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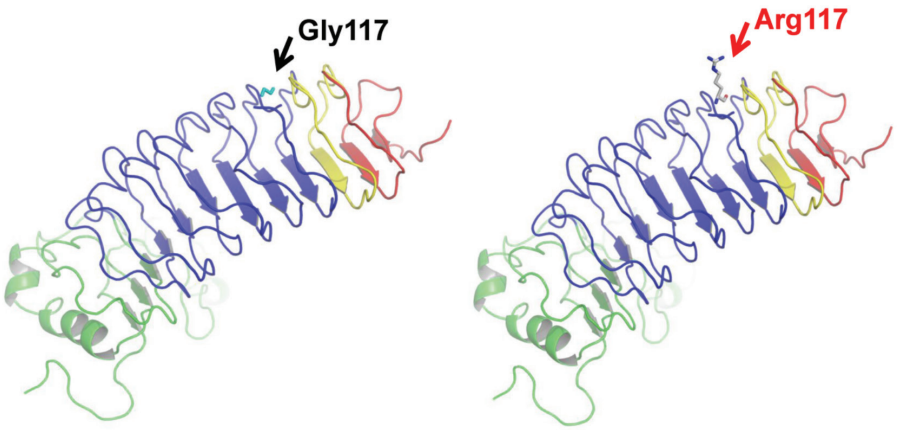

Figure 2. Three-dimensional structure model of the LHCGR protein. The indicated amino acid (p.117, colored arrow: black, wild-type; red, variant) is located in the first leucine-rich repeat domain of the LHCGR protein

