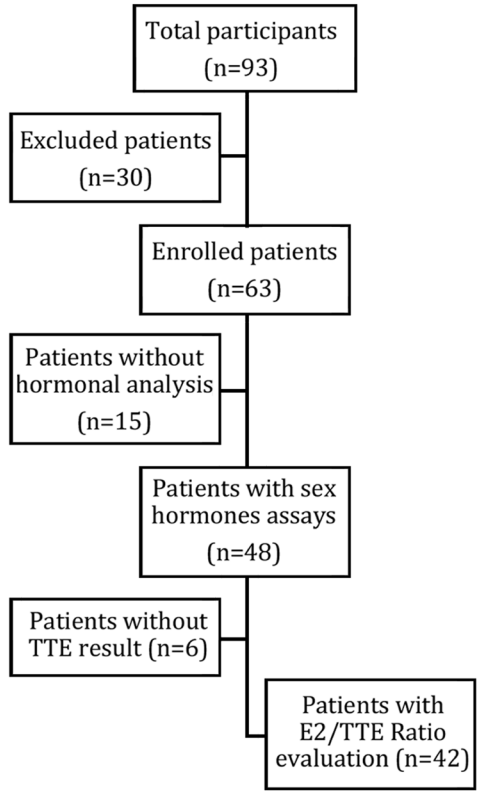

Figure 1. Flow chart of patients included in E2/TTE ratio evaluation

E2/TTE: estrogen to testosterone

to understand these concerns in order to provide proper management. The suggested diagnostic approach and treatment strategies for gynecomastia consist of expert opinion, case series and follow-up observation, implying that the quality of evidence is not satisfactory and and that an unequivocal management appraoch regarding this problem is lacking.

This article reviews the validity of calculating E2/TTE ratio as a diagnostic aid in management of gynecomastia in young and adolescent boys. Increased E2/TTE ratio has been suggested as the main cause of gynecomastia (28,29,30,31). According to the literature, 25% of cases with gynecomastia are described as idiopathic (14,32). We have observed that no clear etiology for breast enlargement can be established in almost 65% of the patients, while sex hormone disturbances or other identifiable cause were present in 35% of the study subjects.

According to latest recommendations, each patient suspected of gynecomastia on physical examination without an identified cause, should undergo hormonal investigation including determination of blood LH, FSH, PRL, TTE, E2, beta-hCG and TSH (3,4). In our cohort only 2/3 of gynecomastia patients had undergone such an evaluation. Moreover, physicians should pay specific attention to "red flags" suggestive of non-physiologic gynecomastia (2,8,10). We did not observe rapid growth of breasts, breast skin changes, firm breast mass, testicular mass nor other signs of malignancy in this series. Only one boy was followed-up for longer than two years. In this case, we were able to diagnose persistent gynecomastia (>2 years), which is considered a "red flag" of pathological gynecomastia. One boy had a history of galactorrhea, which is also a cause for clinical concern, as nipple discharge may suggest a serious underlying pathological etiology for the of gynecomastia.

The average age of our patients at the first visit was 13.8±2.6 years and their level of pubic hair development which was at Tanner stage 3 were consistent with previous studies (33,34). On the other hand, about 40% of the study subjects were at more advanced Tanner stages for pubic hair and almost 35% had greater testicular volumes (P4G4) than is usually observed (P3G3) at this age (22).

Table 3. Biochemical characteristics of adolescent boys with gynecomastia stratified by Tanner B stage

	B2 stage (n=42)	>B2 stage (n=21)	p value
E2 (pmol/L)	23.7±19.6	26.6±17.6	0.55
TTE (ng/L)	278.8±256.8	242.0±178.3	0.58
E2/TTE ratio	-0.3±1.5	0.8±1.8	0.057
TSH (μU/mL)	2.9±1.7	3.0±1.3	0.82
LH (mIU/mL)	3.5±2.2	3.2±1.7	0.69
PRL (mIU/L)	145.7±168.2	154.8±175.0	0.88

Data are presented as mean±standard deviation. E2: estradiol, TTE: testosterone, TSH: thyroid stimulating hormone, LH: luteinizing hormone, PRL: prolactine

The fact, that most of our patients did not have any abnormalities in basal hormone levels encouraged us to calculate the E2/TTE ratio, which was possible in 42 patients. We found that there was a weak correlation between the imbalance of estrogen and androgen, expressed as E2/TTE ratio, and breast size. This finding suggests that there may be a possibility of sex hormones disturbances in gynecomastia patients, despite E2 and TTE serum concentrations being with the appropriate reference ranges. Nonetheless, lack of differences between the B2 and >B2 groups for E2 and TTE concentrations and in

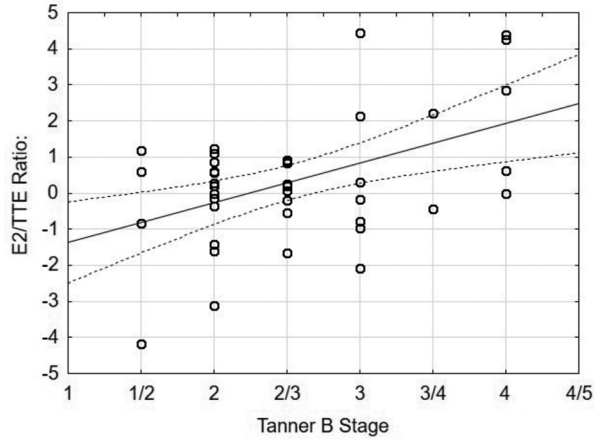

Figure 2. Correlation between E2/TTE ratio and the Tanner scale B stage

E2/TTE: estrogen to testosterone

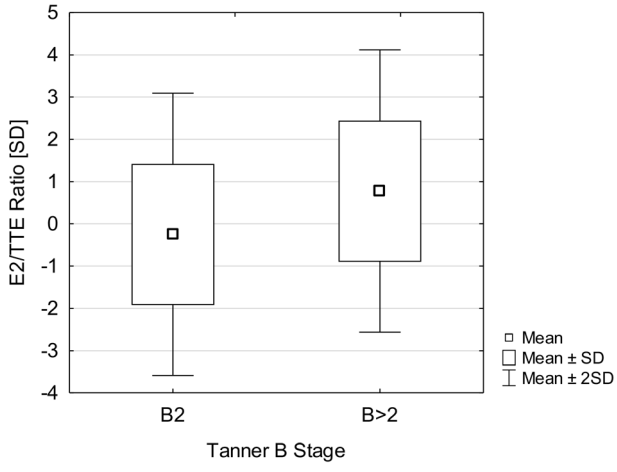

Figure 3. Comparison of E2/TTE ratio stratified by gynecomastia status; p=0.057

E2/TTE: estrogen to testosterone, SD: standard deviation

their respective E2/TTE ratios suggests that caution should be exercised in drawing conclusions.

To the best of our knowledge, there are no specific guidelines on how to calculate E2/TTE ratio and there is no proposed cut off level for this parameter. In our study, the group of boys with higher (+1 SD) E2/TTE ratio values also showed higher E2 levels (40.3 *vs* 24.2 pg/mL) and more advanced breast tissue development. Nonetheless, we did not find any additional causes and features of gynecomastia in this subgroup. The imbalance in the E2/TTE ratio may explain why some adolescents with "normal" hormone levels develop gynecomastia. We propose that a cutoff point needs should be established for E2/TTE ratio following large, well-designed studies for use in clinical practice.

A rapid increase in obesity among children and adolescents results in a higher number of patients presenting with breast enlargement. Despite the fact that obesity causes pseudogynecomastia, that is a proliferation of adipose rather than glandular tissue, true gynecomastia is also associated with higher body weight. Rivera et al. indicated that there is a correlation between pubertal gynecomastia and higher BMI percentiles. Kulshreshtha et al (34) also reported that most of the patients (64%) with breast enlargement were obese as per Coles criteria. In our study, the subjects had higher BMI values than the general population according to Centers for Disease Control growth charts. However, we did not find a relationship between BMI Z-score and breast size. Despite the fact that eight overweight children (57.1%) succeeded in losing some weight, breast size was not reduced in any of them and weight changes did not affect sex hormone levels, as there was no correlation between BMI Z-score and E2/TTE ratio.

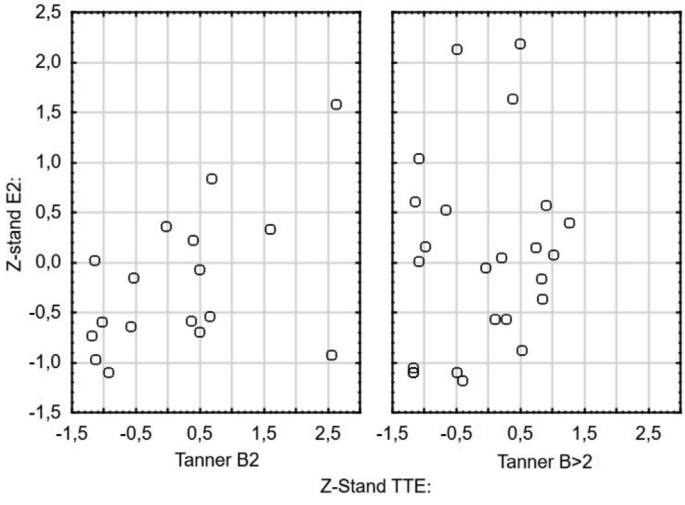

Figure 4. Scatter plot of standarised E2 and TTE values stratified by Tanner B stage

TTE: testosterone, E2: estradiol

This observation, that weight loss alone will not correct true glandular breast enlargement is consistent with that reported by other authors (17,36).

It should be also underlined that in the majority of our patients the breasts did not show an increase in size, while in a minority (19%) progression of breast size was evident. Adolescents with gynecomastia should be encouraged to lose weight, because it may complicate the surgical treatment of long-standing gynecomastia. Handschin et al (37) report that in adults with gynecomastia who are overweight, more severe surgical complications are observed and larger resections are needed.

Further studies should be performed in order to measure whether the same findings are applicable to the active fractions of sex hormones, that is free TTE and free E2 and studies should also take into account sex hormone binding globulin. Such studies might be helpful in providing greater guidance in differentiating true gynecomastia from lipomastia and pseudogynecomastia, especially in obese boys.

Study Limitations

We are aware of the limitations of the retrospective design of our study. As the study was limited to a single center, the number of study subjects was also restricted. The smallness of the sample may have led to overlooking the actual correlations. Moreover, the study was conducted in a single region of Poland, Silesia. Despite these limitations, our study design provides some valuable estimates, as each patient underwent the same process of diagnosis and the hormonal results were established in the same laboratory.

Conclusions

In conclusion, our results show that the E2/TTE ratio may be a helpful tool in diagnosing gynecomastia. We speculate that an altered E2/TTE ratio might be responsible for a portion of the cases previously described as idiopathic. Additionally, weight loss does not imply reduction of breast size in boys. Nonetheless, it should be the first step before further treatment of prolonged gynecomastia.

Ethics

Ethics Committee Approval and Informed Consent: Due to its retrospective design and non-experimental nature a formal consent as well as formal approval by a bioethics committee were not required.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Aneta Gawlik, Concept: Barbara Kalina-Faska, Design: Aleksandra Januszek-Trzciakowska, Data Collection or Processing: Barbara Kalina-Faska, Aleksandra Januszek-Trzciakowska, Analysis or Interpretation: Dominika Tobolska-Lorek, Literature Search: Miłosz Lorek, Dominika Tobolska-Lorek, Writing: Miłosz Lorek.

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Liver Biochemical Abnormalities in Adolescent Patients with Turner Syndrome

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What is already known on this topic?

Elevated liver function tests (LFTs) are common in adult patients with Turner syndrome (TS). Potential causes and mechanisms suggested in the literature are not clear, and may include autoimmunity, venous malformations, obesity and sex hormones replacement therapy (HRT).

What this study adds?

Elevated LFTs are common in children and adolescents with TS. Obesity and HRT do not increase the risk of elevated LFTs.

Abstract

Objective: Elevated liver function tests (LFTs) are common in adult Turner syndrome (TS) patients. Data regarding children and adolescents are lacking. To investigate the prevalence of abnormal LFTs in children and adolescents with TS during several years of observation; to evaluate the potential impact of increased body mass index (BMI) and sex hormone replacement therapy (HRT) on LFTs.

Methods: The analysis included 100 girls with TS, aged 4-16 years, all of whom were receiving recombinant human growth hormone therapy. A longitudinal study was conducted which included 81 patients.

Results: Mean BMI-standard deviation (SD) score of the subjects was 0.63 (SD: 1.53). Forty-four were being treated with HRT. Elevated LFTs were found in 34% of the patients overall (32% not receiving HRT *vs* 36% on HRT). The relative risk of increased LFTs was not higher in obese *vs* normal weight [odds ratio (OR): 0.2; 95% confidence interval (CI): 0.1-0.36, p = 0.38 *vs* OR: 0.16; 95% CI: 0.08-0.3, p = 0.1]. HRT did not increase the risk of abnormal LFTs activity (OR: 0.8; 95% CI: 0.5-1.2, p = 0.37 *vs* OR: 0.7; 95% CI: 0.4-1.1, p = 0.27). During the follow-up period (mean ± SD = 4.31 ± 0.82 years), no patient developed overt liver disease. There was no significant increase nor decrease of abnormal LFT frequency in the subsequent years of follow up.

Conclusion: Constantly elevated LFTs in TS are common in children and adolescents with TS. However the causes and clinical significance remain unclear. This study suggests that obesity and HRT do not increase the risk of elevated LFTs.

Keywords: Turner syndrome, children, liver, estrogen

Introduction

Turner syndrome (TS) affects approximately 1 per 2500 live female births and is one of the most common chromosomal aberration in females (1,2). It is caused by a partial or complete X chromosome monosomy. Conditions often seen in TS include: short stature, ovarian dysgenesis, dysmorphic features and endocrine disturbances such as diabetes mellitus and thyroiditis. Liver involvement indicated by abnormal liver function tests (LFT) seems to be frequent in adult TS patients, with a prevalence of 20 to 80% (3,4,5,6). Data on children and adolescents are lacking. The causes and clinical significance of this phenomenon are unclear. Nevertheless, overt liver diseases are also more common in TS patients than in the general population. The hepatic histological changes reported in TS patients vary and include minimal abnormalities, steatosis, steatohepatitis,

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biliary involvement, nodular regenerative hyperplasia and even cirrhosis (5,6,7,8,9,10,11,12,13,14,15,16,17,18,19). Potential causes and mechanisms are not clear and may include autoimmune processes, venous malformations, obesity and sex hormone replacement therapy (HRT) (5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20). However, some basic and animal studies point to the crucial a role of estrogen deficiency or estrogen receptor malfunction in the development of liver impairment (21,22,23,24,25,26,27).

The aims of this study were to investigate the prevalence of abnormal LFTs in children and adolescents with TS; to analyse LFTs changes and their clinical significance over several years of observation; to evaluate the potential impact of increased body mass index (BMI) and sex HRT on LFTs.

Methods

The analysis included 100 girls with TS, aged 4-16, all of whom were being treated with human recombinant growth hormone. 44 patients were on HRT-estrogen and estrogen/progestin patches. Patients were treated with daily injections of human rekombinant growth hormone, dose 0.33-0.47 mg/kg/week. It was a retrospective analysis plus prospective follow-up period. Blood was collected in the fasting state, in the morning (7.00-9.00), during routine examinations performed in patients with TS.

The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was measured in fresh serum samples using dry chemistry (VITROS® 5.1 FS, Ortho Clinical Diagnostics).

Body height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively, using a stadiometer (Harpenden, UK) and a balanced scale (SECA).

Statistical Analysis

To compare the two sets of data, Student's t-test or two-sided Mann-Whitney U test were used. For a correlation analysis, the correlation coefficient (R) and regression analysis were used. Odds ratio (OR) was calculated using logistic regression analysis. A probability value of less than 0.05 was accepted to be statistically significant

Ethics

The investigation was conducted according to the principles expressed in the Declaration of Helsinki. The participants and/or their parents signed informed consent. The study has been approved by the Jagiellonian University Bioethical Committee (decision number: KBET/102/B/2012);

Results

The longitudinal study included 81 patients (mean follow-up period: 4.31 years, SD: 0.82). Mean BMI-standard deviation (SD) score (SDS) was 0.63 (SD: 1.53).

In the whole group of patients 17 were diagnosed with obesity (9 without HRT and 8 with HRT).

Elevated LFTs were found in 34 patients 34% [in 18 (32%) without HRT *vs* in 16 (36%) on HRT]. Increased AST activity was present in 10 (18%) without HRT; in 5 (11%) on HRT), and elevation of ALT [in 9 (16%) without HRT and in 11 (25%) on HRT]. The mean values of AST in both groups (without HRT and with HRT) were 42.7 IU/L and 44.2 IU/L $p = 0.8$, and the mean value of ALT were 27.5 IU/L and 29.9 IU/L, $p = 0.14$ respectively.

The mean values of AST in patients with obesity and non obese were 47.5 IU/L and 42.5 IU/L, $p = 0.8$. The mean values of ALT in patients with obesity and non obese were 35.6 IU/L and 27 IU/L, $p = 0.037$.

The relative risk of increased LFTs activity was not higher in obese *vs* normal weight [OR: 0.2; 95% confidence interval (CI): 0.1-0.36, $p = 0.38$ *vs* OR: 0.16; 95% CI: 0.08-0.3, $p = 0.1$]. HRT did not increase the risk of abnormal LFTs activity (OR: 0.8; 95% CI: 0.5-1.2, $p = 0.37$ *vs* OR: 0.7; 95% CI: 0.4-1.1, $p = 0.27$). During the follow-up period, no patient developed overt liver disease. There was no significant increase nor decrease of the abnormal LFTs frequency in the subsequent years of follow up ($p > 0.05$) (Figure 1).

Discussion

The reported frequency of elevated LFT activity in TS patients ranges from 20 to 80%, with the highest proportion in older patients (3,4,5,6,27,28). In a recent large study (842

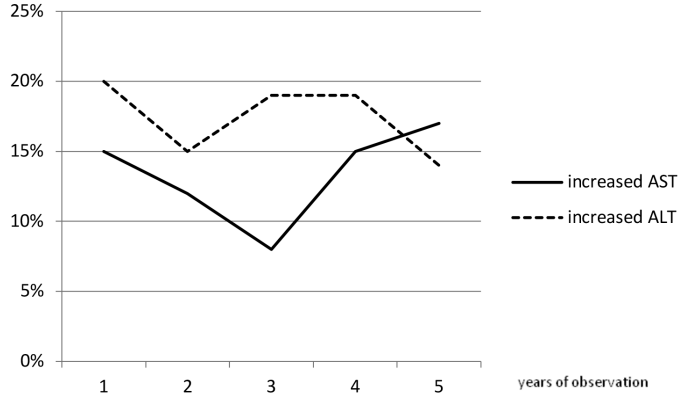

Figure 1. Percentage of abnormal results of liver function tests during subsequent years of observation

AST: aspartate aminotransferase, ALT: alanine aminotransferase

pediatric patients) only 3.4% of 698 examined were found to have abnormal LFT results and in patients younger than 10 years this was only found in five patients (29). Research carried out in older age groups indicates a much more frequent occurrence of abnormal LFTs. In the study of El-Mansoury et al (27) 36% of 218 adult TS patients presented with abnormal levels of one or more liver enzymes at the beginning, and subsequently 23% more developed abnormal LFTs during a 5-year follow-up. In our study, we found a similar proportion of young TS patients with elevated LFTs at the beginning of the study (34%), but we did not observe any progression during the follow-up period. Although liver disease in patients with TS is generally more common than in the general population, so far no direct correlation has been found between the development of liver disease and the occurrence of abnormal LFTs in the preceding period. In most published studies LFTs did not progress to overt liver disease (1,17,18,19). Also, little is known about the factors predisposing to abnormal LFTs. The literature suggests the possible participation of obesity and HRT by analogy to the results of research conducted in various groups of patients (4). As women with TS have short stature and abnormal body proportions, they are more likely to be overweight and obese (4,17,18,19,30). In our present study, no relationship was found between obesity and LFTs. The relative risk of the development of LFTs was comparable in patients with obesity and normal BMI-SDS. This finding is in accordance with some earlier studies in this field, which confirmed obesity as a frequent finding in TS patients, but without correlation to liver impairment (5,27,31). Another potential factor widely considered in older publications as a cause of hepatotoxicity is estrogen replacement therapy (32,33). Estrogen receptors are expressed in the liver and estrogens probably play an important role in hepatic lipid homeostasis (34,35). Despite many studies performed in this field, the causative role of estrogens is not well established. Some reports suggest that estrogen replacement therapy in TS patients can cause deterioration of liver function and in some patients discontinuation of therapy was followed by a decrease in enzyme levels (36). In contrast, some more recent studies point to a potential role of estrogen replacement as a favourable factor improving liver function (21). Although some studies reported alterations in LFTs in TS patients treated with estrogens, these alterations did not improve with the discontinuation of replacement therapy (13,20). More recent studies also found elevated LFTs in young patients before HRT, and some showed a beneficial impact of estrogen replacement (4,28,31). In our study, sex HRT did not increase the risk of elevated ALT and AST. As we examined a group of pediatric TS patients, it can be difficult to compare our results with studies based on results of adult TS patients. More recent observational studies, conducted in post-menopausal women without HRT revealed an increased risk of liver steatosis, in comparison to pre-menopausal women (21,37,38). For this reason, the importance of estrogen in liver function has become the subject of many experimental studies. A number of basic and animal studies have revealed a crucial role of estrogens and estrogen receptor deficiency in the pathogenesis of liver dysfunction. Estrogens can mediate their biologic effects in the liver through a number of mechanisms. The classic mechanism involves its binding to the steroid nuclear hormone receptors, α or β. Both have the classic features of steroid hormone receptors (39). Estrogens can also alter cell signaling via estrogen receptor α or β, localized in the cell membrane. In addition to membrane localized α and β receptors, estrogens can signal through another cell surface receptor, the G-protein coupled estrogen receptor (GPER, also called Gpr30) which is expressed in multiple tissues, including liver (40). It has been shown recently that the loss of receptor α in the liver is associated with hepatic steatosis and inflammation, and its gene expression is lower in patients with non-alcoholic steatohepatitis (41). Zhu et al (22,23) reported that estrogen treatment may reverse aspects of pathway-selective insulin resistance by promoting insulin action on glucose metabolism but limiting hepatic lipid and diacylglycerol deposition. Estrogen treatment reduces liver fat storage on several levels, mainly by blocking insulin signaling to liver acetyl-CoA carboxylase and reducing hepatic apoB100 and phospholipid transfer protein. This protective effect of estrogen treatment requires intact hepatic estrogen signaling through estrogen receptor α. By contrast, hepatic estrogen signaling may not be required for the effects of estrogen treatment on body weight and adiposity (22,23). Moreover, Kao et al (42) found that estrogen receptor α could be an important mediator of liver regeneration. What is more, it has been shown that estrogen receptor β agonist might provide therapeutic benefits in liver steato-hepatititis by directly modulating the bile acid receptors in the liver, which have important functions in the liver, and indirectly, by inhibiting adiposity (43). The mechanisms by which estrogen signaling protects against hepatic steatosis also include reductions in de novo lipogenesis, as reported by Gao et al (25). These mechanisms may be helpful for understanding mechanisms of liver impairment in TS patients and the favourable action of estrogen replacement.

Study Limitations

The main limitation is its retrospective character leading to a lack of long-term observation for the whole group. Due to different models (transdermal/oral) of HRT and various

estradiol doses, the effect of estrogens on LFTs could not be accurately analyzed.

Conclusion

Constantly elevated LFTs in TS are common in children and adolescents with TS. However the causes and clinical significance remain unclear. This study suggests that obesity and HRT do not increase the risk of elevated LFTs.

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Ethics

Ethics Committee Approval: The investigation was conducted according to the principles expressed in the Declaration of Helsinki. The study has been approved by the Jagiellonian University Bioethical Committee (decision number: KBET/102/B/2012).

Informed Consent: The participants and/or their parents signed informed consent.

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Authorship Contributions

Surgical and Medical Practices: Małgorzata Wójcik, Dominika Januś, Anna Ruszała, Concept: Małgorzata Wójcik, Design: Małgorzata Wójcik, Anna Ruszała, Data Collection or Processing: Małgorzata Wójcik, Anna Ruszała, Analysis or Interpretation: Małgorzata Wójcik, Anna Ruszała, Literature Search: Małgorzata Wójcik, Anna Ruszała, Writing: Małgorzata Wójcik, Anna Ruszała, Dominika Januś, Jerzy B. Starzyk.

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Clinical Management and Gene Mutation Analysis of Children with Congenital Hyperinsulinism in South China

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What is already known on this topic?

Congenital hyperinsulinism (CHI) is a rare inherited disease characterized by unregulated insulin secretion and profound hypoglycemia. There are few reports pertaining to patients with CHI from south China.

What this study adds?

This is the first study investigating the clinical features, molecular genetic characteristics and treatment, including the optimal therapeutic approach, in patients with CHI in south China.

Abstract

Objective: To explore the clinical presentation and molecular genetic characteristics of a cohort of congenital hyperinsulinism (CHI) patients from southern China and also to explore the most appropriate therapeutic approaches.
Methods: We retrospectively reviewed a cohort of 65 children with CHI. Mutational analysis was performed for *KCNJ11* and *ABCC8* genes. The *GLUD1* gene was sequenced in patients with hyperammonaemia. *GCK* gene sequencing was performed in those patients with no mutation identified in the *ABCC8*, *KCNJ11* or *GLUD1* genes.
Results: *ABCC8* mutations were identified in 16 (25%) of the cohort, *GLUD1* mutations were identified in five children, and no *KCNJ11* or *GCK* mutations were identified. Moreover, some unique features of *ABCC8* gene mutations in southern Chinese CHI patients were found in this study. The most common mutation was a deletion/insertion mutation p.Thr1042GlnfsX75 was found in five unrelated patients, which possibly represents a relatively common mutation in southern China. Five novel *ABCC8* mutations were detected. The mutations were p.Phe5SerfsX72, p.Gln273ArgfsX85, p.Leu724del, p.Asp1447Gly and IVS 25-1G>T. Five compound heterozygous mutations of *ABCC8* gene were identified in this study, and three of these patients were diazoxide-responsive. Forty patients were diazoxide-responsive, 13 patients were diazoxide-unresponsive and 12 patients received dietary treatment only. A pancreatectomy was performed in 10 patients who were unresponsive to medical treatment.
Conclusion: To the best of our knowledge, this is the first study of CHI in south China. Mutations in *ABCC8* are the most common causes of CHI in this cohort. Diazoxide and dietary treatment were effective in most patients. Multicentre studies are necessary to obtain the long-term follow-up characteristics of such patients at a national level.
Keywords: Congenital hyperinsulinism, clinical management, gene mutation

Introduction

Congenital hyperinsulinism (CHI) is the most frequent cause of persistent hypoglycaemia in neonates and infants. CHI occurs due to the dysregulated and inappropriate secretion of insulin from pancreatic β cells (1). The incidence of CHI is estimated to be 1 in 40,000-50,000 live births in northern Europe (2,3) and 1 in 2,500 births in Saudi Arabia (4). There are no nationwide data regarding the incidence of this disorder in China.

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Inappropriate insulin secretion can suppress the production of ketone bodies, which serve as an alternative fuel during hypoglycemia. The lack of glucose and the deprivation of alternative fuels for the brain will increase the risk of brain damage in these patients (5). To date, mutations in 14 different genes, namely *ABCC8, KCNJ11, GLUD1, GCK, HADH, SLC16A1, UCP2, HNF1A* (6), *HNF4A* (7), *HK1* (8), *PGM1* (9), *PMM2* (10), *CACNA1D* (11), and *FOXA2* (12), that lead to dysregulated secretion of insulin have been described. The most common forms of CHI are due to defects in *ABCC8* and *KCNJ11*, which encode the ATP-sensitive potassium (KATP) channel subunits of the sulfonylurea receptor (SUR1) protein and inwardly rectify potassium channel (Kir6.2) proteins, respectively (13). Both these genes are located on chromosome 11p15.1 (14).