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SAİZEN 8 mg click.easy Enjeksiyonluk Çözelti İçin Toz ve Çözücü
Her bir SAİZEN 8 mg click.easy flakonu, 8 mg Somatropin (rekombinant DNA teknolojisi ile memeli hücrelerinde üretilen insan büyüme hormonu) içerir. 1 kartuş bakteriostatik çözücü ise, 1,37 mL enjeksiyonluk suda %0,3 metakresol içerir. **Terapötik Endikasyonlar:** SAİZEN aşağıdaki tedaviler için endikedir; çocuklarda endojen büyüme hormonu salgısının olmaması veya azlığı ile meydana gelen büyüme gerilği; kromozom analizleri ile teyid edilmiş, gonadal disgenezili kızlarda büyüme gerilği (Turner sendromu); kronik böbrek yetmezliğinden ötürü prepubertal çocuklarda büyüme gerilği (CRF); doğum ağırlığı ve/veya boyu -2 SD altında olan, gestasyonel yaşa göre küçük doğmuş (SGA) ve 4 yaş ya da üzeri itibariyle büyümeyi yakalayamamış (son yıl içinde HV SDS < 0) kısa çocuklarda büyüme bozukluğu (mevcut boy SDS < -2.5 ve ayarlanmış parental boy SDS < -1); yetişkinlerde, büyüme hormonu eksikliğini ölçen tek dinamik test ile teşhis edilen belirgin büyüme hormonu eksikliğinin replasman tedavisi. *Hastalar aşağıdaki kriterlere uymalıdır:* Çocuklukta başlayan; Çocukluk esnasında büyüme hormonu eksikliği teşhis edilmiş hastalar, tekrar test edilmelidir ve SAİZEN ile replasman tedavisine başlamadan önce büyüme hormonu eksiklikleri teyid edilmelidir. 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. **Pozolojі 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 uyumadan önce uygulanması tavsiyeedilmektedir: *Çocuklarda ve ergenlerde:* Endojen büyüme hormonu salgısının yetersiz olduğu büyüme gerilği 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 disgenezisten ötürü büyüme gerilği 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 gerilği 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 gerilği (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 sonunda 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. İntrakranial 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. Ilk neoplazmları için kafa bölgesine radyoterapi uygulanarak tedavi edilen hastalarda, özellikle meninjiyomlar olmak üzere intrakranial tümörler, ikincil neoplazmlar içinde en yaygın olanıdır. Saizen, beraberinde büyüme hormonu eksikliği teşhisi konulmadığı sürece, büyüme gerilği 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 olgularında 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 fundoskopi önerilir. Papilödem varsa, iyi huylu intrakranial basınç artışı (psödotümor serebri) tanısı düşünülmeli ve uygunsa SAİZEN tedavisi kesilmelidir. Halen intrakranial hipertansiyonlu hastalarda klinik karar vermede rehberlik edecek yeterli kant yoktur. Büyüme hormonu tedavisine tekrar başlarsanız, intrakranial hipertansiyon semptomları için dikkatli gözlem gerekir ve intrakranial hipertansiyon tekrarlarsa tedavi kesilmelidir. Nadir görülmesine rağmen, özellikle karın ağrısı 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 sçramaları, 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 gerilği 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 kontrolunu 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 toleransı 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 değerlendirmeye alınabilir. SGA hastalarında, ergenlik başlangıcında tedaviye başlanması konusundaki deneyim sınırlıdır. Bu nedenle puberte döneminde 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 dozaj 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. Oral östrojenler, serum IGF-1'in somatropin tedavisine cevabını azaltabileceğinden, oral östrojen replasmanı alan hastalarda daha yüksek somatropin dozları 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 reproduktif çalışmalarda embriyo veya fetüs için advers reaksiyonların arttığına dair bir bulgu bulunmamaktadır. 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 kadarında, özellikle subkutan yol kullanıldığı zaman, enjeksiyon yerinde kızarıklık ve kaşıntı yaşayabilir. Yetişkinlerde büyüme hormonu replasman tedavisi esnasında sıvı retansiyonu beklenir. Ödem, eklemsişmesi, 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 yavaşlama ilişkilendirilmemiştir. Büyüme hormonu gen komplekisinin 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 lösemi vakaları rapor edilmiştir. Bununla beraber, predispoze faktörlerin yokluğunda büyüme hormonu kullananlarda lösemi insidansı arttığına dair bir bulgu yoktur. Pazarlama sonrası çalışmalarda büyüme hormonu tedavisi sırasında pankreatit bildirilmiştir. İstenmeyen etkiler, ciddiyetleri açısından azalan sıra içinde yer almaktadır. Bağışıklık 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. 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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. **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 arttırabilir, 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 papilldödemi için fundoskopinin yapılması tavsiye edilmektedir. Eğer papilldödemi 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 TİTCK onaylı KÜB 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 yetişkinlerde 5 mg/1,5 mL OMNİTROPE®'un subkütan enjeksiyonundan sonra Cmaks ve tmaks değerleri sırasıyla 72 ± 28 mikrogram/L ve 4 ± 2 saattir. Sağlıklı yetişkinlerde 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 yetişkinlerde 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 saattir, 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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Şüpheli advers reaksiyonların raporlanması: Ruhsatlandırma sonrası şüpheli ilaç advers reaksiyonlarının raporlanması büyük önem taşımaktadır. Raporlama yapılması, ilacın yarar/risk dengesinin sürekli olarak izlenmesine olanak sağlar. Sağlık mesleği mensuplarının herhangi bir şüpheli advers reaksiyonu Türkiye Farmakovijilans Merkezi (TÜFAM)'ne bildirmeleri gerekmektedir (www.titck.gov.tr; e- posta: tufam@titck.gov.tr; tel: 0 800 314 00 08; faks: 0 312 218 35 99).

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Review

110 Pathogenesis of Thalassemia Major-associated Osteoporosis: A Review with Insights from Clinical Experience
Agostino Gaudio, Nancy Morabito, Antonino Catalano, Rosario Rapisarda, Anastasia Xourafa, Antonino Lasco, (Catania, Messina, Turkey)

Original Articles

118 The Glucose Control Resistance Scale
Maria-Eleni Nikita, Helen M. Hendy, Keith E. Williams, Paul L. Mueller, (Maryland, Pennsylvania, United States)

125 Vaspin, a Compensatory Mechanism Against High Glucose Levels Since Birth?
Citlalli E. Hernández-Rodríguez, Cynthia M. Estrada Zúñiga, Manuel E. De la O-Cavazos, Fernando F. Montes-Tapia, Patricia Gerez-Martínez, Fernando J. Lavalle-González, Consuelo Treviño Garza, (Monterrey, Mexico)

132 Evaluation of Normal Thyroid Tissue and Autoimmune Thyroiditis in Children Using Shear Wave Elastography
Figen Bakırtaş Palabıyık, Ercan İnci, Esra Deniz Papatya Çakır, Elif Hocaoğlu, (İstanbul, Turkey)

140 Comparison of Treatment Regimens in Management of Severe Hypercalcemia Due to Vitamin D Intoxication in Children
Korcan Demir, Hakan Döneray, Cengiz Kara, Zeynep Atay, Semra Çetinkaya, Atilla Çayır, Ahmet Anık, Erdal Eren, Ahmet Uçaktürk, Gülay Can Yılmaz, Ayça Törel Ergür, Mustafa Kendirci, Zehra Aycan, Abdullah Bereket, Murat Aydın, Zerrin Orbak, Behzat Özkan, (İzmir, Erzurum, Samsun, İstanbul, Ankara, Aydın, Bursa, Kırıkkale, Kayseri, İzmir, Turkey)

149 Clinical and Laboratory Characteristics of Hyperprolactinemia in Children and Adolescents: National Survey
Erdal Eren, Ayça Törel Ergür, Şükriye Pınar İşgüven, Eda Çelebi Bitkin, Merih Berberoğlu, Zeynep Şıklar, Firdevs Baş, Servet Yel, Serpil Baş, Elif Söbü, Abdullah Bereket, Serap Turan, Halil Sağlam, Zeynep Atay, Oya Ercan, Tülay Güran, Mehmet Emre Atabek, Hüseyin Anıl Korkmaz, Aylin Kılınç Uğurlu, Ayşehan Akıncı, Esra Döğer, Enver Şimşek, Emine Demet Akbaş, Ayhan Abacı, Ülkü Gül, Sezer Acar, Eda Mengen Uçaktürk, Melek Yıldız, Edip Ünal, Ömer Tarım, (Bursa, Kırıkkale, Sakarya, Samsun, Ankara, İstanbul, Van, Konya, Balıkesir, Malatya, Eskişehir, İzmir, Kayseri, Adana, Diyarbakır, Turkey)

157 Urine Levels of Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases in Children with Type 1 Diabetes Mellitus
Zeynep Yürük Yıldırım, Alev Yılmaz, Cemile Pehlivanoğlu, Asuman Gedikbaşı, Mehmet Yıldız, Ahmet Dirican, Rüveyde Bundak, Feyza Darendeliler, Sevinç Emre, Ahmet Nayır, (İstanbul, Turkey)

164 Management of Thyrotoxicosis in Children and Adolescents: A Turkish Multi-center Experience
İhsan Esen, Elvan Bayramoğlu, Melek Yıldız, Murat Aydın, Esin Karakılıç Özturhan, Zehra Aycan, Semih Bolu, Hasan Önal, Yılmaz Kör, Deniz Ökdemir, Edip Ünal, Aşan Önder, Olcay Evliyaoğlu, Atilla Çayır, Mehmet Taştan, Ayşegül Yüksel, Aylin Kılınç, Muammer Büyükinan, Bahar Özcabı, Onur Akın, Çiğdem Binay, Suna Kılınç, Ruken Yıldırım, Emel Hatun Aytaç, Elif Sağsak, (Elazığ, Ankara, İstanbul, Samsun, Düzce, Adana, Diyarbakır, Erzurum, Kocaeli, Konya, Tekirdağ, Gaziantep, Turkey)

173 Evaluation of Unfavorable Cardiovascular and Metabolic Risk Factors in Children and Young Adults with Haemophilia
Melek Yıldız, Nihal Özdemir, Hasan Önal, Basak Koç, Beyza Eliuz Tipici, Bülent Zülfikar, (İstanbul, Turkey)

181 Vitamin D Deficiency and Insufficiency According to the Current Criteria for Children: Vitamin D Status of Elementary School Children in Turkey
F. Sinem Hocaoğlu-Emre, Devrim Sarıbal, Osman Oğuz, (İstanbul, Turkey)

189 Effect of Education on Impaired Hypoglycemia Awareness and Glycemic Variability in Children and Adolescents with Type 1 Diabetes Mellitus
Günay Demir, Samim Özen, Hafize Çetin, Şükran Darcan, Damla Gökşen, (İzmir, Turkey)