The clinical presentation can be varied, ranging from completely asymptomatic to mild or severe disease that is unresponsive to medication and requires surgical intervention (15). Diazoxide is the first-line agent for the treatment of CHI. Diazoxide binds to the SUR1 subunits and opens the KATP channel, thereby preventing depolarization of the β-cell membrane and insulin secretion (16). If necessary, those who are unresponsive to medical therapy may undergo surgical treatment. Histologically, CHI is divided into three subgroups. These are the diffuse, focal and atypical forms of CHI. Children with diffuse CHI may require a near-total pancreatectomy which carries the attendant risk of diabetes mellitus and exocrine pancreatic insufficiency, whereas the focal form will only require a limited, focal lesionectomy. Conventional radiological imaging is often used but is unable to distinguish between the two forms (17). ^{18}F-DOPA positron emission tomography/computed tomography (PET/CT) scanning is an accurate and non-invasive technique to differentiate focal and diffuse types of CHI (18). Unfortunately, this imaging method is not available in southern mainland China. Genetic analysis may provide important diagnostic clues, particularly in the absence of specific imaging modalities, during the investigation of CHI cases. Children with the diffuse form of CHI due to recessive mutations in *ABCC8* and *KCNJ11* usually do not respond to diazoxide. Focal forms are sporadic and associated with a paternally inherited mutation in *ABCC8*/ *KCNJ11* genes (19).

Although the clinical characteristics and genetic aetiology of CHI patients have been described in some studies in China (20,21,22), little is known about CHI in southern China. We first reported our experiences with the management of CHI in 12 children in 2009 (23).

The objectives of the current study were to investigate the clinical presentation and molecular genetic characteristics of a group of patients with CHI in southern China and also to explore the most appropriate therapeutic approaches.

Methods

Subjects

Enrolled patients included those diagnosed with CHI who were hospitalized in the Guangzhou Women and Children's Medical Center from November 2012 to June 2017. Most of the patients with CHI came from the Guangdong, Guangxi, Jiangxi, Hunan, Hubei, Yunnan and Hainan provinces of southern China. Serum ammonia levels were checked in all cases. All infants and children were diagnosed with CHI based on clinical and biochemical criteria which were as follows: whether serum insulin was simultaneously detectable (>2 mU/l) concurrent with hypoglycaemia (blood glucose <2.6 mmol/l); along with evidence of elevated glucose requirements (glucose infusion >8 mg/kg/min) in the absence of ketosis or ketonuria; and an inappropriate glycaemic response to glucagon injection at the time of hypoglycaemia (15). Patients with a secondary cause of hypoglycaemia such as perinatal asphyxia, prematurity, intrauterine growth restriction, maternal diabetes and syndromic forms were excluded. Being responsive to diazoxide was defined as maintaining blood glucose above 3.5 mmol/l after a short period of fasting which varied depending on the age of the patient: 4 hours in neonates; 8 hours in infants; and 12 hours in children (6). Clinical data were obtained from medical records. The study was reviewed and approved by the Ethical Committee of Guangzhou Women and Children's Medical Center (2016022210).

Genetic Analysis

Genomic DNA was extracted from peripheral blood leucocytes, using a kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). All exons and intron-exon boundaries of the *ABCC8* and *KCNJ11* genes were amplified by polymerase chain reaction, purified and sequenced. The sequences were analysed and compared to the wild-type published reference sequences (NM_000525 for *KCNJ11* and NM_000352.3 for *ABCC8*) using Chromas software (Technelysium Pty Ltd, South Brisbane, Australia). The *GLUD1* gene was sequenced in patients with hyperammonaemia (HA), whereas *GCK* gene sequencing was subsequently performed in those patients with no mutation identified in the *ABCC8, KCNJ11* or *GLUD1* genes. The novelty of mutation sites were determined by searching the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk) and the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/clinvar). All variations were identified in this study using the

Single Nucleotide Polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/SNP) and the 1000 Genomes Project database (https://www.internationalgenome.org/1000-genomes-project-publications). Intronic variants were analysed with GenSCAN (http://genes.mit.edu/GENSCAN.html). To test the pathogenicity of novel missense mutations, Polymorphism Phenotyping (PolyPhen, http://genetics.bwh.harvard.edu/pph) and Sorting Intolerant from Tolerant (https://sift.bii.a-star.edu.sg/) were used.

Treatment and Follow-up

Neonatal history, clinical presentation, treatment and complications were analysed in the CHI patients. Intravenous glucose infusion to maintain blood glucose levels of > 2.8 mmol/l, nutritional therapy and diazoxide treatment were initiated immediately upon diagnosis. Nutritional therapy included frequent meals enriched with complex carbohydrates and nasogastric feeds at midnight. A glucose polymer, maltodextrin (Malt Extract, Wakodo, Asahi Group Foods, Ltd., Japan), was utilized in infants younger than six months of age. Subjects older than six months were given supplemental uncooked corn starch through a nasogastric tube between meals, before bedtime and for night-time feeds. Diagnostic tests for protein-sensitive hypoglycemia were performed in five patients with HI/HA syndrome. The blood glucose concentrations of all five patients decreased following the protein load and an age-adjusted daily diet consisting of a protein combination with fat and carbohydrate was started.

Diazoxide was started in a dose of 10 mg/kg/day, given in three divided doses. When diazoxide treatment was effective, the dosage was reduced to the effective minimum. Oral hydrochlorothiazide (1-2 mg/kg/day) was used in conjunction with diazoxide to counteract the fluid-retaining properties of diazoxide. All patients treated with diazoxide were carefully monitored for fluid and sodium retention. In three children who were not responsive to diazoxide, octreotide (5-25 µg/kg/day) injections were administered.

Pancreatectomy was implemented in patients not responding to medical therapy. Clinical follow-up was initiated one month after hospital discharge and continued at intervals of three months subsequently. Self-monitored blood glucose levels were recorded. Brain damage was evaluated by a pediatric neurologist at the time of diagnosis and at each three monthly follow-up.

Statistical Analysis

The results were analysed using the SPSS 17.0 program (IBM Inc., Armonk, NY, USA) and were expressed as the mean ± standard error of the mean (mean ± SE) and in percentages (%). The Student's t-test and the Wilcoxon test were used for statistical analysis of the data. All p values less than 0.05 were considered significant.

Results

A total of 65 patients (47 males and 18 females) with a diagnosis of CHI were included in the study. Age at diagnosis ranged from immediately following birth to seven years old. Twenty-three patients (35.4%) were macrosomic and their mean birth weight was 3,690 g. Sixty-two patients were born at term. The CHI symptoms were first noted during the neonatal period in 29 patients (44.6%), during the infancy period (1-12 months) in 26 patients (40%) and during childhood (> 12 months) in 10 patients (15.4%).

Of the 65 patients, 13 were diazoxide-unresponsive, 40 patients were diazoxide-responsive and 12 received dietary treatment only. Patients were divided into two groups based on diazoxide responsiveness; Group 1, diazoxide-unresponsive and Group 2, responsive to diazoxide or dietary treatment. The clinical and biochemical characteristics of the patients in the two groups are presented in Table 1. Age at onset of CHI was significantly different between these two groups. The neonatal form comprised 92.3% of Group 1, but only 32.7% of Group 2. There was a significantly higher incidence of epilepsy in Group 2 than in Group 1 ($p < 0.05$). The time between symptom manifestation and diagnosis ranged from one day to six years, and the duration was again significantly longer in Group 2. A patient (Case 61) in Group 2 was six years old and was initially misdiagnosed as having a seizure disorder before the hypoglycemia was detected.

In our study, *ABCC8* mutations were identified in 16 children (25% of the cohort), and no *KCNJ11* mutations were identified on KATP channel gene mutation analysis. Five patients with persistent HA had mutations in *GLUD1* (Figure 1). No variants were found in the *GCK* gene. Fifteen different *ABCC8* mutations were discovered, five mutations were compound heterozygous, 11 were heterozygous and none were homozygous (Table 2). Among the children who carried compound heterozygous mutations, diazoxide treatment was effective in three children. Treatment was not effective in one child and one child was regulated with diet.

The most common mutation was a deletion/insertion mutation c.3224-3226delACC ins CAGCCAGGAACTG (p.Thr1042GlnfsX75) found in five unrelated patients, which possibly represents a relatively common mutation in southern China. Five novel *ABCC8* mutations (p.Phe5SerfsX72, p.Gln273ArgfsX85, p.Leu724del,

p.Asp1447Gly and IVS 25-1G > T) were identified. Of the novel mutations, two were frameshift mutations, one was a deletion mutation, one was a missense mutation and one was a splice site mutation. In accordance with the guidelines of the American College of Medical Genetics and Genomics (ACMG) (24), two variants were perceived as "pathogenic" and three variants were predicted as "likely pathogenic". A novel heterozygous variant in *ABCC8* gene was identified in case 22. The patient has now been on therapy with diazoxide for more than one year at a dose of 5 mg/kg/day with normal growth and development. In one case (patient 59), two novel mutations were identified. This girl was macrosomic at birth. Hypoglycemia was first detected on day three after birth at a local hospital, and improved with frequent feedings. However, her parents did not monitor her blood glucose. She was admitted to our hospital at age 14 months for brief generalized convulsive periods. Laboratory tests revealed hypoglycemia (blood glucose: 2.5 mmol/L) HI (plasma insulin level: 5.9 μIU/mL) when she had an episode. Four hourly daytime feeds (solids and cow's milk) and four hourly uncooked cornstarch (1.6 g/kg) could maintain the blood glucose above 3.5 mmol/L. During six months follow-up, there was no episodes of hypoglycemia. However, she had sustained hypoglycaemic brain injury with global developmental delay.

Table 1. Clinical and biochemical characteristics of the congenital hyperinsulinism patients

	Group 1 (n = 13)	Group 2 (n = 52)	p
Sex (Male:Female)	9:4	36:16	
Age of onset			
Neonatal period (0-4 weeks)	n = 12 (92.3%)	n = 17 (32.7%)	< 0.01
Infancy (1-12 months)	n = 1 (7.7%)	n = 25 (48.1%)	< 0.01
Childhood (> 12 months)	0	n = 10 (19.2%)	< 0.01
Birth weight (kg)	4.0 ± 0.6	3.7 ± 1.4	
Seizures	n = 4 (30.8%)	n = 36 (69.2%)	0.02
Other (cyanosis, food refusal, lethargy)	n = 6 (46.2%)	n = 16 (30.8%)	
Time of diagnosis (days)	35 (1-120)	163 (1-2610)	0.02
Blood glucose (mmol/l)	1.89 ± 0.71	1.96 ± 0.56	
Plasma insulin (mmol/l)	32.4 (5.7-96.4)	26.6 (4.8-87.1)	
Blood ammonia level (mmol/l)	50.8 ± 12.1	60.1 ± 28.4	
Neurodevelopmental delay	3 (23.1%)	32 (61.5%)	0.02
Mutation gene			
ABCC8	5	12	
GLUD1	0	5	

Data is presented as means ± standard deviation, median (range) or percentages.

Group 1: diazoxide-unresponsive

Group 2: diazoxide-responsive or dietary treatment

In this study, 16 parents underwent genetic analysis. Five patients (patients 1, 5, 10, 14 and 18) had paternally inherited monoallelic mutations. Of the five patients, three were diazoxide-unresponsive and two were diazoxide-responsive. In the three diazoxide-unresponsive patients (patients 1, 5 and 10), diffuse pancreatic disease was confirmed following surgery. One patient (patient 3) had two heterozygous mutations: one missense mutation c.314A > C (p.His105Pro) in exon 3, inherited from his father and a nonsense mutation c.2800C > T (p.Gln934X) in exon 23 inherited from his mother. He was diazoxide-unresponsive, which had been previously reported (25). The ^{18}F-DOPA PET/CT scan indicated a focal lesion in the head of the pancreas, whereas the histology of the resected pancreas showed atypical form. The enlargement of pancreatic β-cell nuclei were distributed mainly in the head but included the body and tail of the pancreas. The abnormal active endocrine cells were not restricted to a focal lesion nor were they present throughout the entire pancreas.

The *GLUD1* gene was detected in patients with hypoglycaemia, HI and mild HA. Three different heterozygous mutations in the *GLUD1* gene were identified in five patients. The p.Arg322 His mutation was found in patients 54, 55 and 56. Patients 55 and 56 were sisters. The mutation was autosomal and dominantly inherited from their father, who was an asymptomatic carrier. The p.Ser498Leu mutation was found in patient 52 and the p.Asn463Asp mutation in patient 53. All mutations have been previously reported in patients with HI/HA (26,27,28,29). The serum ammonia concentration of this group of patients was 85-184 μmol/l. After a confirmation of the diagnosis of HI/HA syndrome due to a *GLUD1* genetic defect, the patients were started on a low-protein diet (1.5 g/kg/day of natural protein intake). Three patients (patients 54, 55 and 56) were successfully managed by diet alone. They have had no further hypoglycaemic episodes. The other two patients were responsive to diazoxide treatment.

Of 65 patients, 40 (61.5%) achieved long-term stable glycaemic control by diazoxide alone. Octreotide was administered to three children who were not responsive

to diazoxide. Among these three patients, two were unresponsive to octreotide, and one patient discontinued this drug due to severe diarrhoea. Side effects of the diazoxide treatment were observed in 32 (80%) patients. Gastrointestinal disturbances such as nausea, vomiting, severe gastrointestinal upset and poor appetite occurred in 69%. Six patients were fed through a nasogastric tube because of severe gastrointestinal reactions and their blood glucose levels were kept relatively stable. Different degrees of hypertrichosis occurred in 55% (22/40) of patients during clinical follow-up. In one case (patient 43), effective diazoxide therapy had to be stopped because of thrombocytopenic purpura.

Pancreatectomy was performed in 10 patients who were unresponsive to drugs. Nine patients were treated with subtotal pancreatectomy, and one patient underwent pancreatectomy twice. In patient 3, a second resection of the pancreas was required because of sustained hypoglycaemia (25). Histological examination of the resected pancreatic tissue confirmed diffuse disease in nine patients and atypical form in one patient. One patient (patient 7) who underwent surgery at two months of age developed diabetes mellitus at five years of age and was treated with insulin. Case 8 developed diabetes mellitus immediately after surgery and required insulin treatment. Two cases still had mild hypoglycaemia after surgery; one (patient 4) was successfully managed with regular daytime and overnight feedings and the other (patient 9) was treated with diazoxide. Only one case had symptoms of malabsorption.

Diazoxide treatment was stopped in 14 of patients (35%), between the ages of six months and four years, and no recurrence of hypoglycaemia was observed. Eight patients with subtotal resection were able to maintain normal blood glucose and HbA1c levels during the duration of follow-up. There were three diazoxide-unresponsive patients: one patient died of multiorgan failure and two patients abandoned the treatment and died of severe hypoglycemia after three to seven days at home.

Discussion

In this study, the clinical characteristics, laboratory data and genetic features of 65 patients with CHI, the largest CHI cohort from southern China, were reported. Until now, there have been no nationwide data regarding this disorder in China, although several studies have summarized the clinical and genetic characteristics of CHI in northern and eastern China (20,21,22).

In our cohort of patients with CHI, 32.8% were noted to have disease-causing mutations: 16 (25%) patients were positive for *ABCC8* mutations; five (7.8%) were positive for *GLUD1* mutations; and 44 (67%) were negative for *GCK, GLUD1, ABCC8* and *KCNJ11* mutations in the gene analysis. No mutations were found in the *KCNJ11* gene in this study. As described in previous studies, most of the mutations identified have been detected in the KATP channel. The mutation detection rates of *ABCC8* and *KCNJ11* genes reported by Kapoor et al (6) and by Snider et al (26) were 36.3% (109/300) and 69% (288/417), respectively.

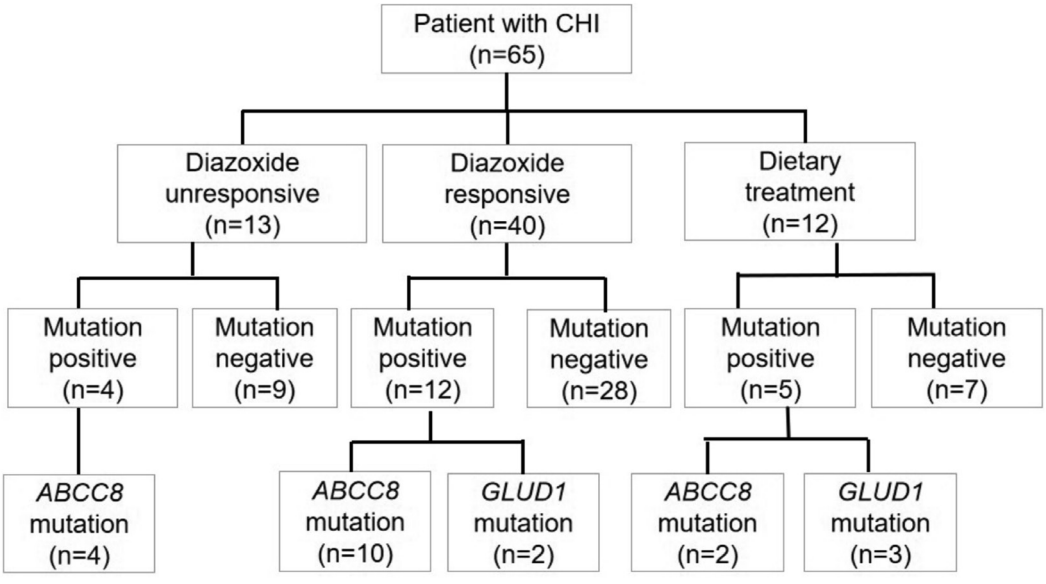

Figure 1. Distribution of patients according to mutation analysis results and treatment choices for patients with congenital hyperinsulinism

CHI: congenital hyperinsulinism

However, in a similar study conducted in a large group in Turkey, it was found that the mutation rate in the *ABCC8/KCNJ11* genes was 17/35 (48.6%) (30). The pick-up rate in our cohort (16/65, 25%) for *ABCC8* and *KCNJ11* gene variants is lower than those in previous reports and also differed from recent studies in China, which reported mutation rates of approximately 44% (12/27) (31) and 67.6% (25/32) (20). Accordingly, the low mutation discovery rate in our study may be due to the differences in genetic background among most of the cases from southern China. In our study, we found the deletion/insertion mutation c.3224-3226delACC ins CAGCCAGGAACTG in five patients, which may be the most common mutation leading to CHI in the southern Chinese population. These findings suggest that a geographical distribution difference may exist in the mutational spectrum of the *ABCC8* gene in the Chinese population. This mutation causes a frameshift and introduces a premature stop codon 75 codons downstream of the mutation, leading to the loss of the functional domain NBD2 (20). Five patients in this study carried compound heterozygous mutations. It was previously demonstrated that harboring compound heterozygous mutations of *ABCC8* gene was usually associated with medically unresponsive CHI (32). However, in this cohort, treatment with diazoxide was effective in three patients and one patient could be regulated with diet alone. Dekel et al (33) reported that some compound heterozygous mutations may cause milder HI which is responsive to diazoxide. Kumaran et al (34) also reported a case of transient hyperinsulinaemic hypoglycaemia due to a compound heterozygous mutation in *ABCC8*. The mechanisms responsible for this clinical variability may be related to background genetic factors and other unknown factors involved in regulating gene expression (35).

Five novel mutations were found in the *ABCC8* gene in five patients. One patient (patient 59) was a compound heterozygote with two novel deletion mutations, P.Gln273ArgfsX85 and P.Leu724del, and dietary treatment alone achieved stable glycaemic control in this patient.

Current medical management for CHI includes diazoxide combined with chlorothiazide as the first-line therapy (36). Diazoxide binds to the SUR1 subunit of the KATP channel and reduces insulin secretion by hyperpolarisation of the pancreatic β-cell plasma membrane (15). After diagnosis, a therapeutic trial with diazoxide was performed immediately. In our cohort, 40 (61.5%) CHI patients were diazoxide-responsive. Similar to our results, Kapoor et al (6) recently reported that 64% of their cohort responded to diazoxide treatment. In the cohort reported by Şıklar and Berberoğlu (19), 71% (100/141) of Turkish patients with CHI were responsive to diazoxide treatment. The recommended dosage of diazoxide is 5-15 mg/kg/day (37) and the effective dosage of diazoxide is believed to always be lower than 15 mg/kg/day. In our study, the initial dosage of diazoxide was 10 mg/kg/day and the minimal effective dosage was sought to maintain the stability of blood glucose. If the patient was unresponsive to a dosage of 10 mg/kg/day of diazoxide, further increasing the dosage did not improve the effect but rather increased the risk of serious complications. Gastrointestinal symptoms, such as vomiting, nausea and poor appetite, were common if dosages higher than 10 mg/kg/day were administered. One patient receiving effective diazoxide therapy had to stop treatment due to serious gastrointestinal symptoms and three cases required nasogastric tube feeding. To improve the effectiveness and reduce side effects, we believe that diazoxide should be used at the minimal effective dosage.

In this study, 35 of 65 patients developed mild mental retardation including four patients associated with HI/HA syndrome. Both low blood glucose and insufficient treatment increased the risk of impairment in neurodevelopment in CHI (38). HI/HA syndrome is caused by activating mutations in the *GLUD1* gene, which encodes the intramitochondrial enzyme glutamate dehydrogenase (GDH) (39). The epilepsy and developmental problems in HI/HA syndrome are thought to be a result of recurrent hypoglycaemia, chronic HA or decreased brain concentrations of the neurotransmitter GABA, due to increased GDH activity (27). The high rate of developmental delay in this study is likely to be due to the delayed diagnosis. Given that the clinical symptoms of this disease were not specific, and their HI may be less severe, more infants with CHI were misdiagnosed and not recognized until they presented with hypoglycaemic seizures, weeks to months later. The earlier determination of blood glucose and serum insulin concentrations will be helpful for diagnosis. Therefore, all neonates, infants and children should be evaluated for hypoglycaemia (40).

Dietary treatment is an important aspect of care for all patients with CHI. In our study, 18% of patients could achieve glycaemic stability with dietary treatment alone. Frequent feedings and specific diets include the provision of adequate carbohydrates to maintain normoglycaemia. The good response to dietary treatment obtained in some cases indicates that this should be the initial treatment for all CHI patients in addition to a trial of diazoxide. To increase the carbohydrate content, glucose polymer and uncooked corn starch were added to the diet of the older infants. Some infants may require a nasogastric tube for regular and frequent feedings. Patients with HI/HA syndrome require a

Table 2. Genetic and biochemical analysis of 21 patients with mutation-positive congenital hyperinsulinism

Patient number	Gender	Gestational age (week)	Birth weight (kg)	Onset age	Glucose levels at the diagnosis (mmol/L)	Insulin levels at the diagnosis (μIU/mL)
22	M	39	3.5	4 months	1.8	8.7
57	F	41	3.5	4 days	2.3	7.1
59	F	40	4.2	3 days	2.5	5.9
21	M	38	4.8	3 days	0.6	42.9
49	M	38	4.1	1 day	2.8	9.9
1	M	38	3.0	1 day	2.4	4.8
3	M	35	4.1	1 day	0.6	38.3
5	M	39	3.5	3 months	2.1	21.6
10	M	38	4.0	1 day	1.3	25.9
14	F	39	2.8	3 days	2.4	30.5
16	M	38	4.1	1 day	2.6	26.7
18	M	37	4.8	3 days	1.6	16.4
23	M	40	4.35	3 days	1.7	5.8
31	M	39	3.55	1 month	2.6	7.3
47	M	34	3.4	1 day	2.2	8.7
48	F	39	4.95	1 day	1.7	13.1
52	M	38	3.6	7 months	2.6	8.3
53	F	Yes	3.8	13 months	2.4	10.7
54	F	Yes	2.5	11 months	2.5	4.8
55	F	Yes	3.2	10 months	2.4	5.7
56	F	Yes	3.3	2 months	2.7	6.9

F: female, M: male

protein-restricted diet. Feeding problems such as difficulty with sucking, swallowing, vomiting and food refusal occur in a significant proportion of children with CHI and continuous feeding through nasogastric tube or gastrostomy may be required (41).

A pancreatectomy was implemented in 10 diazoxide-unresponsive CHI patients, accounting for 15.4% of the whole cohort. During surgery, none of our patients were found to have a focal lesion. A pathological examination of pancreatic tissues revealed the diffuse form of HI in nine of 10 cases.

Our findings were similar to those reported by Bellanné-Chantelot et al (42) (58.7%; 64/109) and Li et al (43) (89.5%). In total, 40% (4/10) of patients with CHI unresponsive to diazoxide had *ABCC8* mutations. In these four patients with the diffuse form of CHI proved by histology, three cases carried a single heterozygous *ABCC8* mutation and one case carried a compound heterozygous mutation. A segregation analysis of both parents in these cases showed that the mutation was paternally inherited in three patients and biparentally inherited in one patient. CHI with a single paternally inherited heterozygous mutation in the *ABCC8* gene has been previously reported to suggest focal

Continuation of Table 2.

Mutations					Treatment
Gene	**Nucleotide**	**Protein**	**Inherited from**	**Previously reported**	
ABCC8	c.4340A > G	p.Asp1447Gly	_	No	Diazoxide
ABCC8	c.12delc	p.Phe5serfsX72	Maternal	No	Dietary
ABCC8	c.817delC c.2169-2171delTCT	p.Gln273Argfsx85 p.Leu724del	No sample	No No	Dietary
ABCC8	c.12delc	p.Phe5SerfsX72	No sample	No	Diazoxide
	c.3224-3226delACC insCAGCCAGGAACTG	p.Thr1042GlnfsX75		Yes	
ABCC8	c.1792C > T	p.Arg598X	No sample	Yes	Diazoxide
	IVS 25-1G > T			No	
ABCC8	c.3224-3226delACC ins CAGCCAGGAACTG	p.Thr1042GlnfsX75	Paternal	Yes	Pancreatectomy
ABCC8	c.314A > G	p.His105 Pro	Bilateral	Yes	Pancreatectomy
	c.2800C > T	p.Gln934X			
ABCC8	c.3224-3226delACCinsCAGCCAGGAACTG	p.Thr1042GlnfsX75	Paternal	Yes	Pancreatectomy
ABCC8	c.2113C > T	p.Arg705X	Paternal	Yes	Pancreatectomy
ABCC8	c.1879delC	p.His627MetfsX20	Paternal	Yes	Diazoxide
ABCC8	c.1990C > T	p.Gln664X	No sample	Yes	Diazoxide
ABCC8	c.3224-3226delACC insCAGCCAGGAACTG	p.Thr1042GlnfsX75	Paternal	Yes	Diazoxide
ABCC8	c.3224-3226delACC insCAGCCAGGAACTG	p.Thr1042GlnfsX75	_	Yes	Diazoxide
ABCC8	c.4213G > A	p.Asp1405Asn	_	Yes	Diazoxide
ABCC8	c.4211T > C	p.Lle1404Thr	Maternal	Yes	Diazoxide
ABCC8	c.1792C > T c.3641G > A	p.Arg598X p.Arg1214Gln	No sample	Yes Yes	Diazoxide
GLUD1	c.1493C > T	p.Ser498Leu	_	Yes	Diazoxide
GLUD1	c.1387A > G	p.Asn463Asp	_	Yes	Diazoxide
GLUD1	c.965G > A	p.Arg322His	Maternal	Yes	Dietary
GLUD1	c.965G > A	p.Arg322His	Paternal	Yes	Dietary
GLUD1	c.965G > A	p.Arg322His	Paternal	Yes	Dietary

disease (29,37). However, Chandran et al (44) reported that heterozygous paternal mutations may also cause diffuse CHI. Paternal mutations causing diffuse disease may act via a different mechanism from that of recessive mutations (45).

Study Limitations

Our study has limitations. Firstly, our research is a single-center study and cross-sectional design. Future multicentre studies are necessary to obtain the long-term follow-up characteristics of such patients at the national level. Secondly, other genes associated with CHI were not tested in this study. A targeted gene panel for CHI or whole-exome sequencing (WES) analysis could be applied in these patients in the future.

Conclusion

In summary, a genetic diagnosis was made in 32% of CHI patients in this large cohort. Mutations in the *ABCC8* gene were the most common identifiable cause with a minority of variants found in the *GLUD1* gene. No mutations were identified in either *KCNJ11* or *GCK* genes. Some unique features of *ABCC8* gene mutations in southern Chinese

CHI patients with more novel and hot-spot mutations were identified. Diazoxide and dietary treatment were effective in most patients. In the remaining 68% of the patients, the genetic cause of hypoglycaemia remains unknown. A targeted gene panel for CHI or WES analysis could be applied in these patients.

Ethics

Ethics Committee Approval: The study was approved by the Ethical Committee of Guangzhou Women and Children's Medical Center (approval number: 2016022210).

Informed Consent: Consent form was filled out by all parents and participants.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Zhe Wen, Aijing Xu, Zhihong Zhou, Concept: Li Liu, Xiuzhen Li, Aijing Xu, Design: Aijing Xu, Jing Cheng, Data Collection or Processing: Zhihong Zhou, Chunhua Zeng, Cuiling Li, Analysis or Interpretation: Huiying Sheng, Yunting Lin, Yongxian Shao, Literature Search: Aijing Xu, Jing Cheng, Huiying Sheng, Writing: Aijing Xu, Jing Cheng, Xiuzhen Li.

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ORIGINAL ARTICLE

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Subclinical Myocardial Dysfunction Demonstrated by Speckle Tracking Echocardiography in Children with Euthyroid Hashimoto's Thyroiditis

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What is already known on this topic?

The cardiovascular system is affected by abnormal thyroid hormone levels, which are detected in overt hyperthyroidism, hypothyroidism and states of subclinical thyroid dysfunction. However, the effect on cardiovascular function in thyroid patients who are euthyroid on treatement is unclear.

What this study adds?

Impairment of global left ventricle myocardial function is present in children with Hashimoto's thyroiditis who are euthyroid on treatment and conventional echocardiography is inadequate to determine these changes. In this study, we demonstrated that speckle tracking echocardiography is a useful method in the early detection of myocardial dysfunction in children with euthyroid hashimoto's thyroiditis.

Abstract

Objective: Thyroid hormones have an important role in the regulation of the cardiovascular system. The aim of this study was to investigate the presence of subclinical myocardial dysfunction in children with euthyroid Hashimoto's thyroiditis (eHT) without evident heart disease using tissue doppler imaging (TDI) and speckle tracking echocardiography (STE) methods.

Methods: TDI and STE were peformed in 50 children with eHT and in 35 healthy children. To assess myocardial velocities and time intervals, including peak systolic velocity (S_m), peak early diastolic velocity (E_m), peak late diastolic velocity (A_m), isovolumetric contraction time (IVCT), isovolumetric relaxation time (IVRT) and ejection time (ET), TDI was performed at the base of the interventricular septum (IVS) and in the left and right ventricles (LV and RV, respectively). Analysis of myocardial deformation by STE including strain (S) and strain rate (SR) was performed globally in two planes, longitudinal (L) and mid-circumferential (C) in LV [LV global longitudinal strain (LVGLS), LV global longitudinal strain rate (LVGLSR), LV global circumferential strain (LVGCS), LV global circumferential strain rate (LVGCSR)] and RV [(RV global longitudinal strain (RVGLS), RV global longitudinal strain rate (RVGLSR)].

Results: Among TDI parameters, ET at LV and IVS were significantly lower, IVRT and myocardial performance index at LV and IVS were significantly higher in the eHT group compared to controls ($p = 0.001$). There were no significant differences in S_m, E_m, A_m and IVCT values between patients and controls. LVGLS, LVGLSR, LVGCS and LVGCSR values were significantly lower in patients than controls ($p = 0.01$). There was a negative correlation between thyroid antibody levels and LV global longitudinal and circumferential strain and strain rate values (TPO-Ab and Tg-Ab between LVGLS, LVGLSR, LVGCS and LVGCSR; $r = -411$, $p < 0.001$; $r = -541$, $p < 0.001$; $r = -430$, $p < .0.001$; $r = -502$, $r < 0.01$ and $r = -397$, $p < 0.001$; $r = -473$, $p < 0.001$; $r = -519$, $p < 0.001$; $r = -421$, $p < 0.00$, respectively)

Conclusion: The results show that myocardial function in children with eHT is impaired in the absence of any clinical symptoms and that conventional echocardiography is inadequate to determine these changes.

Keywords: Hashimoto's thyroiditis, myocardial function, speckle tracking echocardiography, children

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Introduction

Abnormal thyroid hormone levels in states of overt hyperthyroidism, hypothyroidism and subclinical thyroid dysfunction affect many biological functions including the cardiovascular system. However it is unclear if, changes in cardiac performance associated with overt thyroid dysfunction are the result of alterations in myocardial contractility or loading conditions or both remains unclear (1,2,3,4). Hashimoto's thyroiditis is the most commonly encountered, acquired thyroid function disorder in children (5). However, the cardiovascular effects of euthyroid Hashimoto's thyroiditis (eHT) are unclear. Current studies indicate that eHT may be associated with left and right ventricular myocardial dysfunction. It has been suggested that the cardiovascular effects of eHT might be related to the abnormal inflammatory state associated with autoimmunity as well as to endocrine effects (3,4,6,7,8,9,10).

The aim of this study was to evaluate myocardial function using tissue doppler imaging (TDI) and speckle tracking echocardiography (STE) methods in children with eHT with no obvious heart disease. STE is a method that has been recently developed that evaluates parameters of myocardial deformation, even in the absence of clinical signs of abnormal cardiac function (3,6,8). To our knowledge, there is no study which used both TDI and STE to assess both left ventricle (LV) and right ventricle (RV) function in children with eHT.

Assessment of myocardial parameters in eHT with normal LV ejection fraction (EF) may be informative because these echocardiographic indices assess the multidirectional function of the entire myocardium of the LV and RV.

Methods

In this study, TDI and STE for both LV and RV were performed in children with eHT and in healthy children. The relationship between changes in left ventricular myocardial mechanics and laboratory markers was also investigated.

Study Population

This cross-sectional and case-controlled study was conducted from January to December 2016. A total of 50 patients with eHT, aged 5-18 years were recruited from the Pediatric Endocrinology Outpatient Clinics of Ankara Children's Hematology and Oncology Research and Training Hospital. Detection of goiter was the reason for referral to the endocrinology department. The diagnosis of Hashimoto's thyroiditis was based on estimation of thyroid stimulating hormone (TSH), free triiodothyronine (fT3), free thyroxine (fT4), antithyroglobulin antibody (Tg-Ab) levels and antithyroid peroxidase antibody (TPO-Ab) levels, supported by ultrasonographic findings of thyroid parenchymal heterogeneity. The study included patients who presented to the pediatric endocrinology outpatient clinic with goiter and were diagnosed as eHT and remained euthyroid by clinical and laboratory findings for at least six months of follow-up. Inclusion criteria were positive antibodies against thyroid TPO-Ab and/or Tg-Ab, euthyroid function (TSH <6.0 mU/L, normal values for fT3 and fT4), Hashimoto's thyroiditis duration ≥6 months, normal LV EF (≥60 %), good metabolic control. Patients with a normal TSH levels and positive thyroid autoantibodies were evaluated once more after six months, and were included in the study if their TSH levels were still within normal levels and two thyroid autoantibodies were positive. None of the patients had any other systemic or autoimmune disease and were not on any medication. Patients who had congenital and organic heart disease, arrhythmia and anemia were excluded from the study. There were no children receiving thyroid hormone replacement therapy because hormone levels were within normal ranges in all patients.

The control group consisted of 35 age and gender matched, healthy children who had presented to the pediatric cardiology clinic for evaluation of innocent heart murmurs. The same physical examinations and laboratory investigations were performed in the control group. Children with abnormal findings on laboratory testing, electrocardiograms and echocardiography were excluded.

Clinical Data

Anthropometry and blood pressure measurements were carried out in both eHT patients and controls. Goiter staging was performed according to the definition proposed by Perez et al (11). After 12 hours of fasting, venous blood samples were taken to measure fT3, fT4, TSH concentrations and Tg-Ab and TPO-Ab levels by Elecsys Analyzer (Roche, Mannheim, Germany) using the electrochemiluminescence immunoassay method. Reference ranges used for thyroid hormones were: fT3: 0.18-0.44 ng/dL; fT4: 0.8-2.2 ng/dL; TSH: 0.27-4.2 μ IU/mL; Tg-Ab: 0-4 IU/mL; and TPO-Ab: 0-9 IU/mL. Systolic and diastolic blood pressure were measured using a standard mercury sphygmomanometer after 20 minutes of rest.

Thyroid Imaging Methods

Thyroid ultrasonography was performed by using a 10 MHz linear transducer (General Electric, Logic 7, Horten, Norway) by experienced radiologists. In all patients, findings of thyroid ultrasonography (size of thyroid glands,

parenchymal echogenicity) were recorded. Thyroid volume was calculated for each lobe by using the following formula: height x width x depth x 0.529 (11). Thyroid gland volume and volume standard deviation score were calculated using ÇEDD Çözüm Software (TPEDS Metrics) (12,13). Thyroid gland volume was assessed by comparison with age- and sex-adjusted thyroid volumes established by the World Health Organization (14).

Echocardiographic Examination

Conventional Echocardiography

A commercially available ultrasound system (iE33, Philips, The Netherlands, Eindhoven), equipped with a broadband (1-5 MHz) S5 transducer was used to obtain 2D grayscale harmonic images at a frame rate of 60-80 frames per second (frames/s). Two-dimensional and M-mode echocardiography was used to measure left ventricular end-diastolic and end-systolic diameter, end-diastolic septal and posterior wall thickness, EF and shortening fraction (FS), according to the guidelines of the American Society of Echocardiography (15).

Tissue Doppler Imaging

TDI measurements were performed on the basal septum and on the LV and RV lateral walls. Filters were set to exclude high frequency signals. Gain was minimized to obtain clear signals, and images were recorded at a velocity of 100 mm/s. The maximal systolic myocardial velocity (S_m), and early and late diastolic myocardial velocity (E_m and A_m) were measured. The isovolumetric contraction time (IVCT) was calculated from the beginning of QRS in the echocardiogram until the beginning of the S_m wave. Isovolumetric relaxation time (IVRT) was calculated from the end of the S_m wave until the beginning of the E_m wave. Ejection time (ET) was measured from the beginning to the end of the S_m wave. Mean values were recorded by averaging the results of three consecutive measurements. The myocardial performance index (MPI; Tei index), which is a doppler-derived index including both systolic and diastolic time intervals to generate a combined index of global ventricular function, was calculated according to the formula; (IVCT + IVRT)/ET (16).

Speckle Tracking Echocardiography

All two-dimensional STE analyses were performed by the same investigator to avoid inter-observer variability. Myocardial deformation parameters (S and SR) were measured using commercially available software (QLAB Advanced Quantification Software, version 6.0, TMQ, Philips Medical systems, Best, The Netherlands, Eindhoven) on standard 2D grayscale LV images from the standard apical 4-chamber view (AP4) for longitudinal strain and standard parasternal short axis at the papillary muscle level (PML) for circumferential strain. Two consecutive beats synchronized to a continous electrocardiography (ECG) were recorded with frame rate set to > 60 frames/s. The data were transferred to the QLAB software system for off-line analysis. The endocardial borders were identified manually to include the entire myocardium in all view areas. The following peak systolic LV and RV STE parameters were measured:

- LVGLS: Left ventricular global longitudinal strain at AP4,

- LVGLSR: Left ventricular global longitudinal strain rate at AP4,

- LVGCS: Left ventricular global circumferential strain at PML,

- LVGCSR: Left ventricular global circumferential strain rate at PML,

- RVGLS: Right ventricular global longitudinal strain at AP4,

- RVGLSR: Right ventricular global longitudinal strain rate at AP4.

Statistical Analysis

SPSS for Windows (version 18; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Kolmogorov-Smirnov test was used to analyze the distribution of continuous variables. Numeric variables are expressed as the mean ± standard deviation. Chi-square analysis were used to compare continuous and categorical variables between groups. Comparisons of demographic data and echocardiographic parameters between patients and controls were performed using Mann-Whitney U test for non-normally distributed variables. A difference was considered statistically significant at a p value of < 0.05. Spearman's correlation coefficient was used to disclose possible correlations between thyroid volumes, Tg-Ab, TPO-Ab and all echocardiographic data.

The number of patients that should be included in the study was calculated by Russ Lenth's power analysis software (www.stat.uiowa.edu/~rlenth/Power/). The control group of the study was smaller than the study group. For this reason the Power analysis was based on the "mean LVGLS levels" as main outcome, when the mean levels for the study and control groups was given as -23 and -25, respectively, and a common standard deviation of 3. The difference between the two groups can be compared with 34 cases in each group (total 68 cases) using the Independent Samples t-test with an effect size of 0.7 (medium), a two-sided p value of 0.05, and a power of 81 %.

Results

Clinical Characteristics of the Study Population

A total of 50 patients with eHT and 35 healthy controls were evaluated. The mean age of the patients was 12.5 ± 3.2 years. Of the patients, 37 were girls (74%) and 13 boys (26%). There was no significant difference in age, gender and body mass index (body mass index; kg/m^2) between the eHT group and the controls. Heart rate, systolic and diastolic blood pressure values were similar in both groups. No significant differences in fT3, fT4 and TSH levels were found between the groups. Compared to the control group, patients with eHT had significantly higher Tg and TPO antibody levels ($p < 0.001$). A stage 1a goiter in 16 (32%) and stage 1b goiter in 34 patients (68%) were detected. The mean thyroid volume in the patient cohort (n = 50) was 10.1 ± 3.5 mL (range: 4.9-16.0 mL). The patient and control groups had normal ECG findings. Baseline characteristics and laboratory results of study groups are given in Table 1.

Association of Thyroid Volume and Tg and TPO Antibody Levels

There was no correlation between Tg-Ab, TPO-Ab levels and thyroid volume in patients with eHT.

Conventional Echocardiographic Findings

The eHT and control groups were not significantly different for LV end-diastolic diameter, diastolic thickness of the interventricular septum and LV posterior wall or for left ventricular FS and EF. FS and EF were within normal limits in both groups. Conventional echocardiographic findings are summarized in Table 1.

Tissue Doppler Imaging Findings

TDI assessment of LV showed statistically significantly higher values of IVRT and MPI at IVS and LV in the eHT group compared to the control group. Additionally, ET values at LV were significantly lower in patients with eHT. There was no significant differences in S_m, E_m and A_m values between the groups ($p < 0.05$, Table 2). There were no significant differences in TDI values at RV in the eHT group compared to controls.

Speckle Tracking Echocardiographic Findings

The eHT group had statistically significantly lower LVGLS and LVGCS values compared to controls. Also LVGLSR and LVGCSR values were significantly lower in the eHT patients. There were no statistically significant differences for RVGLS and RVGLSR values between patients and controls (Figures 1, 2, Table 3).

Association of LV STE Parameters with Laboratory Markers and Thyroid Volume

There was a negative correlation between Tg-Ab, TPO-Ab levels and LV global longitudinal and circumferential strain and strain rates (Figures 3, 4, Table 4). However, there was no correlation between Tg-Ab, TPO-Ab levels and RVGLS and RVGLSR. In addition, thyroid volume showed no significant correlation with left ventricular global longitudinal and circumferential strain and strain rates (Table 4).

Discussion

Thyroid hormones exert significant effects on the cardiovascular system. Thyroid dysfunction is a condition which affects cardiac performance and it is related with the risk of heart failure. There are two main thyroid hormone receptor genes in the human heart. The receptors are encoded by two genes (TRα and TRβ), each of which

Table 1. Demographic and laboratory variables, thyroid volume and conventional echocardiographic findings in the study groups

	eHT group	Controls	p value
*Age, years	12.5 ± 3.2	12.8 ± 3.1	0.74
Female/male	37/13	22/13	0.45
*BMI, kg/m^2	20.2 ± 3.1	20.4 ± 2.3	0.51
*BSA, m^2	1.4 ± 0.3	1.3 ± 0.2	0.78
*HR, beats/min	79 ± 13	82 ± 14	0.69
*SBP, mmHg	110 ± 9	106 ± 8.6	0.25
*DBP, mmHg	69 ± 8	66 ± 7	0.36
*fT3, pg/mL	0.98 ± 0.5	1.1 ± 0.4	0.86
*fT4, pg/mL	0.95 ± 0.3	1.03 ± 0.4	0.81
*TSH, mIU/L	3.06 ± 1.3	2.6 ± 0.9	0.17
*Tg-Ab, IU/mL	147.1 ± 192.9	2.3 ± 1.4	0.001
*TPO-Ab, IU/mL	244 ± 279	2.6 ± 1.7	0.001
*Thyroid volume, mL	10.1 ± 3.3	-	-
*Thyroid volume, SDS	1.9 ± 1.3	-	-
*EF, %	68.7 ± 3.1	69 ± 2.7	0.21
*FS, %	35.9 ± 2.6	36.6 ± 2.1	0.08
*LVDd, mm	36.9 ± 5.9	37.3 ± 3.6	0.81
*IVSd, mm	7.2 ± 0.8	7.4 ± 0.8	0.14
*LVPWd, mm	7.3 ± 1.1	7.6 ± 0.8	0.49

*Values are presented as mean ± standard deviation.

eHT: euthyroid Hashimoto's thyroiditis, BMI: body mass index, BSA: body surface area, HR: heart rate, fT3: free triiodothyronine, fT4: free thyroxine, TSH: thyroid-stimulating hormone, TPO-Ab: thyroid peroxidase antibody, Tg-Ab: thyroglobulin antibody, EF: left ventricular ejection fraction, FS: left ventricular fractional shortening, LVDd: left ventricular end-diastolic diameter, IVSd: interventricular septum diastolic thickness, LVPWd: left ventricular posterior wall diastolic thickness, SBP: systolic blood pressure, DBP: diastolic blood pressure, SDS: standard deviation score

Table 2. Tissue doppler echocardiography measurements in the study groups

		eHT group	Controls	*p value
IVS	S_m, cm/s	7.8 ± 0.9	7.6 ± 0.7	0.51
	E_m, cm/s	13.6 ± 1.6	14.1 ± 2.3	0.07
	A_m, cm/s	6.4 ± 0.9	6.6 ± 0.9	0.13
	IVCT, ms	56.1 ± 6.7	57.8 ± 3.3	0.07
	IVRT, ms	59.9 ± 4.2	57.9 ± 3.9	0.001
	ET, ms	253 ± 19.6	261 ± 20.3	0.10
	MPI	0.48 ± 0.05	0.41 ± 0.04	0.001
LV	S_m, cm/s	9.4 ± 1.7	8.8 ± 1.7	0.22
	E_m, cm/s	15.7 ± 1.6	16.1 ± 1.2	0.37
	A_m, cm/s	7.3 ± 1.2	7.4 ± 0.8	0.33
	IVCT, ms	58.1 ± 7.1	59.7 ± 4.2	0.35
	IVRT, ms	59.8 ± 3.9	57.6 ± 2.8	0.001
	ET, ms	250 ± 24.3	274 ± 21.7	0.001
	MPI	0.49 ± 0.06	0.43 ± 0.03	0.001
RV	S_m, cm/s	10.4 ± 2.1	9.5 ± 1.4	0.06
	E_m, cm/s	14.4 ± 1.5	14.7 ± 1.1	0.29
	A_m, cm/s	7.1 ± 0.9	7.4 ± 1.3	0.09
	IVCT, ms	57.1 ± 4.9	58.5 ± 3.2	0.06
	IVRT, ms	55.8 ± 8.5	57.6 ± 2.7	0.09
	ET, ms	245 ± 24.8	255 ± 16.8	0.09
	MPI	0.47 ± 0.06	0.46 ± 0.03	0.84

Values are presented as mean ± standard deviation.

*p < 0.05 for statistical significance.

eHT: euthyroid Hashimoto's thyroiditis, IVS: interventricular septum, LV: left ventricle, RV: right ventricle, S_m: peak systolic myocardial velocity, E_m: peak early diastolic myocardial velocity, A_m: peak late diastolic myocardial velocity, ET: ejection time, IVCT: isovolumetric contraction time, IVRT: isovolumetric relaxation time, MPI: myocardial performance index

Table 3. Speckle tracking echocardiography measurements in the study groups

	eHT group	Controls	*p value
LVGLS, %	-20.7 ± 2.7	-24.1 ± 3.1	0.01
LVGLSR, s^{-1}	-0.8 ± 0.2	-1.1 ± 0.2	0.01
LVGCS, %	-20.8 ± 4.1	-25.4 ± 3.4	0.01
LVGCSR, s^{-1}	-0.9 ± 0.2	-1.1 ± 0.2	0.01
RVGLS, %	-23.5 ± 2.3	-24.2 ± 2.2	0.12
RVGLSR, s^{-1}	-1.1 ± 0.3	-1.2 ± 0.1	0.07

Values are presented as mean ± standard deviation.

*p < 0.05 for statistical significance.

eHT: euthyroid Hashimoto's thyroiditis, LVGLS: left ventricle global longitudinal strain, LVGLSR: left ventricle global longitudinal strain rate, LVGCS: left ventricle global circumferential strain, LVGCSR: left ventricle global circumferential strain rate, RVGLS: right ventricle global longitudinal strain, RVGLSR: right ventricle global longitudinal strain rate

undergoes alternate splicing to generate receptor subtypes with differing tissue distributions. The TRα has been shown to play an important role in regulation of cardiac genes. T3 is the biologically active form of thyroid hormone and effects the heart by increasing some of these genes (1,2,3,4,17,18,19,20). The impact of hyperthyroidism or hypothyroidisim on the cardiovascular system is well known. Hyperthyroid patients have an increased heart rate and stroke volume that result in a high cardiac output state. An increased prevalence of LV hypertrophy and increased LV contractility has been reported in patients with overt hyperthyroidism. In contrast hypothyroid patients have low heart rate and low stroke volume that results in low cardiac output. Additionally, overt hypothyroidism has been reported as associated with decreased cardiac contractility (15,16,17,18). A recent study showed that long-term thyroid hormone replacement in euthyroid patients after myocardial infarction significantly improved LV contractility (21,22,23).

The cardiovascular effect of eHT in adults have been extensively studied (6,10). However, reasons for changes in cardiac performance in euthyroid patients remain unclear and children with eHT may be at higher risk for developing cardiovascular diseases (10,24).

Deleterious effects of eHT on the LV and RV systolic and diastolic functions have been reported, indicating that Hashimoto's thyroiditis affects myocardial function regardless of thyroid hormone levels (6,10). Conventional echocardiography and TDI can be used to evaluate both the systolic and diastolic function of the heart in hypothyroid and hyperthyroid state, but the diagnostic value of conventional echocardiography is limited in the early phase

Table 4. Correlation between left ventricular speckle tracking echocardiography parameters and thyroid antibody levels and volume in euthyroid Hashimoto's thyroiditis group

	TPO-Ab	Tg-Ab	Thyroid volume
LVGLS	r = -411; p < 0.001	r = -397; p < 0.001	**r = -0.09; p = 0.256**
LVGLSR	r = -541; p < 0.001	r = -473; p < 0.001	**r = -0.59; p = 0.684**
LVGCS	r = -430; p < 0.001	r = -519; p < 0.001	**r = -0.75; p = 0.602**
LVGCSR	r = -502; p < 0.001	r = -421; p < 0.001	**r = -0,094; p = 0.517**

r = correlation coefficient, p = significance level (p < 0.05 for statistical significance).

LVGLS: left ventricle global longitudinal strain, LVGLSR: left ventricle global longitudinal strain rate, LVGCS: left ventricle global circumferential strain, LVGCSR: left ventricle global circumferential strain rate

of cardiac dysfunction (25,26). The impact of Hashimoto's thyroiditis on myocardial systolic and diastolic functions has been studied using TDI in some previous studies (5,6,10). In some recently reported studies, evaluation of left ventricular systolic function with conventional echocardiographic method were found to be normal but left ventricular systolic dysfunction was demonstrated by TDI and STE methods even in euthyroid stage of patients with Hashimato's thyroiditis. Furthermore STE is found to be a more sensitive parameter that shows left ventricular function (6,16,20). In this study, TDI of the IVS showed significant longer IVRT and shorter ET, consequently a higher Tei index. Additionally, LV-Tei index was significantly increased in the eHT group and this increase is related more to prolongation of IVRT than to shortening of ET, thus reflecting the impairment in both systolic and diastolic functions. Tei index was found to be more sensitive in the evaluation of diastolic relaxation than parameters such as deceleration time and E/A ratio, as previously reported (23,24). Akgul et al (6) also reported an impairment of global LV performance in adult patients with eHT. They showed an impaired Tei index and TDI-derived diastolic parameters despite normal findings by conventional echocardiography.

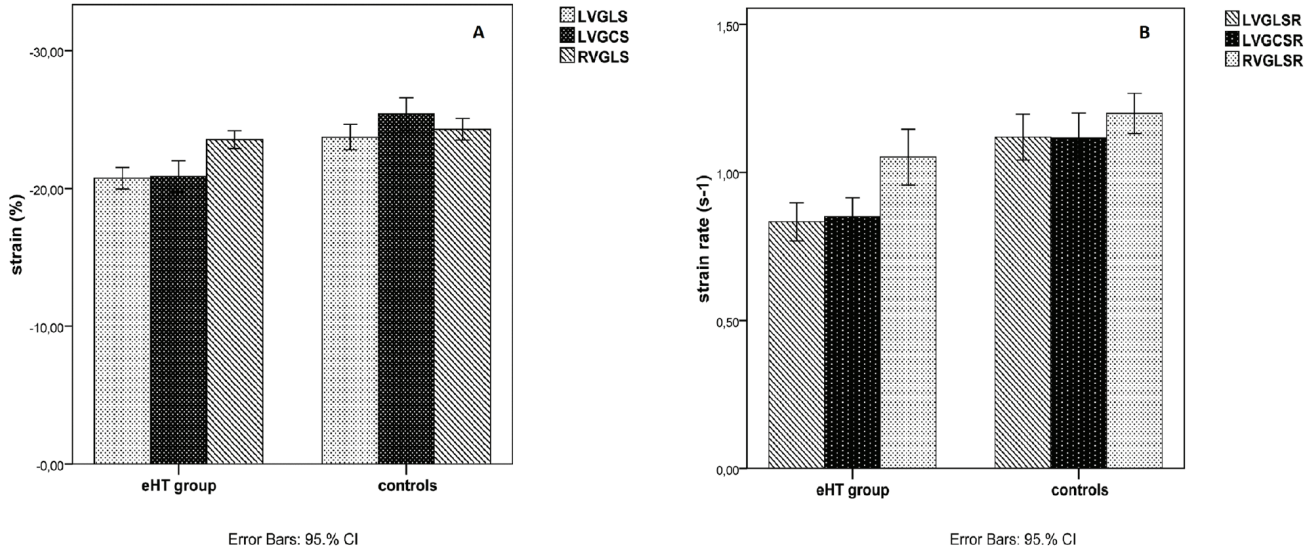

Figure 1. Myocardial strain and strain rate values. A) LVGLS: Left ventricle global longitudinal strain, LVGCS: Left ventricle global circumferential strain, RVGLS: Right ventricle global longitudinal strain. B) LVGLSR: Left ventricle global longitudinal strain rate, LVGCSR: Left ventricle global circumferential strain rate, RVGLSR: Right ventricle global longitudinal strain, RVGLSR: Right ventricle global longitudinal strain rate

eHT: euthyroid Hashimoto's thyroiditis

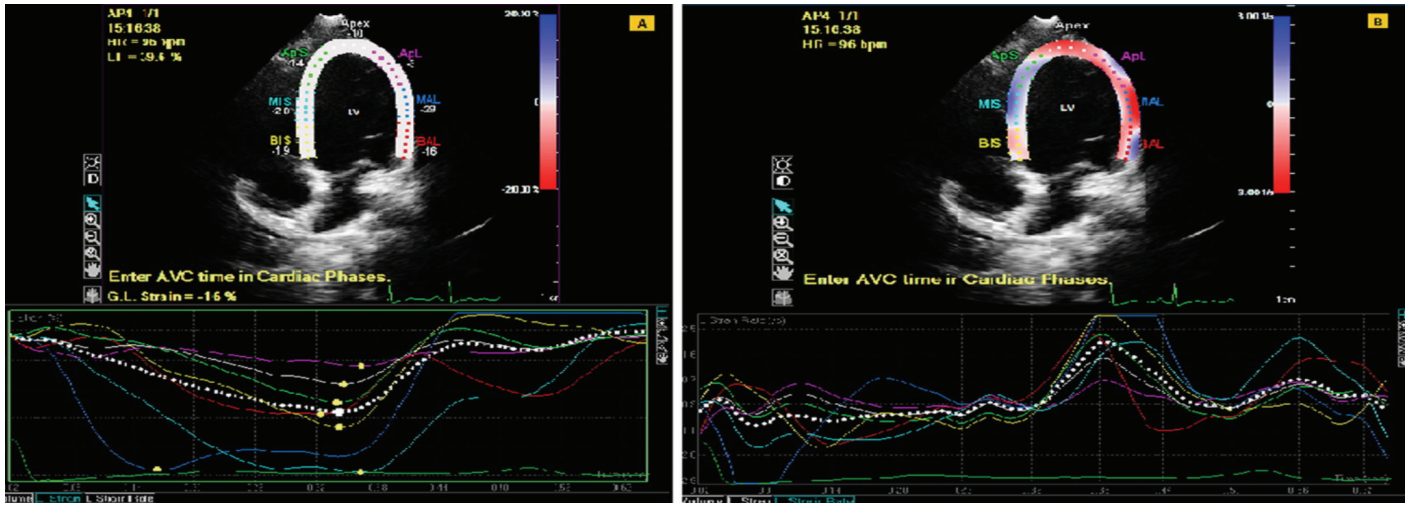

Figure 2. Two dimensional strain and strain rate analysis through speckle tracking echocardiography imaging of euthyroid Hashimoto's thyroiditis. A) Strain analysis, B) strain rate analysis

Recently new imaging techniques have been introduced to evaluate myocardial mechanics. STE is a novel echocardiographic method and strain and strain rate obtained by STE provides an opportunity for quantitative assessment of cardiac function. STE can be used as a diagnostic method in the early stages of many cardiomyopathic diseases. Myocardial global longitudinal strain values were shown to have reduced without any changes in conventional echocardiographic parameters (27). Subclinical myocardial dysfunction can be detected early by TDI and STE methods. STE is a more recent technique that provides a global approach to ventricular myocardial mechanics and cardiac deformation and appears to be a sensitive diagnostic method for early detection of myocardial involvement in asymptomatic patients (6,8,27). We are not aware of any studies that have investigated myocardial functions by STE in children with eHT. This present study aimed to detect myocardial involvement in the euthyroid stage of HT. Recent studies showed that eHT is associated with an increased pulsed-wave velocity, independent of arterial atheromatosis indicating a direct impact of this disorder on arterial stiffening (6,7,16,28,29). Akgul et al (6), concluded that heart rate variability is significantly reduced in Hashimoto's thyroiditis patients as a result of cardiac autonomic dysfunction, even at the euthyroid stage. Therefore, mechanisms that may explain cardiac autonomic and functional changes in eHT are probably related with abnormal cytokine profiles. However, the molecular, physiological and clinical evidence is still controversial (2,17,18,28,29).