CONTENTS

JCRPE Journal of Clinical Research in Pediatric Endocrinology
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Case Reports

196 A Novel Homozygous CYP19A1 Gene Mutation: Aromatase Deficiency Mimicking Congenital Adrenal Hyperplasia in an Infant without Obvious Maternal Virilisation
Fatma Dursun, Serdar Ceylaner, (İstanbul, Ankara, Turkey)

202 Efficiency of Single Dose of Tolvaptan Treatment During the Triphasic Episode After Surgery for Craniopharyngioma
Fatih Gürbüz, Mehmet Taştan, İhsan Turan, Bilgin Yüksel, (Adana, Turkey)

207 A Child with Prostaglandin I_2-associated Thyrotoxicosis: Case Report
Yuri Sonoda, Kenichiro Yamamura, Kanako Ishii, Kazuhiro Ohkubo, Kenji Ihara, Yasunari Sakai, Shouichi Ohga, (Fukuoka, Oita, Japan)

211 Novel Compound Heterozygous Variants in the *LHCGR* Gene in a Genetically Male Patient with Female External Genitalia
Mei Yan, Julaiti Dilihuma, Yanfei Luo, Baoerhan Reyilanmu, Yiping Shen, Maimaiti Mireguli, (Xinjiang Uygur Autonomous Region, China, Massachusetts, United States)

Letter to the Editor

218 Diazoxide Causality Assessment of a Pericardial Effusion in a Child with Kabuki Syndrome
Irene Maffre, Marie Vincenti, Fabienne Dalla Vale, Cyril Amouroux, Oscar Werner, Alexandra Meilhac, Gaelle de Barry, Pascal Amedro (Montpellier, France)

CONTENTS

REVIEW

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Pathogenesis of Thalassemia Major-associated Osteoporosis: A Review with Insights from Clinical Experience

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Abstract

Due to increasing life expectancy in thalassemia major (TM), osteoporosis is emerging as a significant problem. Its aetiology is multifactorial, culminating in increased bone resorption and impaired remodelling. Hypogonadism and marrow expansion seem to play an important role, but iron overload, deferoxamine toxicity, a defective growth hormone-insulin-like growth factor-1 axis and multiple endocrinopathies may represent additional causes of bone damage. Many of these patients, though under appropriate treatment programs, do not achieve normal peak bone mass. The receptor activator of nuclear factor kappa-ß (RANK)/RANK ligand/osteoprotegerin and the Wnt/β-catenin systems work as major mediators of imbalanced bone turnover and bone loss. Additional genetic factors, such as collagen type 1 alpha 1 and vitamin D receptor gene polymorphisms, may exert some influence on the enhanced fracture risk observed in TM. To date, in spite of adequate hormone replacement, chelating therapy and acceptable haemoglobin levels, subjects with TM display impaired bone density and imbalanced bone turnover, thus the puzzle of the pathogenesis of TM-induced osteoporosis remains far from being solved.

Keywords: Osteoporosis, thalassemia major, hypogonadism, marrow expansion, bone turnover

Introduction

Thalassemia major (TM) is a hereditary disease caused by defective globin synthesis, resulting in abnormal as well as a decreased quantity of globin chains, ineffective erythropoiesis, haemolysis and increased red blood cell turnover. Cooley et al (1) described the first patients with anaemia, splenomegaly and cranial and facial bone enlargement. These bone changes were due to the marked expansion of the bone marrow, secondary to anaemia and ineffective erythropoiesis (2,3). Although optimised blood transfusions and iron chelation programs have greatly increased the life expectancy of TM patients and prevented these severe bone alterations, osteoporosis and osteopenia remain serious complications, even in well-transfused and well-iron chelated patients (4).

The pathogenesis of bone changes in TM is not fully clarified. Several studies have shown that multiple factors may act in concert to produce bone disease in TM including bone marrow expansion (5), hypogonadism (6,7,8), defective growth hormone-insulin-like growth factor-1 (GH-IGF-1) axis (9,10,11,12), altered pattern of cytokines (13), iron deposit in bone (5,14,15), deferoxamine bone toxicity (16,17) and vitamin D deficiency (18). Some of these pathogenic factors, directly and/or indirectly, affect osteoblastic population, leading to depressed bone formation, while others often increase osteoclastic bone resorption.