The underlying pathophysiologic mechanism leading to the cardiovascular effects of eHT have not yet been fully understood. Some mechanisms leading to cardiovascular system involvement have been reported previously in patients with Hashimoto thyroiditis.

Firstly, the majority of eHT patients are in a state of slow, progressive thyroid dysfunction. It is widely acknowledged that most of these patients will progress to a state of hypothyroidism. Thus, it may be hypothesized that the insidious progression to thyroid dysfunction in Hashimoto's thyroiditis may be responsible for the cardiovascular adverse effects, even in subjects with normal serum thyroid hormone levels (7,28,29).

Secondly, the spectrum of clinical signs may change during the course of HT. Thus, eHT patients may have been hypothyroid or hyperthyroid previously even though they are euthyroid at the time of assessment and LV and RV functional changes might be due to a previous hypothyroid or a hyperthyroid phase (24). In the present study, we have shown that Hashimoto's thyroiditis is associated with subclinical LV systolic and diastolic dysfunction, even when the patients are euthyroid. Conventional echocardiography does not exclude subclinical left ventricular wall motion abnormalities in patients with eHT. The myocardial dysfunction could be identified as a reduction of LV global and circumferential strain and strain rate and TDI derived Tei index. Accordingly, we showed that children with eHT had a significantly lower left ventricular strain and strain rate values, as well as decreased IVS and LV Tei index values compared to controls.

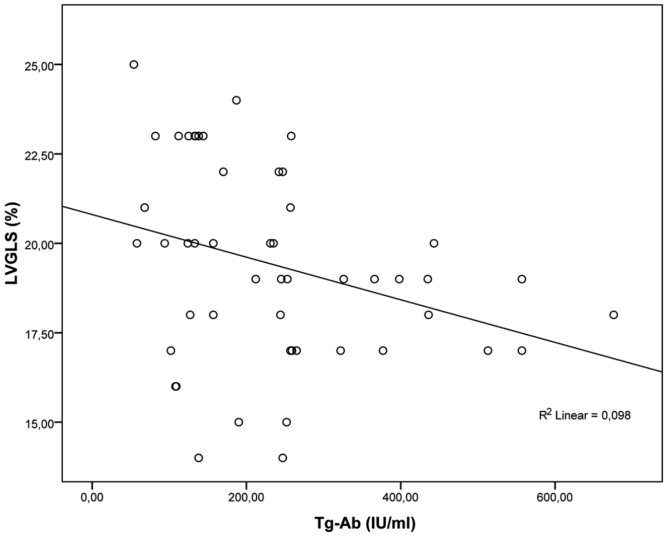

Figure 3. The correlation between myocardial strain and Tg-Ab levels

LVGLS: left ventricle global longitudinal strain, Tg-Ab: antithyroglobulin antibody

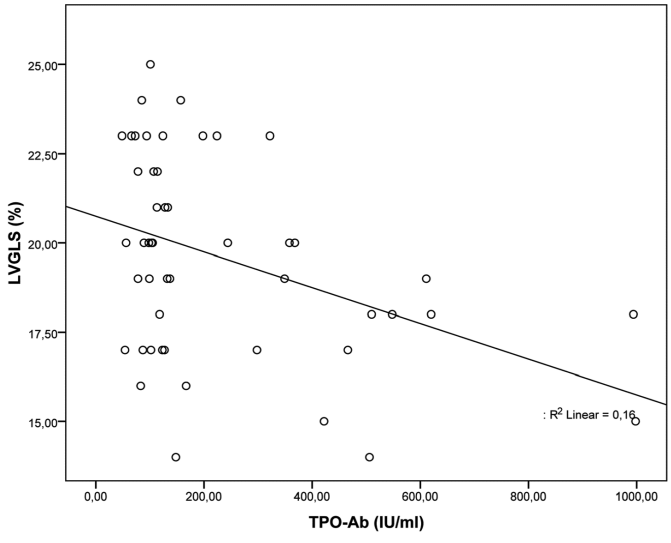

Figure 4. The correlation between myocardial strain and TPO-Ab levels

LVGLS: left ventricle global longitudinal strain, TPO-Ab: antithyroid peroxidase antibody

Thirdly, the autoimmune state associated with Hashimoto's thyroiditis could be the responsible for cardiovascular changes, rather than the effects of secreted hormones. Autoimmunity induced endothelial dysfunction and inflammation may have an important role in the pathogenesis of cardiovascular conditions seen in these patients, such as hypertension, atherosclerosis and myocardial dysfunction (6,10,28,29). In Hashimoto's thyroiditis, it has been reported that goiter may be present either due to lymphocytic infiltration of the thyroid gland or to increased TSH levels caused by hypothyroidism. However, there is a controversy regarding the role of antibodies in the development of goiter (30). In our study, no significant correlation was detected between serum level of thyroid antibodies and thyroid volume. In addition, no significant correlation was detected between thyroid volume and STE parameters. In these patients, there is typical heterogeneous hypoechogenic parenchyma on thyroid sonography. In our cases, serum TSH concentrations were within normal range. In fact, it may be more relevant to evaluate the relationship between serum antibody level and heterogeneity of thyroid gland parenchyma rather than volume. Moreover, we found a correlation between serum thyroid antibody levels and STE paramaters in our patients. Subclinical systolic and diastolic dysfunction of the LV appeared to be significantly related to TPO-Ab and Tg-Ab levels. So, our findings suggest that the autoimmune state associated with Hashimoto's thyroiditis, rather than abnormal secreted hormone concentrations, could be responsible for the cardiovascular effects.

Study Limitations

Our study has several limitations. Firstly, the relatively small number of patients could be considered as a limitation. Secondly, we did not investigate the effect of thyroid replacement therapy on LV and RV functions in eHT patients.

Further clinical research is needed with larger patient groups to investigate the mechanisms on myocardial dysfunction with normal LV EF in eHT patients.

Conclusion

In this study, it was demonstrated that STE is useful in the early detection of myocardial dysfunction in patients with eHT. Impairment of global LV myocardial function is present in children with Hashimoto's thyroiditis who are euthyroid and replacement therapy naive. In addition, conventional echocardiography was inadequate to detect these changes. It is important to increase the data available in this field, particularly prospective data. There is also a need for additional prospective data related to cardiac function in eHT patients. Subclinical myocardial dysfunction in the early disease may be considered as an indication for initiation of thyroid replacement treatment even in euthyroid patients.

Ethics

Ethics Committe Approval: All procedures performed in the study involving patients were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was started after the approval of the Ethics Committee of University of Health Sciences, Ankara Child Health and Diseases Hematology Oncology Training and Research Hospital (number of ethical approval: 2016/071).

Informed Consent: Written informed consent was obtained from parents of all patients included in the study.

Peer-review: Externally peer reviewed.

Authorship Contributions

Consept: Emine Azak, İbrahim İlker Çetin, Seyit Ahmet Uçaktürk, Design: Emine Azak, İbrahim İlker Çetin, Seyit Ahmet Uçaktürk, Data Collection and Processing: Emine Azak, Eda Mengen, Utku Pamuk, Analysis and Interpretation: Emine Azak, İbrahim İlker Çetin, Seyit Ahmet Uçaktürk, Literature Research: Emine Azak, Eda Mengen, Hazım Alper Gürsu, Writing: Emine Azak, İbrahim İlker Çetin.

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CASE REPORT

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A Novel Nonsense Mutation of *PHF6* in a Female with Extended Phenotypes of Borjeson-Forssman-Lehmann Syndrome

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What is already known on this topic?

Borjeson-Forssman-Lehmann syndrome (BFLS) is a rare X-linked disease caused by *PHF6* mutations. The features of classic BFLS include intellectual disability, developmental delay, obesity, epilepsy, characteristic face and anomalies of fingers and toes. Endocrine deregulation in BFLS has been reported but not well delineated.

What this study adds?

We report a female with a novel nonsense mutation c.673C > T (p.R225X) in the *PHF6* gene. She exhibited certain features beyond the classic BFLS, including complete deficiency of growth hormone and a horseshoe kidney. Adverse effects were elicited after growth hormone treatment in this patient, which has not been previously reported and suggests caution in the use of growth hormone in this condition. We also reviewed all the BFLS case reports and summarized data on their endocrine presentations and treatment.

Abstract

Borjeson-Forssman-Lehmann syndrome (BFLS) is a rare X-linked disease caused by *PHF6* mutations. Classic BFLS has been associated with intellectual disability (ID), developmental delay (DD), obesity, epilepsy, typical facial features and anomalies of fingers and toes. Endocrinological phenotypes and outcome of treatment in this condition remain to be delineated. Here we report a patient who exhibited complete growth hormone deficiency who responded to hormonal treatment but with adverse effects. Horseshoe kidney was present in this patient, which is also atypical in BFLS. A heterozygous nonsense mutation c.673C > T (p.R225X) of *PHF6* gene was identified in the patient, inherited from her unaffected mother. Both the patient and her mother showed highly skewed X-inactivation. We reviewed the phenotypes of all reported BFLS cases, and summarized their endocrine presentations. This first report of an Asian patient with BFLS further delineated the genetic and phenotypic spectrum of the syndrome. The adverse effect experienced by the patient suggests caution in the use of growth hormone treatment in this condition.

Keywords: Borjeson-Forssman-Lehmann syndrome, *PHF6*, X-inactivation, growth hormone deficiency, rhGH treatment, hypogonadism

Introduction

Borjeson-Forssman-Lehmann syndrome (BFLS), first described in 1962, is a rare X-linked disease (1). So far, about 33 families or sporadic cases have been reported, with 64 patients total (2,3,4). It is characterized by moderate to severe intellectual disability (ID), developmental delay (DD), obesity, epilepsy, hypogonadism, characteristic face and anomalies of fingers and toes (5). This X-linked condition usually affects males, but mild to severe symptoms are present in female carriers and most of them have highly skewed X-inactivation (6). In 2002,

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Lower et al (7) identified *plant homeodomain finger 6 (PHF6)* as the causal gene of BFLS. Since then, 29 different mutations have been reported in *PHF6* and, among these, 14 mutations were identified in affected females (2,3,4,8,9,10,11,12). All the patients and variants identified were of European ethnicity. In addition, 27 of the BFLS patients were reported to have endocrine abnormalities (10,13,14,15,16,17,18,19,20,21,22, 23,24,25). These hormonal abnormalities have not been well summarized so far.

Here we report a Chinese female with a nonsense mutation in the *PHF6* gene, inherited from her mother. Following a thorough review of all reported BFLS cases, we identified some features in this patient beyond those typical of BFLS. In addition, the endocrine aspect of BFLS patients were reviewed and summarized for the first time in the relevant literature. The genetic and phenotypic spectrum of BFLS is discussed.

Case Report

A 9 year 1 month old girl presented to the Genetic Endocrinology Clinic with complaints of ID and short stature. Her height was 123 cm [-2 standard deviation (SD)]; height age was 7 years. Her weight was 23 kg (-1.3 SD), and body mass index was 15.2 (25^{th}-50^{th} percentile). The height of her father and mother were 168 cm and 157 cm respectively, and the familial target height of the patient was 156 cm (-0.85 SD). She was born by caesarean section post-term, with no history of asphyxia. Her birth weight and body length were 4.25 kg and 50 cm respectively. Severe DD was noticed at toddler stage; she walked alone at the age of three years and could speak a few simple words at the age of five years. She presented with the typical facial features of BFLS, including coarse face, sparse hair, narrow forehead, ptosis, deep-set eyes, broad nasal tip, short nose, malformed teeth and large ears with earlobes of moderate size (Figure 1A, 1B, 1C). She had tapering fingers and fifth curved fingers bilaterally (Figure 1D). She also had flat feet and the fourth toes were shorter than the fifth (Figure 1E). Extensive hyperpigmentation was observed all over the body, but especially on the lower limbs. No secondary sexual characteristics were present at the time of examination. Breast and pubic hair were at stage B1 and P1 respectively (according to the Tanner scale).

Thyroid and liver function tests revealed normal results, but she suffered from a complete deficiency of growth hormone (see Table 1). Her stature was below the 3^{rd} percentile and her bone age was $7^{10/12}$ years. Due to the complete lack of growth hormone, recombinant human growth hormone (rhGH) injections were commenced at a dose of 0.036 mg/kg/day. However, after three weeks she developed edema in both lower extremities, and the hormonal treatment was ended.

Ultrasonography showed that she had fused kidneys at the lower end (horseshoe kidney). Brain MRI revealed periventricular leukomalacia and hyaline compartment formation. The pituitary appeared thinner than girls of the same age, though definitive measurement of the pituitary size was not performed. Her karyotype was 46,XX and chromosomal microarray did not reveal pathogenic variants. Her mother was unaffected, at least no obvious signs of symptom based on the reports of the family, though no formal evaluation was performed.

Clinical information concerning the patient was collected in Shanghai Children's Medical Center in 2012 (see Table 2). Written consent was obtained from the patient's parents.

For whole exome sequencing, genomic DNA was extracted from ethylene diamine tetra acetic acid-treated peripheral blood. Library preparation was performed on the proband with xGen Exome research panel v1.0 (Integrated DNA Technologies Inc, Coralville, Iowa, USA). The captured DNA fragments were subsequently sequenced by HiSeq 4000 (Illumina Inc, San Diego, California, USA). The data were analyzed as previously described (26). The pattern of X-chromosome inactivation in our patient and her mother was evaluated by assays of differential methylation in the genes between the active and the inactive chromosome X based on methylation-specific polymerase chain reaction (PCR) (27).

Results

The clinical features of the proband are presented in Figure 1A, 1B, 1C, 1D and 1E and Table 2. For comparison with previously reported phenotypes, we reviewed the

Table 1. Results of endocrine tests

Thyroid function			Growth hormone stimulation test (ng/mL)	
FT3	**6.99 pmol/L (3.8-9.4)**		**Arginine**	**Clonidine**
FT4	16.72 pmol/L (7.9-16.0)	0 min	1.902	0.450
TSH	3.28 uIU/mL (0.3-5.6)	30 min	0.362	0.083
IGF-1	IGF-BP3	60 min	0.122	1.005
48.5 ng/mL (84-495)	3.4 ug/mL (3.4-11.8)	90 min	0.260	0.347

TSH: thyroid stimulating hormone, IGF-1: insulin-like growth factor-1, IGF-BP3: insulin-like growth factor binding protein 3

description of a total of 20 female and 43 male BFLS patients in the literature (Table 2) and summarized the endocrinological presentations (Table 3). Whole exome sequencing revealed a heterozygous nonsense mutation c.673C>T; p.R225X (NM_001015877) of *PHF6* gene in the proband. Sanger sequencing of the proband and her parents demonstrated that the heterozygous mutation was inherited from her mother. No other variant with clinical significance was identified. Methylation-specific PCR of peripheral blood DNA indicated a highly skewed X-inactivation in the patient (98:2) and in her mother (95:5) (Figure 1F).

Discussion

BFLS is an X-linked syndrome caused by variants in *PHF6* (7,8). The most prevalent features, as observed in >80% of reported BFLS cases were: ID, delay in walking, delay in speech, coarse facies, dental abnormalities, large ears and finger deformities in females. Additionally, genital anomalies and gynecomastia have been frequently reported in male BFLS patients.

The phenotypic features of our patient largely conform to the description of BFLS based on patients of European ancestry. However, complete deficiency of growth hormone was not reported in previous cases. Our patient's height was below the 3rd percentile, which has been reported in 14% of female BFLS patients previously (3). She developed edema in the lower extremities after injection of rhGH (before the *PHF6* mutation was identified). Peripheral edema has occurred in 1:100-1:10000 of patients receiving rhGH therapy (28), possibly due to the impact on fluid homeostasis with retention of water and sodium (29). To date, a total of five BFLS patients have been reported

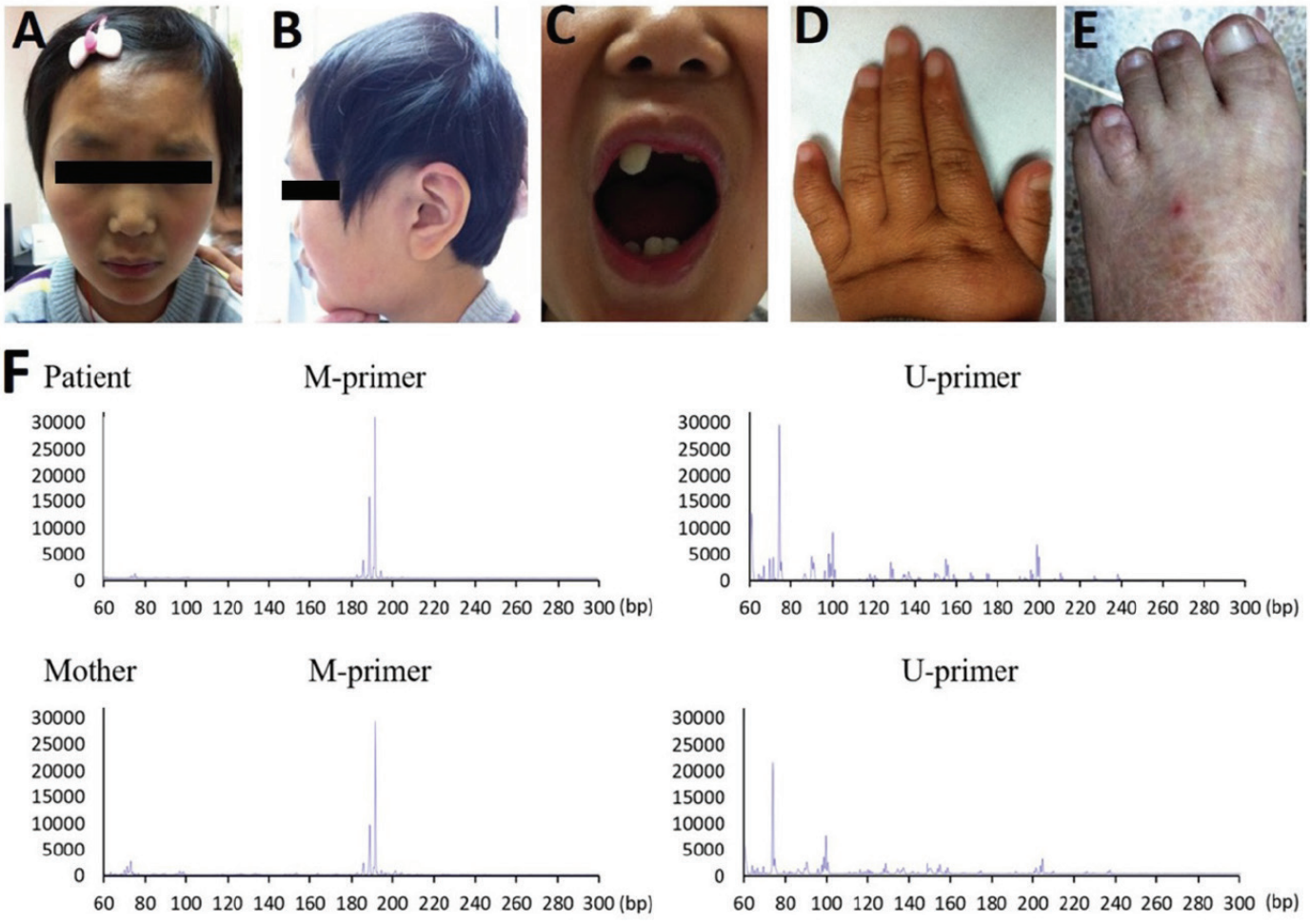

Figure 1. A, B, C, D, E, F) Pictures of our patient at age 9 years. A, B) Facial characteristics, C) Dental abnormalities, D, E) Hand and foot of the patient. The fifth fingers are short and curved, and the fourth toes are short. F) Results of the methylation-specific polymerase chain reaction assay. The inactivated X-chromosome sequence was amplified by the M-primer, the activated X-chromosome sequence was amplified by the U-primer. The result indicated a highly skewed X-inactivation in the patient (98:2) and in her mother (95:5)

Table 2. Clinical information on our patient and on reported Borjeson-Forssman-Lehmann syndrome patients

Patients	Our patient	Frequency in females	Frequency in males
Gender	Female	21 females affected (among 48 carriers)	43 males
Inheritance	Maternally inherited	9 maternally inherited 12 *de novo*	41 maternally inherited
Age	9y-1m	2y-32y	10m-59y
Growth			
Birth weight	Normal, 4.25 kg	/	/
Birth length	Normal, 50 cm	/	/
Abnormal weight	< P10	0/19 low weight 37% (7/19) obesity	3% (1/32) low weight 72% (23/32) obesity
Short stature	< P3	14% (3/21)	35% (13/37)
Abnormal bone age	+, 7y-10m	/	/
Neurological abnormalities			
Intellectual disability	+	86% (18/21)	100% (43/43)
Delay in walking	+	92% (12/13)	91% (21/23)
Delay in speech	+	91% (10/11)	92% (23/25)
Epilepsy	-	19% (4/21)	8% (3/39)
Behavioral anomalies	-	29% (6/21)	36% (4/11)
Vision anomalies	-	67% (8/12)	/
Hearing loss	-	23% (3/13)	/
Craniofacial features			
Coarse face	+	92% (12/13)	84% (27/32)
Hyperpigmentationn	+	77% (10/13)	/
Sparse hair	+	62% (8/13)	50% (5/10)
Narrow forehead	-	54% (7/13)	10% (1/10)
Ptosis	+	8% (1/13)	/
Synophrys	-	29% (6/21)	/
Deep-set eyes	+	44% (4/9)	100% (31/31)
Thick eyebrows	-	38% (8/21)	/
Arched eyebrows	-	62% (8/13)	/
Eyelid narrow	+	14% (3/21)	71% (5/7)
Broad nasal tip	+	85% (11/13)	64% (7/11)
Short nose	+	85% (11/13)	50% (4/8)
Large mouth	-	15% (2/13)	13% (1/8)
Dental abnormalities	+	92% (11/12)	100% (2/2)
Cleft palate	-	10% (2/21)	0
Large ears	+	86% (18/21)	100% (25/25)
Hirsutism	-	19% (4/21)	0
Skeletal features			
Tapering finger	+	67% (4/6)	75% (6/8)
Deformity of fingers	+	90% (9/10)	89% (8/9)
Deformity of toes	+	57% (12/21)	78% (7/9)
Viscera development			
Genital anomalies	-	19% (4/21)	92% (23/25)
Gynecomastia	/	/	97% (31/32)

Table 2. Continued

Patients	Our patient	Frequency in females	Frequency in males
Abnormal brain MRI	+	55% (6/11)	/
Cardiac defect	-	18% (2/11)	/
Renal anomalies	+	83% (5/6)	/
Skewed X-inactivation in blood	+	94% (17/18) (among patients) 88% (38/43) (among carriers)	/

y: years, m: month, +: positive, -: negative, /: not known, MRI: magnetic resonance imaging

Table 3. Summary of hormone levels in Borjeson-Forssman-Lehmann syndrome patients

Reference	Thyroid function	LH	FSH	E2	T	GH	PRL	Other
Female								
Our patient	-	/	/	/	/	↓	/	
Berland et al (13)	-	↓	↓	-	/	/	/	
Crawford et al (10)	↓	/	/	/	/	/	/	
Birrell et al (15)	↓	/	/	/	/	/	/	
Petridou et al (17)	-	-	-	/	/	-	-	
Matsuo et al (22)	-	-	-	-	/	/	/	
Robinson et al (23) #1	↓	↓	↓	↓	-	↓	↓	
Robinson et al (23) #2	↓	↓	↓	↓	-	↓	↓	
Male								
de Winter et al (14)	/	/	/	/	-	/	/	
Carter et al (16) #1	/	-	-	/	/	/	/	
Carter et al (16) #2	/	-	-	/	↓	/	/	
Birrell et al (15) #1	↓	↓	↓	/	/	↓	/	ACTH↓
Birrell et al (15) #2	↓	↓	↓	/	/	↓	/	ACTH↓
Baumstark et al (18) #1	/	-	↑	/	↓	/	/	
Baumstark et al (18) #2	/	-	-	/	↓	/	/	
Baumstark et al (18) #3	/	-	-	/	↓	/	/	
Baumstark et al (18) #4	/	-	-	/	-	/	/	
Turner et al (19) #1	-	↓	-	/	↓	/	/	
Turner et al (19) #2	/	-	/	/	↓	/	/	
Dereymaeker et al (20)	-	-	-	/	/	-	/	Cortisol-
Ardinger et al (21) #1	/	-	↓	/	↓	/	/	
Ardinger et al (21) #2	/	-	↓	/	↓	/	/	
Ardinger et al (21) #3	/	-	↑	/	↓	/	/	
Ardinger et al (21) #4	/	-	↑	/	↓	/	/	
Ardinger et al (21) #5	/	-	-	/	/	/	/	
Robinson et al (23)	-	↑	↑	-	↓	↓	↑	
Veall et al (24)	-	/	/	/	-	-	-	
Weber et al (25)	-	-	-	/	↓	/	/	Cortisol-

-: normal, ↑: increase, ↓: decrease, /: not known

LH: luteinizing hormone, FSH: follicle-stimulating hormone, E2: estradiol, T: testosterone, GH: growth hormone, PRL: prolactin, ACTH: adrenocorticotropic hormone

to have growth hormone deficiency (Table 3) and two of these presented with multiple pituitary hormone deficiency. The authors reported no improvement of stature after GH treatment (15). Considering the adverse effect in our patient, GH use in this condition may not be helpful and should be administered with caution. This is compounded by recent

research showing that *PHF6* mutation may be associated with pediatric leukemia (30).

Genital anomalies were reported in 59% (27/46) of patients. Early literature reported that hypogonadism was caused by hypophyseal dysfunction (1), but recent publications reporting a male patient with low testosterone and elevated LH and FSH, and another patient with abnormal testicular tissue, suggested that both central and gonadal deregulation may occur (23). Review of the literature reveals that the concentration of estradiol was reduced in 2/4 of the female patients and that of testosterone in 12/15 of the male patients. Gonadotrophin concentrations were found to be below reference values in 8/23 patients and hypothyroidism was reported to be present in 6/15 patients, also suggesting that both central and end-organ dysfunction may play a role in BFLS.

As reported in previous studies, hyperpigmentation is common in female BFLS patients with 10 of 13 female patients being hyperpigmented (3,13). Most of these patients were reported to have linear pigmentation in the extremities or individual spots in the armpit (3,13). However in our patient the hyperpigmentation was extensively distributed over the feet and legs. Mosaicism may be the cause for the different presentation in this case. The exact mechanism of hyperpigmentation in BFLS is unknown.

One additional feature of our patient that does not fit the description of classic BFLS is presence of a horseshoe kidney. In an earlier report, clinical phenotypes of BFLS were noted to partially overlap with the Coffin-Siris syndrome (CSS) (12), particularly in infancy among female patients (4). CSS is characterized by ID, typical facial features, hypoplasia/aplasia of the fifth digit of finger/toenail, and organ malformations including horseshoe kidney (4,12). Our patient exhibited many phenotypes overlapping with CSS, including presence of horseshoe kidney. We specifically reviewed the variants of CSS-related genes identifed in our patients, and no pathogenic variant was found. Therefore the similar phenotypes should not be attributed to CSS-related variants. It is well established that *PHF6* interacts with the nucleosome remodeling and deacetylation complex, implicated in chromatin remodeling, and thus functional interaction may exist between *PHF6* and SWI/SNF complex proteins, which are the main factors responsible for CSS (3). This may explain the overlapping features of these two syndromes.

As indicated in Table 2, the penetrance in female carriers is about 44% (21/48). 38/43 of females with *PHF6* mutations had highly skewed X-inactivation, but only 18 of them were affected. Our patient and her mother had the same genotype and similar skewing in X-inactivation. However, their clinical manifestations were quite different, suggesting mosaicism as a contributing factor to the variable expression of the phenotype (12). At the same time, this phenomenon suggests that in obligate carriers of *PHF6* mutations, the level of X-inactivation skewing measured in peripheral blood cells may not be a reliable predictor of the expression of BFLS phenotypes (5).

The limitation of this report is that the manifestation of complete GH deficiency and horseshoe kidney was based on only one patient. Reports of more cases would help to clarify the risk involved in rhGH treatment in this condition.

In conclusion, we report a female with a novel nonsense mutation c.673C > T (p.R225X) of the *PHF6* gene. The patient exhibited certain features beyond classic BFLS, including horseshoe kidney and complete deficiency of growth hormone. An adverse effect was elicited with GH treatment, suggesting caution in the use of GH in this condition. Both the patient and her unaffected mother had skewing of X-inactivation indicating that X-inactivation assay may not reliably predict the expression of BFLS phenotypes. These clinical and genetic findings may contribute to improve our understanding of BFLS and also aid in the diagnosis and genetic counseling of the condition.

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Ethics

Informed Consent: Written consent was obtained from the patient's parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Clinical Data Collection: Yongguo Yu, Xuefan Gu, Genetic Testing and Analysis: Xia Zhang, Yanjie Fan, Xiaomin Liu, Yu Sun, Yunjuan He, Xiantao Ye, X-chromosome Inactivation Assay: Hui Yan, Design: Yanjie Fan, Yongguo Yu, Writing: Xia Zhang, Yanjie Fan, Ming-Ang Zhu.

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CASE REPORT

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Isolated Growth Hormone Deficiency Type 2 due to a novel *GH1* Mutation: A Case Report

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What is already known on this topic?

Dominantly inherited isolated growth hormone deficiency (IGHD) can be caused by multiple defects of the *GH1* gene. Affected individuals show a good growth response to recombinant human GH and can develop multiple pituitary deficiency.

What this study adds?

A novel *GH1* gene mutation was found in an Indonesian infant with the classical presentation of IGHD type 2.

Abstract

Isolated growth hormone (GH) deficiency (IGHD) type 2 is a rare autosomal dominant disorder characterized by severe short stature with low GH level. Timely diagnosis is important for optimal results of recombinant human GH (rhGH) treatment and detection of additional pituitary deficiencies in affected relatives. A male child presented at the age of one year with severe, proportionate short stature [-4.9 standard deviation score (SDS)] and with a normal body mass index (-1.1 SDS). Physical examination revealed frontal bossing, midfacial hypoplasia, normal external genitalia and no dysmorphic features. Paternal and maternal heights were -6.1 and -1.9 SDS. Serum insulin-like growth factor-1 (IGF-1) and IGF-binding protein-3 were undetectable and the peak GH concentration by clonidine stimulation test was extremely low (0.18 ng/mL). Brain magnetic resonance imaging showed anterior pituitary hypoplasia. Genetic analysis identified a novel heterozygous mutation (c.291 + 2T > G) expected to lead to splicing out exon 3 of GH1. rhGH from age 2.4 years led to appropriate catch-up. In conclusion, we identified a novel *GH1* gene mutation in an infant with classical IGHD type 2 presentation.
Keywords: Growth hormone, *GH1*, short stature, isolated growth hormone deficiency

Introduction

Growth hormone (GH) deficiency (GHD) is characterized by decreased GH secretion as assessed by one or two GH provocation tests in addition to low serum insulin-like growth factor-1 (IGF-I) and IGF-binding protein-3 (IGFBP-3) concentrations and clinical features including linear growth failure, typical features at physical examination and bone age retardation (1). GHD can be either isolated GHD (IGHD) or part of multiple pituitary hormone deficiency (MPHD) and can be congenital or acquired. The reported incidence of congenital GHD is 1 in 4,000 to 1 in 10,000 live births with male predominance (2,3).

When IGHD is suspected, further evaluation is urgently needed (4). Establishing the diagnosis is a multistep process involving a careful medical history, detailed physical examination including accurate measurements of stature and analysis of the growth curve, biochemical testing, pituitary imaging and genetic screening in severe and/or familial cases (4,5,6,7,8,9).

Genetic causes of IGHD can be found in 3-30% of patients and are typically classified into four types according to the inheritance pattern: autosomal recessive inheritance (IGHD types 1A and 1B), autosomal dominant (IGHD type 2), and X-linked inheritance (IGHD type 3) (2,3,5). Mutations of the genes encoding GH (*GH1*), GHRH receptor (*GHRHR*), the

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GH secretagogue receptor (*GHSR*) and several transcription factors involved in pituitary development have been described to cause IGHD (5,10).

Here we report a case of genetically proven, autosomal dominant IGHD type 2 caused by a novel mutation of *GH1* at a position where previously two other mutations have been found (10).

Case Report

A male infant, the 0.99 year old son of non-consanguineous parents was referred to our pediatric endocrinology clinic because of severe short stature. His father's height was 132 cm [-6.1 standard deviation score (SDS)] and maternal height was 151 cm (-1.86 SDS). Pregnancy and the perinatal period were uneventful. Birth weight and length were 3.3 kg and 48 cm after 38 weeks of pregnancy (-0.1 and -1.0 SDS, respectively). There were no indications of any chronic disease and psychomotor development was normal.

Length and weight with SDS calculations based on the World Health Organization growth charts at first presentation were 64 cm (-4.9 SDS) and 6.3 kg (-4.8 SDS), respectively (11), body mass index was 15.4 kg/m^2 (-1.1 SDS) and head circumference 44 cm (-1.6 SDS). Physical examination revealed frontal bossing, midfacial hypoplasia, normal external genitalia and no dysmorphic features (Figure 1). Further anthropometric data revealed a proportionate short stature with a sitting height/height ratio of 0.65 (0.1 SDS) (12). The growth velocity foregoing the first observation was 3 cm over six months (-3.5 SDS) (11). Bone age was 6 months at a chronological age of 1.0 year.

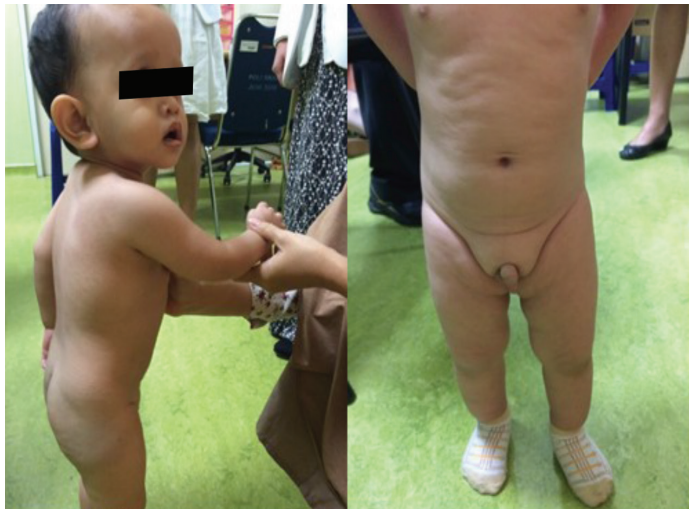

Figure 1. Characteristic clinical features of the patient. Frontal bossing, midfacial hypoplasia, lobulated subcutaneous fat and normal genitalia are noted

Laboratory examination revealed a normal free thyroxine (fT4) level (fT4, 1.23 ng/dL) and thyroid stimulating hormone (TSH) (TSH; 2.74 µU/mL) and undetectable levels of IGF-1 (< 25 ng/mL) and IGFBP-3 (< 0.5 mg/L). The patient's father also had a low serum IGF-I (< 25 ng/mL).

The pedigree of the family is shown in Figure 2. The heights of the paternal grandfather and grandmother were reported as approximately 165 cm (≈-1.6 SDS) and 150 cm (-2.0 SDS), respectively.

The patient then underwent a GH stimulation test using clonidine 0.15 mg/m^2. Peak GH level was extremely low (0.18 ng/mL). An magnetic resonance imaging (MRI) of the brain showed anterior pituitary hypoplasia (Figure 3). Due to financial constraints it took more than a year before recombinant human GH (rhGH) (Saizen, Merck-Serono)

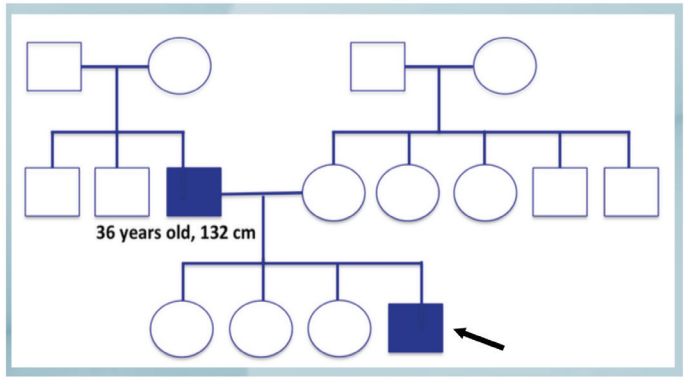

Figure 2. The pedigree of the family of the index patient with autosomal dominant type 2 growth hormone deficiency. Filled squares indicate affected members [the index patient (arrow) and the father]

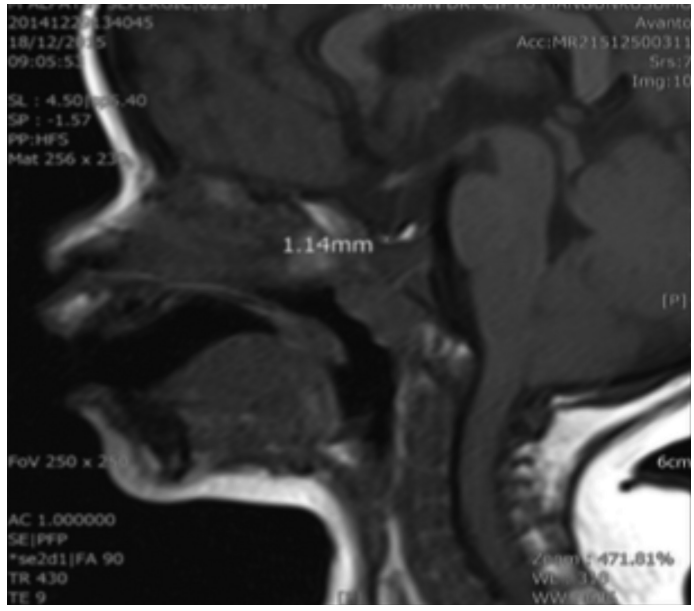

Figure 3. Brain magnetic resonance of the index case, demonstrating anterior pituitary hypoplasia

replacement therapy could be started at the age of 2 years and 5 months at a daily dose of 20-24 μg/kg body weight. This resulted in a appropriate catch-up growth (Table 1, Figure 4). Growth velocity after 1.5 years of treatment was 9.5 cm/year over a 13 month period. Screening for deficiencies of other pituitary hormones including follicle stimulating hormone (FSH), luteinizing hormone (LH), TSH and morning cortisol, showed normal results. Screening the father for other pituitary and related hormones including FSH, LH, testosterone, fT4, TSH, prolactin, adrenocorticotropin hormone (ACTH) and cortisol, also yielded normal results.

Sanger sequencing of *GH1* was performed in the laboratory of Centogene AG (Rostock, Germany) and showed a novel heterozygous mutation (c.291 + 2T > G) expected to lead to splicing out of exon 3. Mutation analysis of the father's DNA has not been performed, but the extremely short stature and low IGF-1 make it highly likely that he carries the same mutation, which appears to be *de novo* according to the normal heights of the paternal grandparents and the father's brothers.

All clinical investigations were conducted in accordance with the guidelines by the Declaration of Helsinki. The parents gave informed consent to clinical and genetic studies, as well as for publication of the clinical information and pictures.

Discussion

In this report we describe a novel splice site mutation of *GH1* leading to severe short stature in the index patient and his father, a characteristic finding for type 2 IGHD. No other relatives with severe short stature are known in this family, so we have assumed that the mutation occurred *de novo* in the patient's father. The mutation is located at a base known to be vital for correct splicing, since previous mutations c.291 + 2T > A and > C have been discovered with an autosomally inherited and similarly severe phenotype (13,14,15), with lower GH peaks upon provocation compared with those with missense mutations (13). The hypoplastic anterior pituitary in the patient is consistent with previous observations reported in 60 % of patients with splice spite

Table 1. Summary of antropometric data of the patient during recombinant human growth hormone therapy

Age (years)	Height (cm)	Height SDS	Weight (kg)	Weight SDS	HC (cm)	HC SDS	HV	HV SDS
2.41	78	-3.68	8.64	-5.14	47.5	-0.97	18 cm/year	14
2.75	85	-2.43	10.1	-3.24	48	-0.87	21 cm/year	20.5
3.08	87	-2.36	10.21	-3.72	48.5	-0.73	12 cm/year	-3
3.33	91	-1.69	13	-1.28	49	-0.52	14 cm/year	21
3.75	96	-1.12	14.8	0.05	49.5	-0.38	9.5 cm/year	10

HC: head circumference, HV: height velocity, SDS: standard deviation score

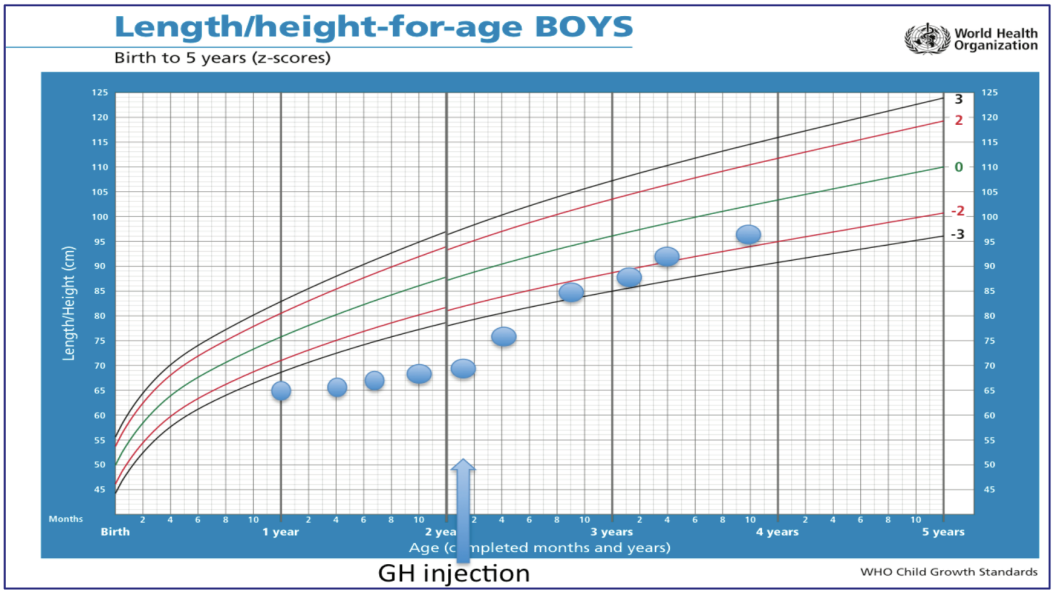

Figure 4. Height data of the patient plotted on the World Health Organization growth chart. The arrow indicates the beginning of recombinant human growth hormone injections

GH: growth hormone

mutations (13). The severe IGHD with early onset is thought to be caused by a disturbance of GH storage and secretion due to misfolded, mutant GH (16).

The combination of early-onset severe proportionate growth failure, bone age delay and classical physical signs (midfacial hypoplasia and frontal bossing) makes the *a priori* likelihood of congenital GHD very high. This should always lead to laboratory testing (serum IGF-I and IGFBP-3 and one or more GH stimulation tests) and MRI of the hypothalamic-pituitary region (8). If one parent is very short and GH deficient, a diagnosis of type 2 IGHD is almost certain, but it is still important to confirm this by genetic testing. In such cases, rhGH treatment in a substitution dose is highly effective in leading to rapid catch-up growth followed by a normal growth pattern and a normal adult height (6,9,14).

Infants with severe congenital GHD can present with neonatal hypoglycaemia, prolonged postpartum hyperbilirubinemia, elevated liver function tests and microphallus (1,4). Although data on blood glucose during the neonatal period were not available in our patient, the absence of reported neonatal seizures argue against a past history of hypoglycaemia. Neonatal hypoglycaemia is less frequent in isolated GHD than in MPHD (17,18).

While in this and similar cases the dominant inheritance and the classical phenotype made the diagnosis of type 2 IGHD straightforward, the diagnosis of less severe IGHD is much more challenging. In such cases the clinician has to make an assessment of the likelihood of IGHD based on the growth pattern, bone age delay, observations at physical examination and the results of the screening test (serum IGF-1) (6,8,9,19,20). If the likelihood appears sufficiently high, the next step is a GH stimulation test, which should be repeated if a low GH peak is observed, to exclude the possibility of false positive results (1,21). With regard to the growth pattern of children with GHD, height velocity can be very low in severe cases, particularly in the first years of life, but in other cases height SDS can stabilize for a number of years at or below the -2 SDS line of the population (but considerably below target height SDS), so that height velocity appears normal for the height SDS position. While in most cases height SDS is lower than TH SDS, the dominant form of IGHD, such as was present in our patient and other type 2 IGHD patients, can present with a height SDS close to the height SDS of one of the parents, so that for this subtype of IGHD the distance to TH is not a strong predictor (6,9,21).

Due to its pulsatile nature, physiological and pharmacological GH provocation tests are the key to assess GH secretion (9). The average GH response to various stimuli is slightly different and the level of adiposity is an important determinant of the GH peak, but usually a single cut-off is still used (1,21). Over time, this response moved upwards from 7 to 10 ng/mL (1,16), but due to the increased potency of GH standards a more rational cut-off may be at 7 ng/mL (22). Although few comparative studies have been performed, clonidine (through its stimulation of GHRH release) is thought to be a powerful stimulant for GH secretion, to a similar degree as insulin (1,23).

Each patient with a congenital GHD needs also to be evaluated with a brain MRI to search for anatomic abnormalities of the pituitary gland (24). MRI is an important tool to forecast future endocrine dysfunction, since individuals with abnormal pituitary anatomy are more likely to have or develop multiple endocrinopathies (25). MRI imaging in our patient demonstrated anterior pituitary hypoplasia, in line with the majority of patients with type 2 IGHD. The specific genetic diagnosis (splicing defect of *GH1*) increases the likelihood that with time other pituitary defects may develop (26).

It has been reported that 3-30% of individuals with isolated GHD have a genetic basis, but the likelihood of a genetic cause is considerably higher in children with a positive family history and/or in those with severe short stature (5). Mutations of relevant candidate genes have been identified in 11% of patients with severe IGHD and in frequencies as high as 38% in familial cases (13). Thus, genetic testing is recommeded in children with severe and/or familial IGHD (13,27,28). Children with proportionate short stature and a low peak GH after stimulation, without additional pituitary deficiency, should be considered for mutation screening for *GHRHR* and *GH1*. Another potential genetic cause is a *GHSR* mutation, although the wide phenotypic spectrum of published patients with such mutations do not allow for strong statements about their pathogenicity (28). While it was previously thought that GHD is almost always associated with a normal birth weight and length (1,19,21), it has recently become clear that average birth size of GHD infants is decreased (18). A positive family history of severe short stature in one of the parents strongly suggests an autosomal dominant inheritance pattern, which makes type 2 IGHD very likely, so that full gene sequencing of *GH1* is indicated, as was done in our patient (10,13,27,28,29).

In IGHD type 2, GH secretion is very low but usually still detectable and associated with heterozygous splice site, missense, splice enhancer mutations or intronic deletions in *GH1* (5,10,27,28,29). Most patients, such as ours, with type 2 IGHD have mutations within the first six nucleotides of intron 3 of *GH1*, resulting in skipping of exon 3. The result is the production of the 17.5-kDa isoform, which lacks amino acids 32-71 and, hence, the loop that connects helix

1 and helix 2 in the tertiary structure of GH. This isoform exerts a dominant negative effect upon secretion of the full-length GH molecule and may disturb the secretion of other pituitary hormones, such as TSH, LH and prolactin (5,10,29,30,31,32). Pre-treatment thyroid hormones, as well as other anterior pituitary hormones were normal in our patient. These values were also normal 18 months after start of rhGH treatment. The probability of having other pituitary hormone deficiencies in IGHD increases around puberty, and the first hormone to be affected is ACTH at around eight years of age (33). The normal results of pituitary testing in the patient's father suggest that the risk of additional pituitary insufficiencies in this family may be limited.

In summary, we report a novel mutation in *GH1* leading to type 2 IGHD in an Indonesian child with a classical phenotype. Genetic testing is indicated in severe and or familial IGHD, particularly if one parent is also affected.

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Ethics

Informed Consent: The parents gave informed consent to clinical and genetic studies, as well as for publication of the clinical information and pictures.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ahmad Kautsar, Aman Pulungan, Concept: Ahmad Kautsar, Aman Pulungan, Design: Ahmad Kautsar, Jan M. Wit, Aman Pulungan, Data Collection or Processing: Ahmad Kautsar, Aman Pulungan, Analysis or Interpretation: Jan M. Wit, Aman Pulungan, Literature Search: Ahmad Kautsar, Aman Pulungan, Writing: Ahmad Kautsar, Aman Pulungan, Jan M. Wit.

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CASE REPORT

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A Novel Homozygous Mutation of the Acid-Labile Subunit *(IGFALS)* Gene in a Male Adolescent

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What is already known on this topic?

Patients with acid-labile subunit (ALS) *(IGFALS)* mutations have markedly decreased insulin like growth factor-1 (IGF-1), and extremely low IGF-binding protein-3 levels. Although patients with ALS deficiency show moderate short stature, the phenotype of ALS deficiency is quite variable. Microcephaly, delay in puberty, insulin resistance and reduced bone mineral density (BMD) have been shown in some patients.

What this study adds?

A novel homozygous frameshift mutation in *IGFALS* (p.Ser555Thrfs.19) causes short stature and delayed puberty but ultimately, with obvious pubertal growth acceleration and good pubertal height gain, resulting in a normal adult height, comparable to the target height. Heterozygous carriers of this mutation have normal prenatal growth, puberty, insulin metabolism and BMD.

Abstract

Acid-labile subunit (ALS) forms ternary complexes with insulin like growth factor-1 (IGF-1) and IGF-binding protein-3 (IGFBP-3) and is essential for normal circulating IGF-1 levels. The *IGFALS* gene encodes the ALS and mutations in *IGFALS* cause ALS deficiency. We describe a patient with ALS deficiency with a novel homozygous frameshift mutation in *IGFALS* presenting with short stature and delayed puberty but ultimately achieving an adult height (AH) comparable to his target height (TH). A 15.25 year old boy presented with short stature (149.9 cm, -3.04 standard deviation score). The patient had a low circulating IGF-1 concentration, extremely low IGFBP-3 concentration, insulin resistance and osteopenia. The peak growth hormone (GH) response to GH stimulation test was high (31.6 ng/mL). Sequencing of *IGFALS* revealed a novel, homozygous, frameshift mutation (p.Ser555Thrfs.19). His mother and elder sister were heterozygous carriers. Although he had delayed puberty and short stature at the onset of puberty, he reached his TH and an AH similar to those of his heterozygous mother and sister. The heterozygous carriers had normal or low IGF-1 concentrations and low IGFBP-3 concentrations but not as markedly low as that of the patient. They had normally timed puberty, insulin metabolism and bone mineral density (BMD). The phenotype of ALS deficiency is quite variable. Despite short stature and delayed puberty, patients can achieve normal pubertal growth and AH. ALS deficiency may cause osteopenia and hyperinsulinemia. Heterozygous carriers may have normal prenatal growth, puberty, insulin metabolism and BMD.

Keywords: Short stature, acid-labile subunit deficiency, *IGFALS* gene mutation, primary IGF-1 deficiency

Introduction

The majority of circulating insulin like growth factor-1 (IGF-1) is bound to IGF-binding proteins (IGFBP), mainly to IGFBP-3 and the acid-labile subunit (ALS). ALS has a major role in stabilizing the 150-kDa ternary complex. The ternary complex extends the half-life of IGF-1 from 10 minutes in the free form to more than 12 hours (1,2). Therefore, ALS is necessary to maintain normal circulating IGF-1 and IGFBP-3 levels. Patients with ALS gene (*IGFALS*) mutations have markedly decreased IGF-1 and extremely low IGFBP-3

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concentrations, associated with normal or compensatory elevated growth hormone (GH) levels (3).

Patients typically show moderate short stature in contrast to other, more severe causes of primary IGF-1 deficiency. In addition to short stature, some other features have been reported in the phenotype of ALS deficiency. Although some of the clinical and laboratory features of these patients remain controversial, microcephaly, delay in puberty, insulin resistance and reduced bone mineral density (BMD) have been shown in some patients (4,5,6,7,8,9,10,11,12, 13,14,15).

IGFALS is located on chromosome 16p13.3 and encodes the 85-kDa ALS glycoprotein. ALS is produced by the liver under GH stimulation (1,3). Homozygous or compound heterozygous mutations in *IGFALS* lead to ALS deficiency. *IGFALS* consists of two exons. To date, at least 22 different inactivating mutations of *IGFALS* have been reported (4,5,6,7,8,9,10,11,12,13,14,15). Similar to patients with homozygous or compound heterozygous mutations, heterozygous carriers were reported to be shorter than wild-type carriers (5,9,16,17).

We report the genotype and phenotype of a patient with ALS deficiency with a novel, homozygous, frameshift mutation in *IGFALS*, presenting with short stature and reaching an adult height (AH) similar to that of heterozygous carriers of this mutation.

Methods

Molecular Studies

Informed consent was obtained from the patient and his sister and mother. The genetic analyses were performed at the Cincinnati Center for Growth Disorders, Cincinnati Children's Hospital Medical Center. Genomic DNA was extracted from peripheral blood leukocytes.

Auxology

Height and weight were measured using a Harpenden stadiometer and electronic scale respectively and head circumference (HC) with a tape measure. Small for gestational age (SGA) was defined as birth weight and/or length standard deviation (SD) score (SDS) <-2.0. SDS for height, weight, sitting height/height SDS and HC calculated according to Turkish standards (18,19). Target height (TH) was calculated using the following equation TH = [father's height (cm) + mother's height (cm)]/2 -6.5 cm (girls) or +6.5 cm (boys) (20). The onset of puberty was defined according to Tanner standards as attainment of testicular volume ≥4 mL in boys (21). Bone age was estimated by the Greulich and Pyle method and height prediction was calculated by Bayley-Pinneau method (22).

Serum Hormone Assays

Serum concentrations of IGF-1 and IGFBP-3 were measured by an automated immunochemiluminescence assay (Immulite 2000 XPi; Siemens Medical Solutions Diagnostics, Erlangen, Germany).

Case Report

A 15.25 year old boy was referred to the pediatric endocrinology clinic for evaluation of short stature. His height was 149.9 cm (-3.04 SDS) and his weight 52.3 kg (-2.3 SDS) with a HC 53.8 cm (-2.0 SDS) at presentation. He was 2.05 SD shorter than his TH. Clinical and laboratory characteristics of the patient at presentation and during follow-up are given in Table 1.

At presentation, there were no dysmorphic features noted and no body disproportion. He was prepubertal; his testicular volumes were 3 mL bilaterally. He was born SGA

Table 1. Clinical characteristics of the patient at presentation and during follow-up

	At presentation	At onset of puberty	At adult height
Age (years)	15.25	15.7	20.1
Height (cm) (SDS)	149.9 (-3.04)	153.2 (-2.96)	169.5 (-1.08)
Weight (kg) (SDS)	43.7 (-2.3)	48 (-2.06)	68.8 (-0.31)
BMI (kg/m^2) (SDS)	19.5 (-0.72)	20.45 (-0.5)	23.9 (0.28)
Head circumference (cm) (SDS)	54.1 (-1.8)	54.4 (-1.83)	56.2 (-1.0)
Sitting height/height (SDS)	0.53 (0.0)	0.53 (-0.84)	0.52 (-0.6)
Testis volume (mL)	3/3	6/6	20/20
Bone age (years)	13.5	13.5	18
Target height (cm) (SDS)	169.3 (-1.12)	169.3 (-1.12)	169.3 (-1.12)
Predicted adult height (cm) (SDS)	166.2 (-1.62)	169.8 (-1.04)	170.2 (-0.97)

SDS: standard deviation score, BMI: body mass index

at 40 weeks of gestation, with a weight of 2400 g (-2.7 SDS). Neuromotor development was normal. His medical history was otherwise unremarkable.