In this review, in the light of our experience, we analysed the alterations of bone metabolism and the acquired and genetic factors that could be responsible for the development of osteopenia/osteoporosis in TM patients.

Bone Metabolism in TM Patients

Osteoporosis is a skeletal disorder characterized by compromised bone strength, predisposing to an increased risk of fracture (19). According to the World Health Organization, diagnosis of osteoporosis is based on the T-score for bone mineral density (BMD), assessed at the lumbar spine or the femoral neck. Osteoporosis is defined

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by a BMD that is 2.5 standard deviations (SD) or more below the mean value for a young adult female (T-score less than or equal to -2.5 SD) (20). The Z-score describes the number of SDs by which the BMD in an individual differs from the mean value expected for a given age and sex. The diagnosis of osteoporosis in children and adolescents should not be made on the basis of densitometric criteria alone. In the absence of vertebral compression (crush) fractures, the diagnosis of osteoporosis is indicated by the presence of both a clinically significant fracture history and BMD Z-score less than or equal to -2.0 SD (21). In some studies (22,23), in order to reduce the influence of bone size on BMD measurements in the growing skeleton, the apparent volumetric density of the lumbar spine has been calculated using a specific formula (24).

In TM patients, it is very common to find low BMD values (osteopenia or osteoporosis) and in some studies up to 90%, even in optimally transfused and chelated patients, as is shown in Table 1 (8,25,26,27,28,29,30).

Prevalence of fractures in TM patients is depicted in Table 2 and ranges from 16% to 49%, depending on study population and method of data collection (3,31,32,33,34,35). Extremity fractures are the most common (26), in particular at the upper extremity (31). Vertebral fractures are usually underestimated, and their prevalence varies from 2.6% to 13% (26,36).

TM patients, in spite of following a regular transfusional regimen, and receiving adequate sex hormone replacement and chelating therapy, show imbalanced bone turnover with an increased resorptive phase that is not followed by an appropriate neoformation rate, resulting in a decreased BMD, particularly at the vertebral level, where trabecular bone is mostly represented (22,26,37,38,39). In previous studies (12,22), we described a decreased neoformation phase in accordance with Mahachoklertwattana et al (23) and histomorphometric studies performed by De Vernejoul et al (40).

The depression of bone formation, even if slight, is surprising because an increase in resorption is generally followed by a corresponding increase in bone formation due to coupling of bone turnover. Numerous acquired factors could lead to the inhibition of osteoblastic activity, such as a defective GH-IGF-1 axis, iron deposits in bone, or deferoxamine toxicity (12,22). Many studies (12,22,37,38,41,42) have

Table 1. Osteoporosis/osteopenia prevalence in thalassemia major patients

Study	Subjects (n)	Gender	Mean age (year)	Osteoporosis (%)	Osteopenia (%)	Criteria for diagnosis of osteoporosis
Jensen et al (8)	82	38 males 44 females	25 (male) 27 (female)	51.2%	45.1%	Osteopenia: Z-score between -1 and -2.5 SD Osteoporosis: Z-score below -2.5 SD.
Vogiatzi et al (25)	31	14 males 17 females	15.3	61.3%	22.6%	Osteopenia: Z-score between -1 and -2 SD Osteoporosis: Z-score below -2 SD
Vogiatzi et al (26)	236	116 males 120 females	24.4	49.1%	30.5%	Osteopenia: Z-score between -1 and -2 SD Osteoporosis: Z-score below - 2 SD
Pirinççioğlu et al (27)	47	23 males 22 females	7.42	62%	NA	Osteopenia: Z-score between -1 and -2.5 SD Osteoporosis: Z-score below -2.5 SD
Aslan et al (28)	47	25 males 22 females	NA	53.1%	44.6%	Osteopenia: Z-score between -1 and -2.5 SD Osteoporosis: Z-score below -2.5 SD
Izadyar et al (29)	40	21 males 19 females	23.0	12.5% (femoral level) 37.5% (lumbar level)	37.5% (femoral level) 47.5% (lumbar level)	Osteopenia: Z-score between -1 and -2.5 SD Osteoporosis: Z-score below -2.5 SD
Tzoulis et al (30)	99	49 males 50 females	36	55.5%	NA	Osteoporosis: Z-score below - 2 SD

NA: not available, SD: standard deviation

shown increased osteoclast activation in these patients, measuring markers of bone resorption such as urinary levels of N-telopeptide of collagen type 1, serum levels of tartrate resistant acid phosphatase isoform-5b, and urinary pyridinium cross-links. The mechanism responsible for this osteoclast activation in well-treated thalassemic patients could be related to the altered cytokines network, which is often observed in these patients.