His parents were unrelated but originated from the same village. Table 2 shows clinical and laboratory characteristics of the patient and his mother and sister. Mother's height was 155.6 cm (-1.28 SDS). She reported achieving menarche at 13 years. Father passed away due to chronic renal failure. His reported height was approximately 170 cm (-0.9 SDS). There was no information on the father's pubertal history. TH of the patient was 169.3 cm (-0.99 SDS). His elder sister's birth weight was 3000 g at 40 weeks of gestation (-0.9 SDS) and height was 157.1 cm (-1.02 SDS) at 21 years of age. Her age at menarche was 12 years.

Serum IGF-1 concentration of the patient was markedly reduced at 68.6 ng/mL (normal: 193-731 ng/mL). IGFBP-3 concentration was extremely low at <0.5 ng/mL (normal: 3.2-8.7 ng/mL). Thyroid function was normal. The peak GH response to GH stimulation test was high at 31.6 ng/mL. Bone age was 13.5 years at presentation. IGF generation test showed a response of serum IGF-1 from 58.4 ng/mL to 100 ng/mL, but no response of serum IGFBP-3 which did not change from baseline of <0.5 ng/mL.

During follow-up, fasting glucose concentrations of the patient were within the normal range. An oral glucose tolerance test, performed when he was 20.1 years old, showed insulin resistance (Table 2). He did not have bone pain or any fractures. His spine (L1-L4) BMD, determined by DXA at the age of 20.1 years was -3.5 SDS, indicating osteoporosis. Serum calcium, phosphate, alkaline phosphatase, parathyroid hormone and 25-OH vitamin D levels were normal.

Puberty of the patient started at 15.9 years. Testes volumes were 6 mL/6 mL (Tanner stage G2). His height was -3.0 SDS at onset of puberty. His bone age was retarded by approximately two years when compared to his chronological age. His peak height velocity was 7 cm/year during progression of puberty (Tanner stage G3; Figure 1) and total height gain during puberty was 19.6 cm.

At his last follow up visit at age 20.1 years, his height SDS, weight SDS and HC SDS values were -1.08, -0.31 and -1.0, respectively. He reached his TH (Table 1). Puberty was completed and testicular volumes were 20/20 mL at age 20.1 years. His bone age was 18 years. Serum LH and FSH concentrations were 2.77 mIU/mL (normal: 1.7-15.3) and 3.88 mIU/mL (normal: 1.5-12.4), respectively, with a testosterone level of 5.17 ng/mL (normal: 2.18-9.06 ng/mL).

His sister's IGF-1 concentration was low but his mother's was normal. Although their IGFBP-3 concentrations were decreased they were higher than that of the patient. His sister and mother had normal fasting insulin and glucose levels. Their L1-L4 BMDs were also normal (Table 2).

Sequencing of the *IGFALS* gene revealed a novel homozygous mutation in exon 2 at c.1663-1664delTC (p.Ser555Thrfs.19). This frameshift point mutation resulted in a substitution

Table 2. Clinical and laboratory characteristics of the patient and heterozygous carriers at last visit

	Patient	Sister	Mother
Age (years)	20.1	21.0	51.3
Height (cm) (SDS)	169.5 (-1.08)	157.1 (-1.02)	155.6 (-1.28)
Weight (kg)	68.6 (-0.31)	49.5 (-1.48)	64.1 (0.88)
BMI (kg/m^2)	24.2 (0.36)	20.06 (-0.94)	26.48 (1.75)
Head circumference (cm) (SDS)	56.2 (-1.0)	54.9 (-0.97)	55.1 (-0.81)
Sitting height/height (SDS)	0.52 (-0.6)	0.53 (-1.0)	0.54 (0.1)
BMD L1-L4 Z-score	-3.6	-0.2	0.2
IGF-1 (ng/mL) (SDS)	68.6 (-2.9) (N: 193-731)	64.7 (-3.0) (N: 117-323)	76.8 (-1.3) (N: 48.1-209)
IGFBP-3 (ng/mL) (SDS)	<0.5 (<-4.0) (N: 3.2-8.7)	1.92 (-2.8) (N: 2.9-7.3)	1.88 (-3.7) (N: 3.4-6.8)
Fasting glucose (mg/dL)	77	77	98
Insulin (µU/mL)	27.49	6.4	9.85
Oral glucose tolerance test	0' 30' 60' 90' 120'		
Glucose (mg/dL)	77 144 110 99 62	-	-
Insulin (µU/mL)	27.5 487.6 462.5 292.2 113.2		

SDS: standard deviation score, BMD: bone mineral density, IGF-1: insulin like growth factor-1, IGFBP-3: insulin like growth factor-binding protein-3, BMI: body mass index

of a serine for a threonine at position 555 of the protein leading to an early stop codon, 19 codons later (Figure 2). His mother and elder sister were heterozygous mutation carriers.

Discussion

In our patient, low IGF-1, an extremely low IGFBP-3 concentration and moderate short stature at presentation pointed to the possibility of ALS deficiency. Molecular genetics analysis for *IGFALS* revealed a homozygous mutation in exon 2 (c.1663-1664delTC and p.Ser555Thrfs.19). The mutation is located towards the last third of the ALS protein, within LRR 20 (leucine rich repeat). The LRRs would be replaced by 19 new amino acids. These LRRs are critical to the interaction between ALS and IGFBP-3 (23,24). The majority of mutations in *IGFALS* gene result in defects within the LRR region of the protein (3). Different mutations (missense, deletion and insertion) in the ALS protein have been reported and all of the mutations reported to date are located in exon 2. This frameshift mutation in our patient is predicted to cause early protein termination and likely destabilize ALS, thus leading to nonsense-mediated decay of the truncated mRNA, resulting in ALS deficiency in our patient. Serum IGF-1 and IGFBP-3 concentrations were not so profoundly low in family members who were heterozygous carriers, similar to other heterozygous carriers reported previously (9,16,17).

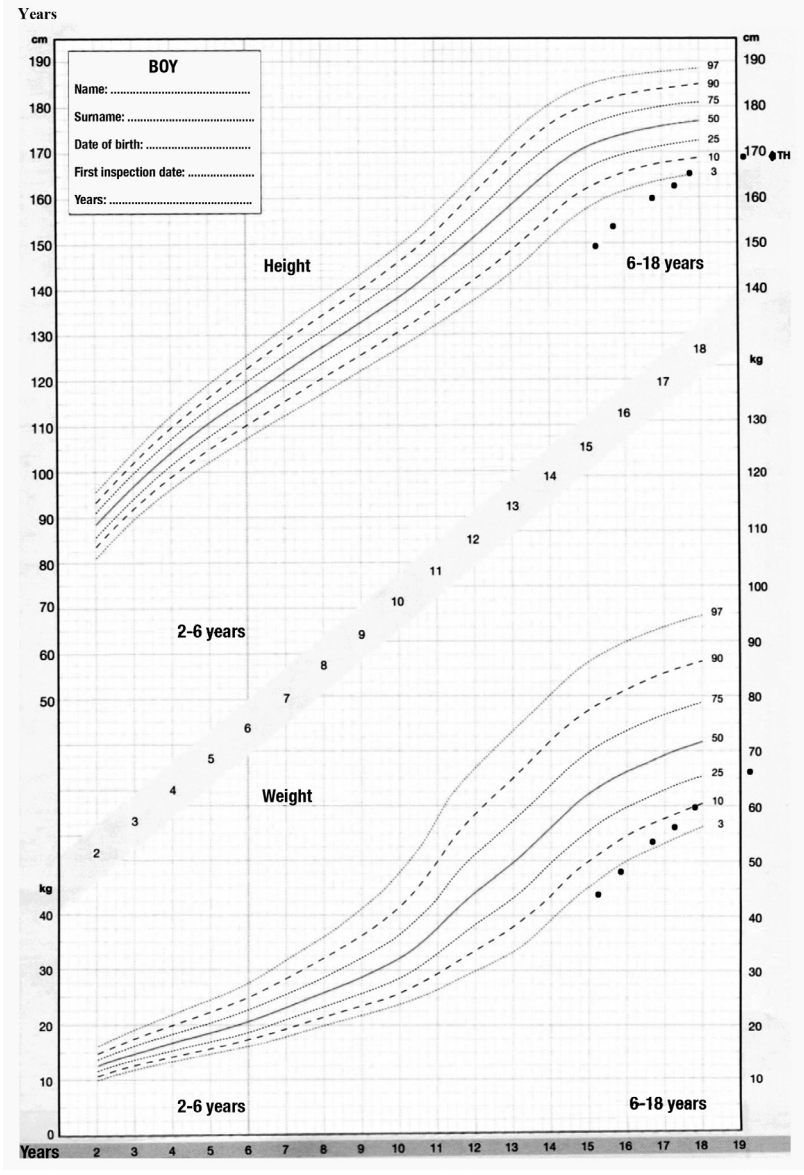

Figure 1. Growth chart of patient for height (at the upper panel) and weight (at the lower panel) plotted on growth chart for Turkish children (18)

In keeping with most reported cases, the main clinical feature of our patient is moderate short stature before puberty. His height SDS at presentation (-3.04 SDS) is also consistent with previous reports in ALS deficient individuals (16). During follow-up, our patient showed a normal growth pattern in puberty and reached his TH, which contrasts with most of the cases reported in the literature in which AH was approximately 1.3 to 1.5 SD below their TH SDS (4,5,6,10). Phenotypic variations between patients who are homozygous for *IGFALS* mutation have been reported. Even a degree of phenotypic variation between two siblings was demonstrated (12). Schreiner et al (8) reported an ALS deficient patient with normal height (-0.19 SDS) and growth pattern with a difference of approximately 0.5 SDS between AH SDS and TH SDS. Although van Duyvenvoorde et al (5) reported that the sitting height/height ratio was in the upper normal range in most cases, sitting height/height ratio of our patient was normal.

AH SDS of our patient is similar to the heights of his heterozygote mother and sister. The height SDSs of heterozygous carriers in our family are approximately 1 SD lower than population mean. This is consistent with previous reports indicating that heterozygous carriers are 1.0 SDS shorter than wild type subjects (5,9,17). The heterozygous carriers have a milder phenotype compared to cases with homozygous mutations (9,12,17). It is proposed that there could be a possible gene dosage effect (9,16).

Our patient and other family members who are heterozygous carriers do not have microcephaly and their HC SDSs were similar. Microcephaly was previously reported in some patients but not present in other reported cases (5,6,7,9,12). It was reported that three siblings with an *IGFALS* mutation had HCs that were lower than those of heterozygous and wild-type carriers and mean HC SDS of heterozygous carriers was 0.7 SD lower than those of non-carriers. It has been speculated that microcephaly may be related to the low IGF-1 levels, due to ALS deficiency, during fetal life (5).

Our patient showed decreased BMD Z-score but BMD Z-scores of heterozygous family members were within the normal range. There was no history of bone pain or fracture. There is conflicting evidence about the prevalence of low BMD in patients with ALS deficiency (5,6,7,11,16,17).

Our patient was born SGA and his birth size was significantly smaller than his heterozygous carrier sister. Although he was SGA, he reached an AH comparable to that of his appropriate-for-gestational age sister. It has been reported that some cases

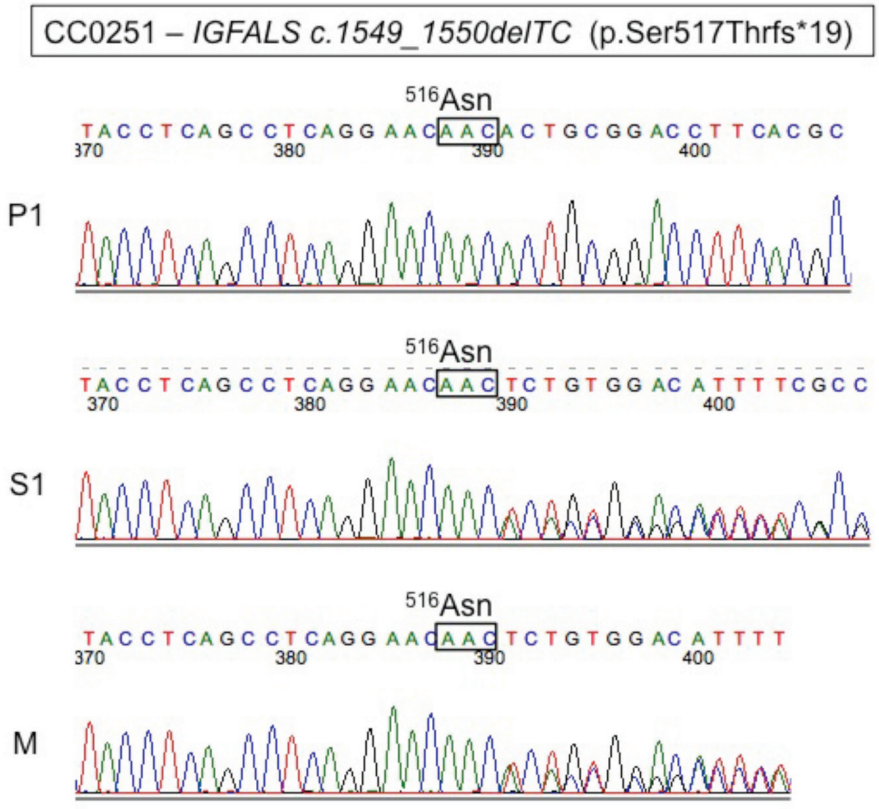

Figure 2. Sequence chromatograms for patient (P), his sister (S) and mother (M). The two nucleotide deletions are located immediately downstream of boxed triplex. The patient is homozygous. His sister and mother are heterozygous

with ALS deficiency are born SGA (7,11,12,15,16). The effect of being SGA on AH in these patients needs to be investigated.

Although our patient had pubertal delay, he demonstrated an obvious pubertal growth acceleration and good pubertal height gain and reached an AH comparable to his TH. The effect of ALS deficiency on pubertal development remains controversial. Age of pubertal onset and growth pattern are still unclear in these patients. Delayed puberty was reported in 50% of males with ALS deficiency, however, normal pubertal growth has been reported in some patients with ALS deficiency (4,6,8,10,11). An adolescent female was reported with a novel homozygous mutation of the *IGFALS* gene with absent pubertal growth spurt and a slow pubertal progression, despite a normal onset of puberty (7).

Our patient had insulin resistance but heterozygous carriers in this family have normal fasting glucose and insulin levels. Insulin resistance has previously been reported in some patients with ALS deficiency (4,6,11,16). It was suggested that this may be related to the increased GH levels and the low IGF-1 levels (25).

In conclusion, we report a novel *IGFALS* mutation identified in a patient with biochemical signs of ALS deficiency and short stature, born SGA, with delayed puberty but normal growth and an AH comparable to TH. It is important to know all of the phenotypic features of ALS deficiency in order to ensure proper follow up of these patients. Although heterozygosity for ALS affects height, it seems to show no effect on prenatal growth, puberty, insulin metabolism and BMD.

Ethics

Informed Consent: Informed consent was obtained from the patient, mother and sister.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Şükran Poyrazoğlu, Feyza Darendeliler, Design: Şükran Poyrazoğlu, Feyza Darendeliler, Data Collection and Processing: Şükran Poyrazoğlu, Vivian Hwa, Firdevs Baş, Andrew Dauber, Ron Rosenfeld, Feyza Darendeliler, Analysis and Interpretations: Şükran Poyrazoğlu, Vivian Hwa, Firdevs Baş, Andrew Dauber, Ron Rosenfeld, Feyza Darendeliler, Literature Search: Şükran Poyrazoğlu, Feyza Darendeliler, Writing: Şükran Poyrazoğlu, Feyza Darendeliler.

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CASE REPORT

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A Case of Autosomal Dominant Osteopetrosis Type 2 with a *CLCN7* Gene Mutation

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What is already known on this topic?

Autosomal dominant osteopetrosis type 2 (ADO-2) is the benign form of osteopetrosis, which is characterized primarily by vertebral endplate thickening and includes increased cortical bone volume with fragile bones and multiple fractures later in life. In families with ADO-2, the penetrance ranges from 60 to 90%.

What this study adds?

This is the first case of autosomal dominant ADO-2 with a confirmed mutation in the *CLCN7* gene in Korea. The patients showed typical radiologic findings of ADO-2 with hearing loss. However, the father with the same mutation was asymptomatic, with no clinical or radiologic signs. Thus, we conclude that the exact prevalence is not known.

Abstract

Osteopetrosis is a rare genetic disease characterized by increased bone density and bone fractures due to defective osteoclast function. Autosomal dominant osteopetrosis type 2 (ADO-2), Albers-Schonberg disease, is characterized by the sclerosis of bones, predominantly involving the spine, pelvis and the base of the skull. Here, we report a typical case of osteopetrosis in a 17.7-year-old male who carries a heterozygous c.746C > T mutation in exon 9 in the *chloride voltage-gated channel 7 (CLCN7)* gene. The patient's spine showed multiple sclerotic changes including sandwich vertebra. His father had the same mutation but his skeletal radiographs were normal. This is the first reported case of ADO-2, confirmed by genetic testing in a Korean patient.

Keywords: Osteopetrosis, bone density, osteoclast, sclerosis, mutation

Introduction

Osteopetrosis, also known as marble bone disease, is an extremely rare bone disease characterized by increased bone mineral density, where bones are prone to be fractured due to defective osteoclast function despite the increased bone mineral density (1,2,3). According to The Nosology Group of the International Skeletal Dysplasia Society, osteopetrosis is classified based on clinical features, mode of inheritance and molecular mechanism (2). There are various clinical features and genes involved in different types of osteopetrosis. Inheritance type can be autosomal recessive, autosomal dominant or X-linked. Autosomal recessive osteopetrosis (ARO), which is a malignant form of osteopetrosis that can be seen in infancy, can result in growth failure and increased frequency of fractures. Patients with ARO suffer from anemia and recurrent infections. It is thought that this is due to expansion of the bone which leads to narrowing of the bone marrow space and results in extramedullary hematopoises. Some patients with ARO also suffer from blindness, facial paralysis, and deafness, due to pressure on the cranial nerves by the narrowing of spaces due to bone expansion (1,2,3).

However, patients with autosomal dominant osteopetrosis (ADO), which is the benign form of osteopetrosis, may present with no symptoms of ADO and most of them are found incidentally. ADO type 2 (ADO-2), also known as Albers-Schonberg disease, is characterized primarily by vertebral endplate thickening "sandwich vertebrae" and includes increased cortical but normal cancellous bone

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volume, and fragile bones with multiple fractures later in life (4,5,6,7). The most common cause of ADO-2 is the presence of inactivating mutations in the *chloride channel 7* (*CLCN7*) gene, which results in ineffective, osteoclast-mediated bone resorption through disrupted acidification of the osteoclast resorption lacunae, that in turn prevents degradation of the mineral component of the bone (3). Here, we present a case of ADO-2 in an adolescent male, who carried a heterozygous gene mutation in *CLCN7*.

Case Report

A 17.7-year-old male was referred to our hospital due to sclerotic changes in bony structures. Approximately one month prior to referral, the patient started complaining of pain in the right shin. X-rays in a local clinic revealed a generalized increase in bone density.

The patient's history revealed that he weighed 3.8 kg (75th percentile) at birth. He had no history of chronic diseases such as hypertension, diabetes or hepatitis. The patient and his family, including his parents and younger sister had no history of bone fractures. His aunt was suspected of having a bone-related disease, but she did not present for examination. The patient suffered from chronic otitis media and was diagnosed with partial hearing loss when he was 16 years old. On physical and neurological examination, no specific findings were noted. His current height and weight were 170.6 cm (50th percentile) and 69.0 kg (75th percentile), respectively.

Plain radiographs showed a generalized increase in bone density involving the skull, vertebrae and pelvis. X-rays of the skull showed thickening and increased skull-base density (Figure 1A). X-rays of the spine showed typical end-plate thickening and sclerosis producing the classic "sandwich vertebrae" appearance (Figure 1B). Sandwich vertebra is a radiologic finding in which the endplates are densely sclerotic, resulting in the sandwich appearance. X-rays of the pelvis showed the "bone-within-bone" appearance, primarily in the iliac wings (Figure 1C). The other family members, including his younger sister, mother and father, showed normal bone density. Figure 1D shows normal bone appearance in the patient's father. Bone mineral densitometry (BMD) of the antero-posterior lumbar spine vertebrae, L1-L4, was measured as 2.466 g/

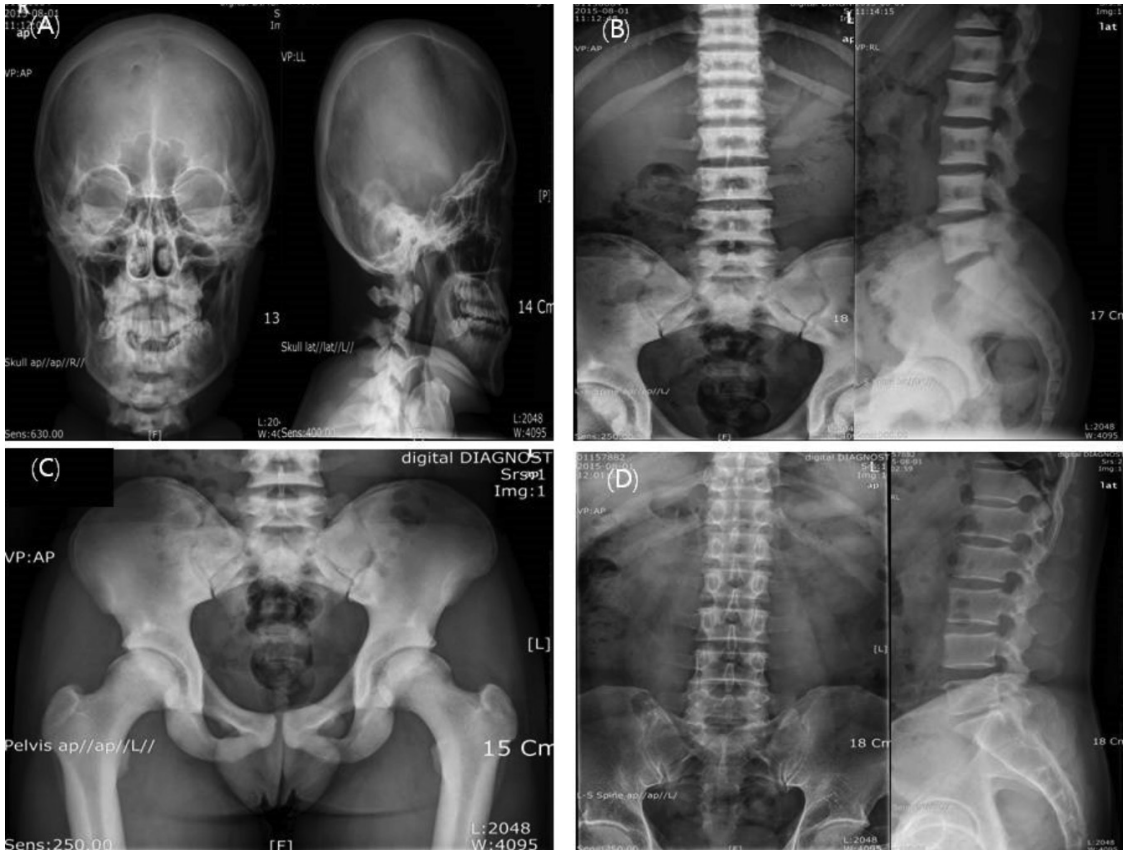

Figure 1. A) X-rays of the skull showing generalized increase in bone density. The sclerosis is more prominent in the base of the skull. B) Typical end-plate thickening and sclerosis producing the classic "sandwich vertebrae" appearance. C) Sclerosis in the iliac wings, acetabuli and femur heads. However, typical "bone-within-bone" appearance cannot be noted in the patient. D) The patient's father showed normal bone density

cm^2 (Z-score = 10.7) by dual-energy X-ray absorptiometry on a Lunar Prodigy (Lunar, Madison, WI, USA). The BMD of the left femoral neck, trochanter and Ward's triangle were measured as 1.966 g/cm^2 (Z-score = 7.0) (8), 1.825 g/cm^2, and 1.943 g/cm^2, respectively. Blood chemistry showed the following: serum albumin 4.4 g/dL (reference range 3.5-5.2 g/dL), total calcium 9.5 mg/dL (8.6-10.2 mg/dL), elevated phosphorus 5.0 mg/dL (2.7-4.5 mg/dL), ionized calcium 4.81 mg/dL (4.48-4.92 mg/dL), alkaline phosphatase: 108 U/L (40-129 U/L), sodium 145 mmol/L, potassium at 4.4 mmol/L, chloride 105 mmol/L and bicarbonate 28.4 mmol/L. The intact parathyroid hormone level was slightly elevated, being 79.5 pg/mL (reference range: 14-72 pg/mL), 25-hydroxy-vitamin D_3 level was 25.7 ng/mL (insufficiency range: 10-30 ng/mL) and thyroid stimulating hormone 5.38 uIU/mL (reference range: 0.27-4.20 uIU/mL).

For evaluation of osteopetrosis, targeted gene panel sequencing was performed to check for the presence of pathogenic variants of multiple associated genes responsible for osteopetrosis. After informed consent, 3 mL of blood was obtained from the patient, sister and both parents. A library preparation was performed using the TruSight One Sequencing Panel (Illumina, Inc., San Diego, CA, USA), which enriches a 12-Mb region spanning 62,000 target exons of a total of 4,813 clinically relevant genes. Massively parallel sequencing was performed on the Illumina NextSeq platform. Sequence reads were mapped to UCSC hg19 standard base for comparative analysis. The results of targeted gene panel sequencing revealed heterozygous missense mutation c.746C > T (p.Pro249Leu) in exon 9 of the *CLCN7* gene in the proband, which was previously reported in a patient with ADO-2 (5): There was no pathogenic variant in other genes. Sanger sequencing confirmed the presence of this variant, and the same heterozygous variant was only found in the patient's father (Figure 2). However, the father denied having any complaints including history of fracture, osteomyelitis, visual impairment and hearing problem. Radiographs of his bones were also normal (Figure 1D). We did not evaluate bone mineral density in the patient's father, as his X-rays were of normal appearance.

Discussion

This is the first case of autosomal dominant ADO-2 in Korea, with a confirmed mutation in the *CLCN7* gene. Previously, a case of infantile malignant *CLCN7*-related ARO, with neonatal thrombocytopenia was reported in Korea (9). There are two mutations in that case: (1) a deletion of an A at nucleotide 17631, in the paternally derived allele, causing a frame shift and a premature stop codon at codon 395; and (2) an intronic point mutation G23742A in the maternal allele.

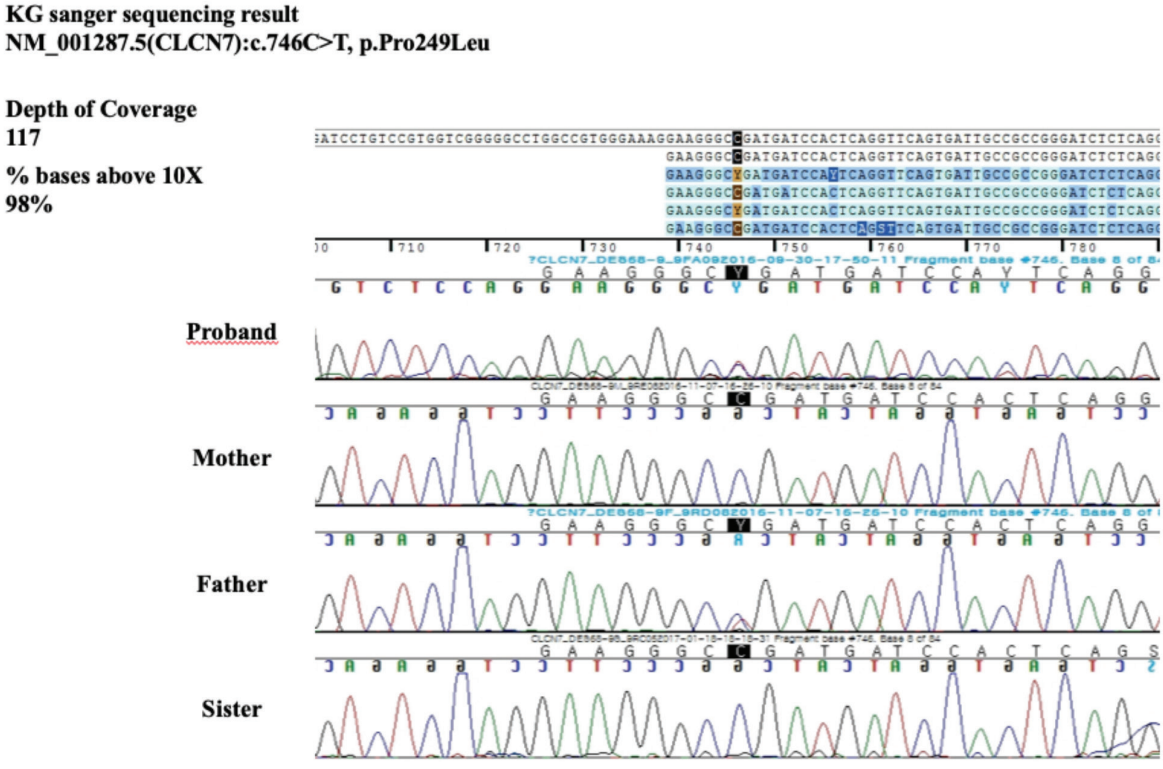

Figure 2. A heterozygous missense mutation was identified in the patient and his father. A heterozygous C to T transition is shown at position 746 in exon nine of *CLCN7* gene, changing a proline to leucine substitution at codon position 249

ADO-2 is the most common form of osteopetrosis and is characterized by sclerosis, predominantly involving the spine, pelvis and base of the skull (4,5,6,7). The fragility of bones and dental abscess are common complications. The gene that is mutated in ADO-2 was reported to be localized on chromosome 16p13.3 and was later identified to be *CLCN7* (5,6). The *CLCN7* gene encodes the chloride channel 7 protein subunit (ClC-7), which consists of 803 amino acids and plays a role in efficient proton pumping in the osteoclast ruffled membrane (3). Thus, patients with the *CLCN7* mutation have reduced bone resorption, which leads to osteopetrosis (10). Previously, over 70 different mutations in *CLCN7* have been identified in ADO-2 families and almost all cases have been associated with heterozygous mutations in the *CLCN7* gene (5,7,11,12).

The spectrum of *CLCN7*-related osteopetrosis includes infantile malignant ARO, intermediate autosomal osteopetrosis and ADO-2 (1). ADO-2 is a benign condition and the disease onset is usually in late childhood or adolescence. The diagnostic criterion for ADO-2 is osteosclerosis of the spine with a "sandwich vertebra" or "rugger-jersey" appearance. Most affected subjects have a "bone-within-bone" appearance, primarily in the iliac wings, but also in other long bones. Erlenmeyer-shaped femoral metaphyses, transverse bands of sclerosis and mild osteosclerosis in the base of the skull are often observed (1,2,3,4,7).

The complications of infantile malignant ARO include poor growth and fractures, with a life expectancy of fewer than 10 years. However, most ADO-2 cases are benign, with normal life expectancy. Long-term complications of ADO-2 include fractures in long bones or vertebrae, scoliosis, hip osteoarthritis and osteomyelitis (4). One study which included longitudinal data, suggested that the course of the ADO clinical phenotype worsens over time, especially with regard to fractures (13). Thus, vigorous physical activities should be avoided to prevent fractures and routine dental examination and oral hygiene are important to prevent osteomyelitis of the mandible. In treating fractures, orthopedic surgeons should pay special attention to delayed union or non-union fractures. Cranial nerve compression is a rare occurrence. Hearing loss and vision loss occur in fewer than 5% of affected subjects. In our case, the patient had hearing loss due to osteopetrosis, but we did not perform further evaluation in order to identify the cause of hearing loss in the right ear.

The prevalence of ADO-2 is estimated to be as low at 0.2-5.5 in 100,000 cases (14,15,16). However, asymptomatic carriers were reported with some mutations, and non-penetrance rates were 24 to 41%, depending on mutations, in families with ADO-2 and most of them are asymptomatic at younger ages (4,15,17). Furthermore, no *CLCN7* mutation could be found in up to 30% of patients presenting with a clinical phenotype of ADO (10,18). Given the reduced penetrance of the ADO phenotype, the spectrum of disease expression can range from radiographically unaffected gene carriers through skeletally affected yet asymptomatic subjects to severely affected patients with fractures which increase in severity over time, the prevalence of ADO-2 is likely to be higher (13). In our case, the father had the same mutation but was asymptomatic, with no clinical or radiologic signs. We believe his lack of symptoms was due to reduced penetrance of phenotype.

Therefore, it is important that *CLCN7* gene mutations be considered when patients have increased bone density, with radiologic findings such as "bone-within-bone" appearance. In future studies, we hope to perform genetic testing to confirm additional cases of asymptomatic Korean ADO-2 cases.

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Ethics

Informed Consent: Korea Cancer Center Institutional Review Board (no: K-1711-002-026) exampt written consent.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Jung Sub Lim, Concept: Jung Sub Lim, Design: Sol Kang, Jung Sub Lim, Data Collection or Processing: Jun Ah Lee, Dong Ho Kim, Young Kyung Kang, Analysis or Interpretation: Sol Kang, Jung Sub Lim, Literature Search: Jun Ah Lee, Dong Ho Kim, Young Kyung Kang, Writing: Jung Sub Lim, Jun Ah Lee.

Financial Disclosure: This study was approved by Korea Cancer Center Institutional Review Board (No: K-1711-002-026).

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CASE REPORT

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Three Siblings with Idiopathic Hypogonadotropic Hypogonadism in a Nonconsanguineous Family: A Novel *KISS1R/GPR54* Loss-of-Function Mutation

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What is already known on this topic?

KISS1 and its receptor, KISS1R, (formerly called GPR54) play key roles in the initiation of puberty. Kisspeptin, a peptide encoded by the *KISS1* gene and its receptor are essential to stimulate gonadotropin releasing hormone secretion from the hypothalamus, which in turn stimulates pituitary gonadotrophin secretion to initiate puberty. Thus, the function of KISS-1 and KISS1R in the hypothalamus is critical for the onset and progression of puberty. Loss of function mutations in *KISS1R* gene can cause normosmic idiopathic hypogonadotropic hypogonadism (NIHH). To date, more than 20 different mutations have been reported. Most of these were loss of function mutations.

What this study adds?

A compound heterozygous mutation of the *KISS1R* gene was found to cause NIHH as well as incomplete puberty. In previous studies, the loss-of-functional mutations of *KISS1R/GPR54* which were inherited in an autosomal recessive manner were reported in consanguineous families. The cases reported herein were from a non-consanguineous family, illustrating a different phenotypic spectrum of KISS1R/GPR54. We recommend genetic counselling for families with *KISS1R* mutations, even when there is no consanguinity.

Abstract

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare disease caused by defects in the secretion of gonadotropin releasing hormone (GnRH) or the action of GnRH on the pituitary gonadotrophes. *KISS1R* is one of the genes which, when mutated, cause IHH and mutations of this gene are responsible for about 2-5% of patients with normosmic IHH (NIHH). In this report, we present three siblings with NIHH due to a compound heterozygous *KISS1R* mutation. Genetic studies were carried out in the 14 year old index case with IHH and three siblings, two of whom were prepubertal. Genomic DNA was extracted from peripheral leukocytes and *KISS1R* gene was sequenced by using standard polymerase chain reaction amplification procedures. In molecular analysis of the index case, a compound heterozygous mutation was determined in *KISS1R* gene c.969C>A (p.Y323X) (known pathogenic) and c.170T>C (p.L57P) (novel). Mutation c.170T>C (p.L57P) was inherited from the mother while c.969C>A (p.Y323X) was inherited from the father. The same genotype was also found in two of the three siblings. A compound heterozygous mutation of the KISS1 gene, including one novel mutation, was found to cause NIHH and also incomplete puberty in a non-consanguineous family.

Keywords: Kisspeptin, KISS1R, hypogonadotrophic hypogonadism, delayed puberty

Introduction

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare genetic disorder, which is caused by defects in the secretion of gonadotropin releasing hormone (GnRH) or the action of GnRH on the pituitary gonadotrophes (1). Increase in frequency and amplitude of the pulsatile secretion of GnRH is essential for the initiation of normal pubertal development. The failure of pulsatile secretion of GnRH from the hypothalamus leads to impairment of pubertal development and reproductive function, the clinical entity IHH. The clinical presentation of IHH may manifest as absent or incomplete puberty, cryptorchidism, small penis and infertility in males and amenorrhea, absence of

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breast development and infertility in females. IHH is divided into two major groups: Kallmann syndrome (KS) which is characterized with delayed puberty and impaired sense of smell and normosmic IHH (NIHH) (2). KS can show a wide variety of additional signs and symptoms. These include a failure of one kidney to develop (unilateral renal agenesis), abnormalities of bones in the fingers or toes, a cleft lip with or without an opening in the roof of the mouth (a cleft palate), abnormal eye movements, hearing loss, and abnormalities of tooth development (3). The incidence of IHH is approximately 10 in 100,000 live births and 60% of these patients have KS (3). NIHH results from the dysfunction of the normally situated GnRH neurons in the hypothalamus. Patients with NIHH typically do not have any accompanying congenital anomaly. To date about 50 genes have been reported to be associated with IHH (2). However, a smaller number of these genes are reported to be responsible in the pathogenesis of NIHH (1,2,4,5). Pathogenic mutations can be detected in about half of IHH cases (1,2). *KISS1R*, which encodes the kisspeptin receptor KISS1R, is one of the genes which causes NIHH, and mutations of this gene are responsible for 2-5% of patients with NIHH (5,6). To date, more than 20 different mutations have been reported. Most of these were loss of function mutations (7).

Kisspeptin is a peptide encoded by the *KISS1* gene and its receptor, KISS1R, are essential to stimulate GnRH release from the hypothalamus which, in turn, stimulates pituitary gonadotrophins secretion to initiate puberty. Thus the genes *KISS-1* and its receptor, *KISS1R*, (formerly called GPR54) play key roles in the initiation of puberty and the respective proteins KISS-1 and KISS1R and their function in the hypothalamus are critical for the onset and progression of puberty.

Here, we present three siblings from a non-consanguineous family with NIHH due to a compound heterozygous mutation including the previously reported c.969C>A (p.Y323X) and a novel c.170T>C (p.L57P) mutation in *KISS1R*.

Case Report

The proband, a 14 year-old boy, was referred to our outpatient clinic due to lack of pubertal development. He was the first child of healthy, non-consanguineous, Turkish parents. He had three sisters whose ages were fourteen, twelve and five years. He was reported to have microphallus and bilateral undescended testicles in the newborn period. Bilateral orchiopexia was performed when he was one and a half years old. On physical examination, height was 165.3 cm [0.14 standard deviation score (SDS)], weight 62 kg (0.94 SDS) and bone age 14.0 years. He had typical signs of complete hypogonadism, including microphallus, enuchoid habitus (upper segment/lower segment ratio <0.9 and arm span >height) and lack of pubic and axillary hair. Both testicles were intrascrotal and the testis sizes were 3 mL, bilaterally. He had a normal sense of smell on olfactometry. No craniofacial stigmata or other morphological abnormalities were detected in the physical examination. His karyotype was 46,XY. Basal serum luteinizing hormone (LH), follicule-stimulating hormone (FSH), plasma testosterone (T), adrenocorticotropin, dehydroepiandrosterone sulfate and cortisol concentrations were determined by electro-chemiluminescence immunoassay (Table 1). An intravenous GnRH-stimulation test was also performed to obtain stimulated FSH and LH levels at 0, 20, 40 and 60 minutes, to confirm a diagnosis of hypogonadotropic hypogonadism. Magnetic resonance imaging of the central nervous system revealed normal findings.

The oldest sister, who was also 14 years old at the time of diagnosis, had breast development corresponding to Tanner stage 2. She had no pubic and axillary hair and was premenarcheal. Her bone age was appropriate at 14 years. Her breast development first appeared at age 10 years and after that no further progression in pubertal stages had occurred. Pelvic sonography revealed a uterus (47x18x11 mm) and two small ovaries (24x18x14 and 20x18x14 mm). Hormone assays were: basal FSH: 4.06 mIU/mL, LH: 1.21 mIU/mL and estradiol: 14 pg/mL.

The second sister of the proband was 12 years old and had no sign of pubertal development. Pelvic sonography showed a small uterus and small ovaries (uterine size was 25x6.5x13 mm; right ovary was 9.5x7x13 mm, left ovary was 6.5x10.5x15 mm). Evaluation of basal and GnRH stimulated hormone levels confirmed incomplete puberty. The youngest sister, who was 5 years old, had Tanner stage 1 breast development and a prepubertal hormone profile.

Karyotype analysis of all three sisters were 46,XX.

Genomic DNA was extracted from peripheral leukocytes and the promoter region, the three coding exons and exon-intron boundries of the *KISS1R* gene (NM_032551) were amplified by polymerase chain reaction and sequenced. In the index case a compound heterozygous mutation in the *KISS1R* was present, comprising a nonsense variant (c.969C>A, p.Y323X) which was known as an inactivating mutation to cause NIHH and a novel missense variant (c.170T>C, p.L57P; see Figure 1). This novel missense variant was evaluated for functional impact using a variety of *in-silico* prediction tools including SIFT, PolyPhen-2 and Mutation Taster which supported a disease-causing effect of this mutation (8,9,10). Molecular analysis of the parents

showed that both parents were heterozygous carriers. While the mutation c.969C > A (p.Y323X) was inherited from the father, the novel c.170T > C (p.L57P) variant was inherited from the mother. Genetic analysis of the older two sisters, who were 12 and 14 years old, revealed the same compound heterozygous mutation, whereas the genetic analysis of the youngest sister revealed a normal karyotype and normal KISS1R sequence. Clinical and hormonal characteristics of all cases, including the proband, are shown in Table 1.

Informed consent from the parents of the patients was obtained for publication.

Discussion

Timing of onset of puberty is related to increased GnRH pulses which in turn, activate the increase in gonadotropin and sex hormone levels. Interaction of kisspeptins and their corresponding receptors has been reported to have a critical role in initiation and development of puberty (2). Inactivating mutations of *KISS1R* lead to NIHH (11,12,13).

Kisspeptin, which is a very potent stimulator of GnRH secretion, is secreted from neurons located in two different parts of the mammalian hypothalamus, the preoptic area and the arcuate nucleus (14). It is not only a potential stimulator of GnRH but also a mediator of positive and negative feedback effects on sex steroids (15).

More than 20 mutations in the *KISS1R* (*GPR54*) gene have been previously described and these mutations have variable clinical manifestations (16,17).

Recently, Topaloglu et al (18) reported an inactivating mutation of *KISS1* causing complete NIHH in a large consanguineous family from Turkey. The probound was 14.9 years-old. She had no breast development and her pelvic ultrasonography revealed a hypoplastic uterus and ovaries lacking follicles. The affected three sisters of the proband had no spontaneous breast development. All four affected sisters were otherwise healthy and had a normal sense of smell.

Table 1. Clinical and hormonal characteristics of the proband and siblings

	Patient 1 (proband)	Sibling (patient 2)	Sibling (patient 3)	Sibling (patient 4)
Age at diagnosis (years)	14	14	12	5
Sex	Male	Female	Female	Female
Physical examination	Tanner stage 1 Stretched penis length 4 cm Size of testes 3 mL/3 mL	Tanner stage 2 amenorrhea	Tanner stage 1	Tanner stage 1
Laboratory findings	**FSH:** 0.9 mIU/mL **LH:** 0.13 mIU/mL **Total T:** 15 ng/dL **ACTH:** 34 g/mL **Cortisol:** 15 µg/dL **17OH** **Progesterone:** 0.11 ng/mL **AMH:** 51.2 ng/mL (2.0-30.7)	**FSH:** 4.06 mIU/mL **LH:** 1.21 mIU/mL **Estradiol:** 14 pg/m	**FSH:** 0.86 mIU/mL **LH:** 0.07 mIU/mL **Estradiol:** < 10 pg/mL	**FSH:** 1.58 mIU/mL **LH:** < 0.07 mIU/mL **Estradiol:** < 10 pg/mL
Imaging	- Testicles are in scrotum bilaterally - Right testis 16x9x9 mm - Left testis 15x8x8 mm	- Pelvic USG: Uterus 47x18x11 mm - Right ovary 24x18x14 mm - Left ovary 20x18x14 mm	- Pelvic USG and pelvic MR: small uterus and ovaries	- Pelvic USG: Uterus 33x15x9 mm - Right ovary 19x15x9.5 mm - Left ovary 22x11x9 mm
Karyotype	46,XY	46,XX	46,XX	46,XX
Genetic analysis	c.969C > A (p.Y323X) and novel c.170T > C (p.L57P) compound heterozygous mutation in *KISS1R* gene	c.969C > A (p.Y323X) and novel c.170T > C (p.L57P) compound heterozygous mutation in *KISS1R* gene	c.969C > A (p.Y323X) and novel c.170T > C (p.L57P) compound heterozygous mutation in *KISS1R* gene	Normal

ACTH: adrenocorticotropin, FSH: follicule-stimulating hormone, LH: luteinizing hormone, T: testosterone, ACTH: adrenocorticotropin, AMH: antimüllerian hormone, USG: ultrasound, MR: magnetic resonance

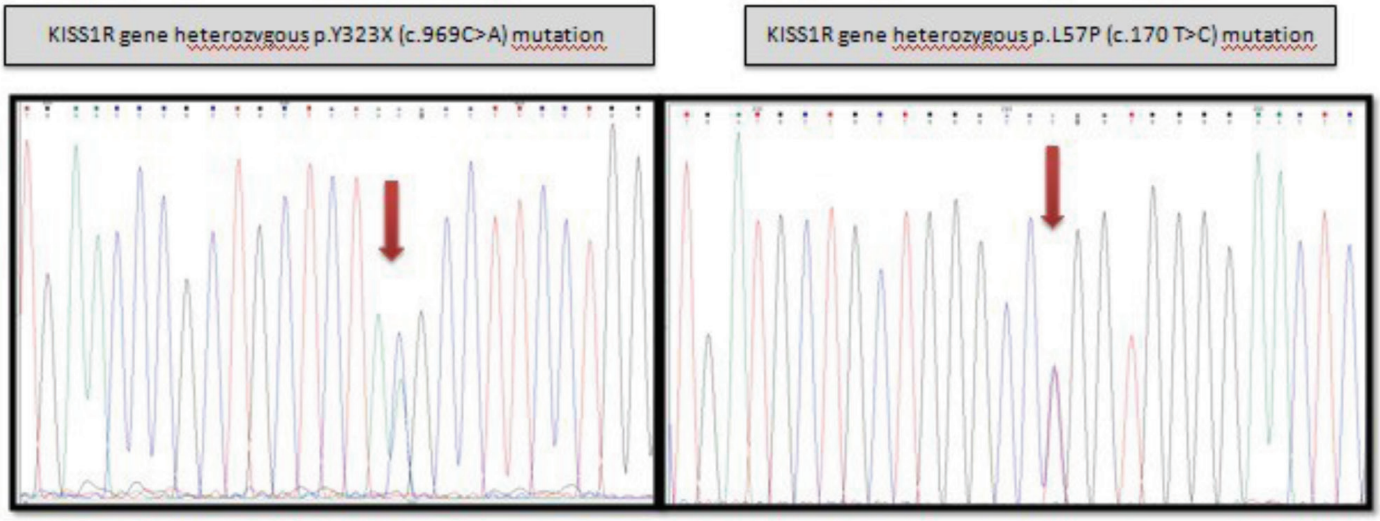

Figure 1. *KISS1* gene mutations detected in the index patient

Demirbilek et al (19) identified a homozygous nonsense mutation, c.C969A (p.Y323X) in the *KISS1R* gene in three non-consanguineous families with NIHH. One male presented with absence of pubertal onset and severe penoscrotal hypospadias and cryptorchidism. Two other males had absence of pubertal onset. Two of four female cases required replacement therapy for pubertal onset, while the other two females had spontaneous pubertal onset but incomplete maturation. A similar nonsense mutation, at position 969 of the nucleotide sequence in the *KISS1R* gene (c.C969>A) located on the short arm of chromosome 19 (19p13.3), has been reported in a case of normosmic IHH in a female patient from a consanguineous family (1). This nonsense mutation results in the creation of a premature stop codon that leads to incomplete production of the kisspeptin receptor. This truncated KISS1R protein fails to signal the release of GnRH from the hypothalamus.

Nimri et al (16) reported two highly consanguineous families of Israeli-Arab origin. Among these, some had evidence for complete hypogonadotropic hypogonadism. Cryptorchidism and a relatively short penile length were noted in all male patients at birth. A novel loss-of-function mutation in the *GPR54* gene in six members of the family was identified (16).

Breuer et al (17) described a novel, severe homozygous *KISS1R* splice site mutation in three siblings in a consanguineous Palestinian family with IHH. They had normal neonatal external genitalia, but presented with no pubertal development, normosmia and a low response to GNRH stimulation.

KISS1R mutations which have been reported previously include point mutation, deletion, insertion, acceptor splice site mutation and missense mutation. Hereby, we described a compound heterozygous mutation in *KISS1R* gene in a non-consanguineous family. One of these was a known pathogenic nonsense variant (c.969C>A, p.Y323X) and the other was a novel missense variant (c.170T>C, p.L57P). The proband had NIHH, whereas his two sisters had incomplete pubertal development and the other sister was prepubertal. Previously described inactivating mutations associated with the *KISS1R* gene have been homozygous from consanguineous marriages. In this report, for the first time, we described *KISS1R* gene mutation in a non-consanguineous family. Thus, we have shown that kisspeptin receptor insufficiency can manifest as different clinical entities. In this study, we report that although three siblings have the same inactivating compound heterozygous mutation, one of them has incomplete puberty and amenorrhea, while the remaining two have NIHH. Different phenotypes can be obtained with the same mutation. In conclusion, we report a compound heterozygous mutation of the *KISS1R* gene causing normosmic IHH and incomplete puberty in siblings. In previous studies, the loss-of-functional mutations of *KISS1R/GPR54*, which were inherited as autosomal recessive mutations, are reported in consanguineous families. We identified these mutations in a non-consanguineous family, a finding which illustrates the different phenotypic spectrum of *KISS1R/GPR54*. We recommend genetic counselling for families with *KISS1R* mutations, even in non-consanguineous families.

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Ethics

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Filiz Hazan, Concept: Behzat Özkan, Design: Özlem Nalbantoğlu, Data Collection or Processing: Semra Gürsoy, Analysis or Interpretation: Özge Köprülü, Literature Search: Gülçin Arslan, Writing: Özlem Nalbantoğlu.

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Catch-up Growth at Term Equivalence in Extremely Premature Small for Gestational Age Infants Compared with Extremely Premature Appropriate for Gestational Age Infants

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Keywords: Nutritional thrift, small gestational age, postnatal weight gain

Dear Editor,

Extremely premature small for gestational age (SGA) children are more prone to medical conditions such as insulin resistance, type 2 diabetes mellitus, precocious puberty, polycystic ovarian syndrome, hypertension, hyperlipidemia and cardiovascular disease (1,2,3,4). There is a balance between prenatal and postnatal weight gain in life. This balance allows the safe storage of fat in the subcutaneous adipose tissue. SGA children have a greater risk of endocrine and metabolic problems if there is mismatch between prenatal and postnatal weight gain (1,2,3,4).

SGA fetuses need to make a metabolic organization for surviving, if they do not have an adequate supply from the placenta and these fetuses tend to economize their resources. Thus, these fetuses send a blood supply to their brain for maintaining their life, while their bodies receive an inadequate blood supply. Their organs (pancreas, liver, kidneys) also receive an inadequate blood supply in the prenatal period (1,2,3). Pancreatic beta cells can not tolerate more energy intake in later life if there is mismatch between prenatal and postnatal weight gain and decreased insulin sensitivity may occur (1,2,3,4). This mismatch is also associated with central adiposity in later life. These infants are also susceptible to precocious puberty, polycystic ovarian syndrome, hypertension, hyperlipidemia. They tend to have a lower risk for insulin resistance and cardiovascular disease, as long as they receive a restricted food supply in later life as in their prenatal period (1,2,3,4). Ng et al (5) reported that extremely premature SGA infants achieved catch up growth with postnatal nutrition, but they tend to have a greater risk of insulin resistance, type 2 diabetes, polycystic ovarian syndrome, hypertension, hyperlipidemia and coronary artery disease because of nutritional thrift.

Catch-up growth is important for reaching higher adult height in extremely premature SGA infants, but nutritional thrift should be considered for prevention of insulin resistance, type 2 diabetes mellitus, polycystic ovarian syndrome, hypertension, hyperlipidemia and cardiovascular disease. Mismatch between prenatal and postnatal weight gain may cause more serious medical disorders than short stature. Nutritional balance should be provided for mitigating the risk of metabolic and endocrine disorders.

Ethics

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Kang S, Kim YM, Lee JA, Kim DH, Lim JS. Early Menarche is a Risk Factor for Short Stature in Young Korean Females: An Epidemiologic Study. J Clin Res Pediatr Endocrinol 2019;11:234-239. Epub 2019 Jan 3.

The Statistical Analysis section line 9 of the article has been corrected as following:

"Linear regression analysis was used to evaluate the predictors of the subject's height as a dependent variable using heights at menarche (????) as predictive variables, controlling for current age." sentence in page 235, Statistical Analysis section, line 9 has been corrected as;

"Linear regression analysis was used to evaluate the predictors of the subject's height as a dependent variable using heights at menarche as predictive variables, controlling for current age."

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Referanslar: 1. Hey-Hadavi J et al. Clin Ther. 2010;32:2036-47. **2.** Genotropin® GoQuick™ 16 IU (5.3 mg) Kısa Ürün Bilgisi. **3.** Genotropin® GoQuick™ 36 IU (12 mg) Kısa Ürün Bilgisi.

Genotropin® Kısa Ürün Bilgisi Özeti: GENOTROPİN GOQUİCK® 16 IU (5.3 mg) - 36 IU (12 mg) enjeksiyonluk solüsyon için toz ve çözücü içeren kullanıma hazır kalem **Formül:** Rekombinant DNA teknolojisiyle Escherichia Coli hücrelerinde üretilmiş 16 IU (5,3 mg) - 36 IU (12 mg) somatropin içerir. **Endikasyonları:** Büyüme hormonunun yetersiz salgılanmasına bağlı çocuklardaki büyüme bozukluklarında; gonadal disgenezi (Turner Sendromu) ile birlikte bulunan büyüme bozukluklarında; kronik böbrek yetersizliği olan prepubertal çocuklardaki büyüme bozukluklarında; SGA tedavisinde – doğum ağırlığı ve/veya uzunluğu -2 SD olan ve 4 yaşı ve sonrasında gerekli büyümeyi yakalayamamış (son 1 yılda yıllık boy kazanımı SDS<0) çocuklarda veya gestasyonel yaşına göre küçük doğmuş olan (SGA) kısa çocuklardaki büyüme bozukluklarında (uzunluk SDS<-2.5 ve ebeveyne uyarlanmış uzunluk SDS<-1) – ; hipotalamus-hipofizer hastalığı saptanan hipofizer cerrahi girişime geçirmiş, kraniyal radyoterapi görmüş veya çocuklukta başlamış büyüme hormonu yetmezliği olan erişkinler ile hipofizde adenomu olan hastalarda büyüme hormonu eksikliği varsa veya büyüme hormonu yetersizliğini düşündüren bulguların bulunması durumunda biyokimyasal tanı testleri ile büyüme hormonu eksikliği kesin olarak saptanan yetişkinlerde, özetle: konjenital veya idiopatik hipofiz hastalıkları, hipotalamus hipofiz tümörleri ve tedavileri sonunda, kraniofarenjioma tedavisinden sonra, cerrahi girişime hasarlarında, Sheehan sendromu ve vasküler sebeple gelişen iskemik sebepli büyüme hormonu yetersizlikleri, radyasyon, travma, kronik otoimmun, bakteriyel veya viral enflamasyonlar ile hemokromatozis ve amiloidoziste görülen hipofizer yetmezliklerde, septo-optik displazide meydana gelebilen aşikâr büyüme hormonu eksikliğinin replasmanı için büyüme hormonu replasman tedavisi endikasyonu vardır. **Pozoloji:** Çocuklardaki büyüme hormonu salgılanma yetersizliğine bağlı büyüme bozukluğunda: Genellikle 0,025 – 0,035 mg/kg veya 0,7 –1,0 mg/m² önerilmektedir. Turner Sendromuna bağlı büyüme bozukluğu: 0,045–0,050 mg/kg veya 1,4 mg/m² önerilir. Kronik böbrek yetmezliğine bağlı büyüme bozukluğu: 0,045–0,050 mg/kg (1,4 mg/m²) önerilir. Büyüme hızı çok düşükse daha yüksek dozlar gerekebilir. Gestasyonel yaşa göre küçük doğmuş (SGA) olan kısa boylu çocukların büyüme bozukluklarında: Final uzunluğa erişinceye kadar genellikle vücut ağırlığına göre günlük 0,035 mg/kg (1,0 mg/m²) önerilmektedir. Yetişkinlerdeki büyüme hormonu eksikliği: Çocukluk çağı BHY sonrasında büyüme hormonu tedavisine devam eden hastalarda önerilen yeniden başlangıç dozu 0,2-0,5 mg/gün' dür. Yetişkin başlangıçlı BHY olan hastalarda tedavi düşük doz (0,15-0,3 mg/gün) ile başlamalıdır. **Uygulama şekli:** Enjeksiyonlar subkütan enjeksiyon şeklinde ve lipoatrofi gelişmesini önleyebilmek için her seferinde yeri değiştirilerek uygulanır. **Kontrendikasyonlar:** Etkin madde veya yardımcı maddelerden herhangi birine karşı aşırı duyarlılık durumunda kullanılmamalıdır. Somatropin, tümör aktivitesini gösteren herhangi bir bulgunun bulunması durumunda kullanılmamalıdır. Büyüme hormonu tedavisine başlanmadan önce intrakraniyal tümörler inaktif olmalı ve antitümör tedavi tamamlanmış olmalıdır. Tümör büyümesine ilişkin kanıt olması halinde tedavi sonlandırılmalıdır. GENOTROPİN GOQUİCK® epifizleri kapanmış çocuklarda büyümenin uyarılması için kullanılmamalıdır. Açık kalp ameliyatı, abdominal cerrahi, kazaya bağlı multipl travma, akut solunum yetmezliği veya benzeri durumları izleyen komplikasyonların bulunduğu akut kritik hastalığı olan hastalara GENOTROPİN GOQUİCK® uygulanmamalıdır. **Özel kullanım uyarıları ve önlemleri:** Hastalığın tanısı ve GENOTROPİN GOQUİCK® tedavisi, terapötik kullanım endikasyonunda; hastaların tanı ve tedavisinde yeterli nitelikte ve tecrübeli doktorlar tarafından başlatılmalı ve takip edilmelidir. Maksimum önerilen günlük doz aşılmamalıdır. Miyozit çok nadir bir advers olaydır ve koruyucu madde metakrezol ile ilişkili olabilir. Somatropin insülin hassasiyetini azaltabilir. Diabetes mellitus olan hastalarda somatropin tedavisine başlandıktan sonra insülin dozunun ayarlanması gerekebilir. Büyüme hormonu T4'ün T3'e tiroit dışı dönüşümünü artırabilir ve bu durum serum T4'ünün azalmasına ve serum T3'ünün artmasına yol açabilir. Malign bir hastalığın tedavisine sekonder büyüme hormonu yetersizliğinde malignitenin relaps belirtilerine dikkat edilmesi önerilmektedir. Çocukluk döneminde kanser sonrası sağkalımlarda, somatropin ile tedavi edilen hastalarda ilk neoplazma sonrası ikinci bir neoplazma gelişiminde risk artışı bildirilmiştir. Büyüme hormonu yetersizliği dahil, endokrin bozukluğu olan hastalarda kalça ekleminde epifiz kayması genel popülasyondan daha sık görülebilir. Şiddetli veya tekrarlayan baş ağrısı, görme sorunları, bulantı ve/veya kusma gelişmesi halinde papilla ödemi için fundoskopi yapılması önerilmektedir. Somatropin içeren ürünlerin hepsinde olduğu gibi, hastaların düşük bir yüzdesinde GENOTROPİN GOQUİCK®'e karşı antikorlar gelişebilir. Seyrek görülmekle birlikte; somatropin ile tedavi edilen hastalarda; özellikle karın ağrısı gelişen çocuklarda pankreatit dikkate alınmalıdır. SGA olarak doğan kısa boylu çocuklarda tedaviye başlamadan önce büyüme bozukluğuna neden olacak diğer tıbbi nedenler veya tedaviler ekarte edilmelidir. Kronik böbrek yetersizliğinde, tedavi başlangıcından önce böbrek fonksiyonu normalin %50 altında olmalıdır. **İlaç Etkileşimleri:** Glukokortikoidlerle eş zamanlı tedavi somatropin içeren ürünlerin büyümeyi tetikleyici etkilerini engelleyebilir. Büyüme hormonu eksikliği olan yetişkinlerde yapılan bir etkileşim çalışmasında somatropin uygulamasının sitokrom P450 izoenzimleriyle metabolize olduğu bilinen bileşiklerin klirensini artırdığı belirtilmektedir. **Gebelik kategorisi:** C. **İstenmeyen etkiler:** Enjeksiyon bölgesi reaksiyonları, artralji, periferik ödem, parestezi, karpal tünel sendromu, miyalji, kas-iskelet sertliği çok yaygın ve yaygın görünen istenmeyen etkilerdir. **Doz aşımı ve tedavisi:** Akut doz aşımı başlangıçta hipoglisemi ve takiben hiperglisemiye neden olabilir. Uzun süreli doz aşımı fazla miktardaki insan büyüme hormonunun bilinen etkilerine benzer belirti ve bulgulara neden olabilir. **Saklama koşulları:** Sulandırılmadan önce: Buzdolabında (2°C - 8°C'de) veya 25°C'nin altında maksimum 1 ay boyunca saklayınız. İki kompartımanlı kartuşu/önceden doldurulmuş kalemi ışıktan korumak için dış kutusunda saklayınız. Sulandırıldıktan sonra: Buzdolabında (2°C - 8°C'de) saklayınız. Dondurmayınız. İki kompartımanlı kartuşu/önceden doldurulmuş kalemi ışıktan korumak için dış kutusunda saklayınız. **Ticari Takdim Şekli ve Ambalaj Muhtevası:** 16 IU, 36 IU GoQuick® enjeksiyonluk solüsyon için toz ve çözücü içeren 1 adet kullanıma hazır kalem. Reçete ile satılır. **Satış Fiyatı:** GoQuick® 16 IU 273,84 TL (19.02.2019), GoQuick® 36 IU 617,43 TL (19.02.2019). Sosyal Güvenlik Kurumu tarafından geri ödenir. Ödeme koşulları ile ilgili detaylı bilgi için Sosyal Güvenlik Kurumu, Sağlık Uygulamaları Talimatına bakınız. **Kısa ürün bilgisi/ kullanma talimatı onay tarihi:** 03.11.2016 **Ruhsat No:** 103/41 **Ruhsat tarihi:** 18.12.1997 **Ruhsat sahibi:** Pfizer İlaçları Ltd. Şti. Muallim Naci Cad. No:55 34347 Ortaköy-İSTANBUL Telefon no: (212) 310 70 00 - Faks no: (212) 310 70 58. Daha geniş bilgi için firmamıza başvurunuz. www.pfizer.com.tr

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Yetişkinlerde başlayan: İzole GH yetmezliğinde, 2 dinamik test ile hipofiz veya hipotalamus hastalığına bağlı durumlarda diğer hipofiz ön lob hormonlarından en az birinin (prolaktin hariç) eksikliği durumunda ise 1 dinamik test ile yetmezliğin kanıtlanması halinde eksikliği yerine koymak amacıyla kullanılır. **Pozoloji ve Uygulama Şekli:** SAİZEN çoklu doz kullanımı için tasarlanmıştır. SAİZEN dozu, vücut yüzey alanı ya da vücut ağırlığı temel alınarak her hasta için bireysel olmalıdır. SAİZEN'in aşağıdaki dozlarda gece uygulanan tavsiyeedilmektedir: *Çocuklarda ve ergenlerde:* Endojen büyüme hormonu salgısının yetersiz olduğu büyüme geriliği gösteren hastalarda: Subkutan uygulama ile günde 0.7-1.0 mg/m^2 vücut yüzey alanı ya da günde 0.025-0.035 mg/kg vücut ağırlığı. *Gonadal disgeneziden ötürü büyüme geriliği gösteren kızlarda (Turner sendromu):* Subkutan uygulama ile günde 1.4 mg/m^2 vücut yüzey alanı ya da günde 0.045-0.050 mg/kg vücut ağırlığı. Turner sendromlu hastalarda non-androjenik anabolik steroidlerle birlikte tedavi büyümeye cevabı artırabilir. *Kronik böbrek yetmezliğinden ötürü büyüme geriliği gösteren prepubertal çocuklarda (CRF):* Subkutan uygulama ile günde 1.4 mg/m^2 vücut yüzey alanı ya da günde 0.045-0.050 mg/kg vücut ağırlığı. *Gestasyonel yaşa göre Küçük doğmuş kısa çocuklarda büyüme geriliği (SGA):* Tavsiye edilen günlük doz, subkutan uygulama yoluyla 0.035 mg/kg vücut ağırlığıdır. (ya da 1 mg/m^2/gün, eşittir 0.1 IU/kg/gün ya da 3 IU/m^2/gün) Uygun yetişkin boya ulaşınca ya da epifizler kapanınca tedavi kesilmelidir. SGA ile doğan kısa çocuklardaki büyüme bozukluğunda, erişkin boya ulaşılıncaya kadar tedaviye devam edilmesi önerilmektedir. Eğer boy uzama hızı SDS'si +1'in altında ise, bir yıl sonrunda tedavi sonlandırılmalıdır. Erişkin boya ulaşıldığında (boy uzama hızı <2 cm/yıl olarak tanımlanmıştır) ve teyit gerekiyorsa epifizyal büyüme plaklarının kapanmasına uygun olarak kemik yaşı >14 (kızlarda) veya >16 (erkeklerde) olduğunda, tedavi sonlandırılmalıdır. *Yetişkinlerde:* büyüme hormonu eksikliğinde: Somatropin tedavisi başlangıcında, günlük subkutan enjeksiyon olarak 0.15-0.3 mg gibi düşük dozlar önerilir. Doz, İnsulin-benzeri Büyüme Faktörü 1 (IGF-1) değerleri ile kontrollu şekilde ayarlanmalıdır. Önerilen en fazla büyüme hormonu dozu nadiren 1.0 mg/gün değerini aşar. Genellikle en düşük etkili doz uygulanmalıdır. Yaşlılarda veya kilolu hastalarda, daha düşük dozlar gerekli olabilir. SAİZEN'i uygulamak için one.click oto-enjektör ya da cool.click iğnesiz oto-enjektör ya da easypod oto-enjektör ile sağlanan kullanım kılavuzunda ve hasta kullanma talimatında verilen talimatları takip ediniz. **Özel Popülasyonlara İlişkin Ek Bilgiler:** *Böbrek/Karaciğer yetmezliği:* Bu hasta grubunda pozoloji üzerine herhangi bir öneri yapılamamaktadır. *Pediyatrik popülasyon:* SAİZEN, büyüme hormonu eksikliği olan çocuklarda endikedir. *Geriyatrik popülasyon:* 60 yaş üstü hastalarda ve uzun süreli kullanımlarla ilgili sınırlı deneyim bulunmaktadır. **Kontrendikasyonlar:** SAİZEN içeriğindeki etkin maddeye ya da yardımcı maddelerden birine karşı aşırı hassasiyeti olan hastalarda kontrendikedir. Somatropin, epifizleri kapanmış çocuklarda büyümeyi artırmak için kullanılmamalıdır. Somatropin, herhangi bir aktif malin tümor bulgusu varlığında kontrendikedir. Tedaviye başlamadan önce, aktif intrakranial tümörler inaktif olmalı ve antitümör tedavi tamamlanmalıdır. Tümör büyümesine dair bir bulgu varlığında tedavi kesilmelidir. Proliferatif veya preproliferatif diyabetik retinopati durumunda Somatropin kullanılmamalıdır. Açık kalp ameliyatı, karın ameliyatı, çoklu kaza travması, akut solunum yetmezliği, ya da benzer durumlarla ilişkili akut hastalığı olan bireylerde somatropin kullanılmamalıdır. Kronik böbrek yetmezliği olan çocuklarda, böbrek nakli durumunda somatropin tedavisi kesilmelidir. **Özel kullanım uyarıları ve önlemleri:** Büyüme hormonu eksikliği olan hastaların teşhis ve tedavisinde deneyimli bir hekimin düzenli gözetimi altında tedavi takip edilmelidir. Önerilen günlük maksimum doz aşılmamalıdır. Büyüme hormonu ile tedavi alan remisyondaki intra ya da ekstrakranial neoplazili hastalar hekim tarafından dikkatle ve düzenli aralıklarda kontrol edilmelidir. Intrakranial tümöre bağlı sekonder büyüme hormonu eksikliği olan hastalar, altta yatan hastalığın herhangi bir ilerleme ya da yineleme durumu için sık sık muayene edilmelidir. Saizen tedavisi sırasında, özellikle pubertal olan ve/veya beraberinde tiroid tedavisi alan çocuklarda, kemik yaşı düzenli olarak takip edilmelidir. Bu şartlar altında, epifiz olgunlaşması hızlı gelişebilir. Epifizal füzyon meydana geldiğinde, büyüme hormonu eksikliği durumunun endokrin testleriyle yeniden konfirme edilmesi önerilir ve eğer varsa, yetişkinlerde büyüme hormonu eksikliği için endike olan dozlarda tedaviye devam edilebilir. Çocuklukta kanser atlatan ve sonrasında büyüme hormonu tedavisi alan hastalarda ikincil neoplasm (tümör) riskinin arttığı bildirilmiştir. İlk neoplazmları için kafa bölgesine radyoterapi uygulanarak tedavi edilen hastalarda, özellikle menenjiyomlar olmak üzere intrakranial tümörler, ikincil neoplazmlar içinde en yaygın olanıdır. Saizen, beraberinde büyüme hormonu eksikliği teşhisi konulmadığı sürece, büyüme geriliği olan, genetik olarak Prader-Willi sendromu olduğu doğrulanmış pediatrik hastaların uzun dönem tedavisi için endike değildir. Ciddi obezite, üst solunum yolu tıkanıklığı öyküsü, uyku apnesi veya tanımlanmamış solunum yolu enfeksiyonu gibi risk faktörlerinden bir veya daha fazlasına sahip olan Prader-Willi sendromlu çocuklarda büyüme hormonu tedavisi sonrasında uyku apnesi ve ani ölüm rapor edilmiştir. Büyüme hormonu ile tedavi edilmiş veya edilmemiş büyüme hormonu eksikliği olan çocuklarda kısıtlı sayıda ölsemi vakaları rapor edilmiştir. Bununla beraber, predispoze faktörlerin yokluğunda büyüme hormonu kullananlarda ölsemi insidansının arttığına dair bir bulgu yoktur. Somatropin, insülin duyarlılığını azaltabileceğinden dolayı, hastalar glukoz intoleransı yönünden gözlenmelidir. Diabetes mellitusu olan hastalarda, somatropin içeren tedaviye başlanması sonrasında insülin dozunu ayarlamak gerekebilir. Diabetes mellitusu ve glukoz intoleransı olan hastaların somatropin tedavisi süresince yakın takip edilmeleri gerekir. İlerlemeyen retinopati oluşlarında somatropin replasman tedavisi kesilmemelidir. Somatropin tedavisi, T4'den T3'e dönüşümü artırır ve başlangıç aşamasındaki hipotiroidizmin fark edilmesini önleyebilir. Bu yüzden somatropin ile tedaviye başladıktan sonra ve doz ayarı sonrası tüm hastalara tiroid fonksiyon testi yapılması tavsiye edilir. Hipopitüitarizm hastalarında somatropin tedavisine başlandığında, standart yerine koyma tedavisi yakından takip edilmelidir. Şiddetli ya da tekrarlayan başağrısı, görsel problemler, bulantı ve/veya kusma durumlarında, papilödem için funduskopi önerilir. Papilödem varsa, iyi huylu intrakraniall basınç artışı (psödotümor serebri) tanısı düşünülmeli ve uygunsa SAİZEN tedavisi kesilmelidir. Halen intrakranial hipertansiyonlu hastalarda klinik kararı vermede rehberlik edecek yeterli kanıt yoktur. Büyüme hormonu tedavisine tekrar başlanırsa, intrakranial hipertansiyon semptomları için dikkatli gözlem gerekir ve intrakranial hipertansiyon tekrarlarsa tedavi kesilmelidir. Nadir görülmesine rağmen, özellikle kanın ağnsı gelişen çocuklarda olmak üzere somatropin ile tedavi edilen hastalarda pankreatit düşünülmelidir. Tüm somatropin içeren preparatlarda olduğu üzere, bazı hastalarda somatropin'e karşı antikorlar oluşabilir. Bu antikorların bağlanma kapasitesi düşük olup büyüme hızına etkileri yoktur. Tedaviye yanıt vermeyen hastalarda somatropin'e karşı antikor üretimi test edilmelidir. Büyüme hormonu eksikliği ve hipotiroidizm gibi endokrin bozukluğu olan hastalarda, femur başı epifizlerinde daha sık kayma meydana gelebilir. Büyüme hormonu ile tedavi edilen çocuklarda, femur başı epifizlerinde kayma ya altta yatan endokrin bozuklukları ya da tedavi ile meydana gelen artmış büyüme hızından ötürüdür. Büyüme sıçramaları, prepubertal büyüme sıçraması esnasında özellikle gerilim altında olan kalça eklemi gibi, eklemle ilgili problem riskini artırabilir. SAİZEN ile tedavi edilen hastalarda diz ağrısı ya da kalça şikayetleri ya da topallama gelişmesine karşı hekimler ve hasta yakınları dikkatli olmalıdır. Kronik böbrek yetmezliğinden ötürü büyüme geriliği olan hastalar, renal osteodistrofi ilerleme bulgusu için periyodik olarak kontrol edilmelidir. Femur başı epifiz kayması ya da femur başının avasküler nekrozları, ilerlemiş renal osteodistrofisi olan çocuklarda görülebilir ve bu problemlerin büyüme hormonu tedavisinden etkilendiği kesin değildir. Tedavi başlamadan önce kalçanın röntgeni alınmalıdır. Kronik böbrek yetmezliği olan çocuklarda, tedaviye başlamadan önce böbrek fonksiyonu normalin %50 altına kadar düşmüş olmalıdır. Büyüme bozukluğunu doğrulamak için, tedaviye başlamadan önce 1 yıl büyüme takip edilmelidir. Böbrek yetmezliği için konservatif tedavi (tedavi başlamadan önce 1 yıl süreyle asidoz, hiperparatiroidizm ve beslenme kontrolünü içerir) saptanmalı ve büyüme hormonu tedavisi esnasında sürdürülmelidir. Tedavi böbrek nakli esnasında kesilmelidir. SGA ile doğan kısa çocuklarda, tedaviye başlamadan önce, büyüme bozukluğunu açıklayabilecek diğer medikal sebeplerin ve tedavilerin ekarte edilmesi gerekmektedir. SGA hastalarında, tedaviye başlamadan önce, açlık kan şekeri ve insülin ölçümlerinin yapılması ve sonrasında yılda bir kez tekrarlanması tavsiye edilmektedir. Diabetes Mellitus için artmış riske sahip hastalarda (örn. ailede diyabet öyküsü), şişmanlık, artmış vücut kitle indeksi, şiddetli insülin direnci, acanthosis nigricans) oral glikoz toleans testi (OGTT) yapılmalıdır. Diyabet varlığı kesin ise, büyüme hormonu uygulanmamalıdır. SGA hastalarında, tedaviye başlamadan önce, IGF-I seviyesi ölçümünün yapılması ve sonrasında yılda iki sefer tekrarlanması tavsiye edilmektedir. Tekrarlanan ölçümlerde IGF-I seviyeleri, yaş ve pubertal durumuna uygun referanslarla karşılaştırıldığında +2 SD aşarsa, doz ayarlaması için IGF-I / IGFBP-3 oranı değerlendirilmeye alınabilir. SGA hastalarında, ergenlik başlangıcında tedaviye başlanması konusundaki deneyim sınırlıdır. Bu nedenle puberte dönemine yakın hastalarda tedaviye başlanması önerilmemektedir. Silver-Russell sendromuna sahip SGA hastalarındaki deneyimler sınırlıdır. SGA ile doğan kısa çocuklarda, eğer erişkin boyuna erişilmeden tedavi sonlandırılırsa, Somatropin tedavisi ile boy uzamasında elde edilen büyüme hızının bir kısmı kaybedilebilir. Yetişkinlerde büyüme hormonu replasman tedavisi esnasında sıvı retansiyonu beklenir. Devamlı ödem ya da şiddetli paraestezi durumunda karpal tünel sendromu gelişmesini önlemek için doza azaltılmalıdır. Akut kritik hastalık gelişen tüm hastalarda, büyüme hormonu ile tedavinin olası yararları; olabilecek potansiyel riskler düşünülerek değerlendirilmelidir. Lipoatrofiyi önlemek için enjeksiyon farklı yerlere yapılmalıdır. Yetişkinlerde büyüme hormonu eksikliği ömür boyu süren ve tedavi edilmesi gereken bir durumdur. Ancak 60 yaş üstü hastalarda ve uzun süreli kullanımlarla ilgili sınırlı deneyim bulunmaktadır. SAİZEN her dozunda 1 mmol (23 mg)'dan daha az sodyum ihtiva eder, yani aslında "sodyum içermez". **Diğer Tıbbi Ürünler ile Etkileşimler ve Diğer Etkileşim Şekilleri:** Eşzamanlı olarak glukokortikoid kullanılması, somatropinin büyümeyi artırıcı etkisini engeller. ACTH eksikliği olan hastalarda büyüme hormonu tedavilerinin engellenmemesi için glukokortikoid tedavileri çok dikkatli ayarlanmalıdır. İlaveten, büyüme hormonu replasmanının başlatılması, inaktif kortizonun kortizole dönüşmesini sağlayan bir enzim olan 11β-hidroksisteroid dehidrojenaz, tip 1 (11β-HSD1)'in aktivitesini azaltarak bazı hastalarda ikincil adrenal yetmezliğin ortaya çıkmasına neden olabilir. Glukokortikoid replasman tedavisi alan hastalarda somatropinin başlatılması, kortizol eksikliğinin ortaya çıkmasına neden olabilir. Glukokortikoid dozunun ayarlanması gerekebilir.Büyüme hormonu eksikliği olan erişkin hastalarda yapılan bir çalışma, büyüme hormonu uygulamasının sitokrom P450 enzim sistemi ile metabolize edilen ilaçların klirensini artırabileceğini göstermektedir. Özellikle, CYP P450 3A4 hepatik enzimler ile metabolize olduğu bilinen ilaçlarla (ör. seks stereoidleri, kortikosteroidler, antikonvülsanlar ve siklosporin) somatropin ile eşzamanlı kullanıldığı zaman, bu ilaçların klirensi artarak plazma seviyelerinde düşmeye sebep olabilir. Bu gözlemin klinik anlamı bilinmemektedir. **Gebelik ve Laktasyon:**Gebelikte kullanım kategorisi C'dir. Somatropin içeren ürünler kontrasepsiyon kullanmayan çocuk doğurma potansiyeline sahip kadınlarda kullanılmamalıdır. Gebelerde yapılmış klinik veri mevcut değildir. Hayvanlarda somatropin içeren ürünlerle ilgili yürütülen reprodüktif çalışmalarda embriyo veya fetüs için advers reaksiyonların arttığına dair bir bulgu bulunmaktadır. Bununla birlikte somatropin içeren ilaçlar gebelikte ve kontrasepsiyon kullanmayan çocuk doğurma potansiyeli olan kadınlarda önerilmez. Somatropinin insan sütüyle atılıp atılmadığı bilinmemektedir. Somatropinin süt ile atılımı emziren kadınlarda araştırılmamıştır. Bu nedenle emziren kadınlarda somatropin uygulandığında dikkatli olunmalıdır. Emzirmenin durdurulup durdurulmayacağına ya da SAİZEN tedavisinin durdurulup durdurulmayacağına/tedaviden kaçınılıp kaçınılmayacağına ilişkin karar verilirken, emzirmenin çocuk açısından faydası ve SAİZEN tedavisinin emziren anne açısından faydası dikkate alınmalıdır. Üreme yeteneği / Fertilite: Klinik dışı toksisite çalışmaları, rekombinant insan büyüme hormonunun erkek ve kadın fertilitesi üzerinde advers etkileri indüklemediğini göstermiştir. Somatropin içeren ilaçların hastanın araç ya da makine kullanma kabiliyeti üzerine etkisi yoktur. **İstenmeyen Etkiler:** Hastaların %10'a kadarı, özellikle subkutan yol kullanıldığı zaman, enjeksiyon yerinde kızarıklık ve kaşıntı yaşayabilir. Yetişkinlerde büyüme hormonu replasman tedavisi esnasında sıvı retansiyonu beklenir. Ödem, eklemişmesi, artralji, myalji, ve paresteziler sıvı retansiyonunun klinik manifestasyonları olabilir. Yine de bu semptomlar genellikle geçicidir ve doza bağlıdır. Çocukluk döneminde büyüme hormonu eksikliği tanısı konmuş yetişkin hastalarda ortaya çıkan istenmeyen etkiler, hastalığın yetişkin dönemde ortaya çıktığı hastalara göre daha azdır. Hastaların küçük bir yüzdesinde somatropin'e karşı antikorlar oluşabilir; bugüne kadar antikorların düşük bağlanma kapasitesine sahip olduğu görülmüş ve bu antikorlar ile, gen delesyonu olan hastalar dışında, büyümede yavaşlamaya ilişkilendirilmemiştir. Büyüme hormonu gen kompleksinin delesyonuna bağlı boy kısalığı gibi nadir görülen durumlarda, büyüme hormonu ile tedavi büyümeyi geriletenantikorları indükleyebilir. Büyüme hormonu ile tedavi edilmiş veya edilmemiş büyüme hormonu eksik çocuklarda bazı ölsemi vakaları rapor edilmiştir. Bununla beraber, predispoze faktörlerin yokluğunda büyüme hormonu kullananlarda ölsemi insidansı arttığına dair bir bulgu yoktur. Pazarlama sonrası çalışmalarda büyüme hormonu tedavisi sırasında pankreatit bildirilmiştir. İstenmeyen etkiler, ciddiyetleri açısından azalan sıra içinde yer almaktadır. Bağokiklik sistemi hastalıkları: Bilinmiyor: Lokal veya genelleşmiş aşırı duyarlılık reaksiyonları. Endokrin hastalıkları: Çok seyrek: Hipotiroidizm. Metabolizma ve beslenme hastalıkları: Yaygın (Yetişkinlerde)Yaygın olmayan (çocuklarda): Sıvı tutulması: periferal ödem, sertlik, artralji, myalji, parestezi, Bilinmiyor: İnsülin rezistansı hiperinsülinizm ve nadiren hiperglisemi ile sonuçlanabilir. Sinir sistemi hastalıkları: Yaygın: Baş ağrısı (özele), karpal tünel sendromu (yetişkinlerde). Yaygın olmayan: İdiopatik intrakraniall hipertansiyon (iyi huylu intrakraniall basınç artışı), karpal tünel sendromu (çocuklarda). Gastrointestinal hastalıklar: Bilinmiyor: Pankreatit Kas-iskelet bozuklukları, bağ doku ve kemik hastalıkları: Çok seyrek: Femur başı epifiz kayması, ya da femur başının avasküler nekrozları. Genel bozukluklar ve uygulama bölgesine ilişkin bozukluklar: Yaygın: Enjeksiyon yeri reaksiyonları, Lokalize lipoatrofi Yaygın olmayan: Jinekomasti **Doz Aşımı ve Tedavisi:** Önerilen dozları aşmak yan etkilere sebep olabilir. Doz aşımı hipoglisemi ve sonrasında hiperglisemiye yol açabilir. Bundan başka, somatropin doz aşımı sıvı retansiyonuna sebep olabilir. **Saklamaya Yönelik Özel Tedbirler:** Raf ömrü 36 aydır. 25ºC altındaki oda sıcaklığında ve ambalajında saklayınız. Dondurmayınız. Rekonstitüye edildiğinde, kartuşda, 2°C-8°C arasında buzdolabında saklanmalı ve en geç 28 gün içinde kullanılmalıdır. Rekonstitüye edilmiş SAİZEN kartuşu içeren easypod ve one.click oto-enjektörler 2°C-8°C buzdolabında arasında saklanmalıdır. Cool.click iğnesiz oto-enjektör kullanıldığı zaman, sadece rekonstitüye edilmiş SAİZEN kartuşu 2°C-8°C arasında buzdolabında saklanmalıdır. Sulandırılmış enjeksiyonluk çözelti partikülsüz berrak olmalıdır. Çözelti partikül içeriyorsa, enjekte edilmemelidir. **Ruhsat Sahibi:** Merck İlaç Ecza ve Kimya Tic.A.Ş. Atatürk Mh. Ertuğrul Gazi Sk. Metropol İstanbul Sit. C2 Apt. No: 2A/20, Ataşehir/İstanbul Tel: 0 216 578 66 00 Fax: 0 216 469 09 22 **KDV dahil Perakende Satış Fiyatı:** 616,67 TL (19.02.2019). **Ruhsat Numarası:** 112/56 **İlk Ruhsat Tarihi:** 29.05.2002 **KÜB Onay Tarihi:** 17.04.2018. **Reçete ile satılır.**Daha geniş bilgi için firmamıza başvurunuz.

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