Cytokines Network

The receptor activator of nuclear factor kappa-ß (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system regulates the activation and proliferation of osteoclast precursors (43). In our previous study (42), and in accordance with others (41), we found that the ratio of RANKL/OPG is increased in patients with TM and osteoporosis, showing that the RANKL/OPG system acts as an important paracrine mediator of bone metabolism also in TM patients. Cytokines other than RANKL and OPG, such as interleukin (IL)-1α, IL-6 and tumor necrosis factor-α, that are largely recognised as important effectors in the pathogenesis of several forms of osteoporosis (44,45,46,47), could have a role in TM-related osteoporosis. Our data (13) clearly showed an increase of circulating pro-osteoclastogenic cytokines associated with several markers of bone turnover and densitometric data, also pointing to their potential role in accelerating bone loss in TM-related osteoporosis. In particular, we observed significantly higher serum concentrations of IL-1α and IL-6 in TM patients and a significant correlation of these cytokines with urinary pyridinium cross-links (13).

Recently, the Wnt/β-catenin canonical pathway has been suggested to be involved in bone remodelling by promoting proliferation and differentiation of osteoblast precursor cells, reducing apoptosis of mature osteoblasts, and promoting the ability of differentiated osteoblasts to inhibit osteoclast differentiation. This pathway has been proposed to participate in the pathogenesis of osteoporosis in TM, and negative modulators of this signalling system, such as Dickkopf-1 and sclerostin, have also been associated with BMD in TM patients (48,49).

Bone Marrow Expansion

Bone marrow expansion (2,3,4,50) is considered by various authors as a major determinant of bone destruction in TM patients. In spite of regular blood transfusions, the ineffective erythropoiesis in TM is not fully suppressed. Expansion of the bone marrow may contribute to the decreased BMD - even if data are contradictory (51) - because transferrin receptor studies have demonstrated increased bone marrow activity, even in patients with low reticulocyte count or marrow hypoplasia (52).

An intimate relationship between bone marrow and the process of remodelling exists, however. This interaction between bone marrow and bone tissue could explain the fact that bone loss in TM largely involves trabecular bone. In fact, the lumbar spine, which consists mostly of trabecular bone and with wide bone marrow spaces, is the most affected site in these patients (23).

It has been speculated that the increased generation of cells of the erythropoietic lineage may adversely affect the proliferation and maturation of cells of the osteogenic lineage. Osteoclasts originate from a hemopoietic granulocyte-macrophage lineage. The cytokines that are involved in haematopoiesis are also involved in the development of osteoclasts (53). Therefore, it is possible that the mechanism that stimulates haematopoiesis in TM

Table 2. Fracture prevalence in thalassemia major patients

Study	Subjects (n)	Gender	Mean age (year)	Fracture prevalence (%)
Exarchou et al (32)	62	36 males 26 females	16.7	32.2%
Finsterbush et al (33)	61	30 males 31 females	16.0	49.1%
Ruggiero and De Sanctis (3)	977	472 males 505 females	NA	19.7%
Vogiatzi et al (34)	379	177 males 202 females	20.2	16.6%
Fung et al (31)	152	80 males 72 females	25.5	38.8%
Sutipornpalangkul et al (35)	136	48 males 88 females	30.8	44.1%

NA: not available

may also stimulate osteoclastic formation and/or activity, which, in turn, increases bone resorption and reduces bone mass.

Iron Overload in Endocrine Glands

A regular transfusional regimen is a cornerstone of TM treatment, but theis results in significant iron overload. Excessive iron is deposited in almost all tissues but primarily in the liver, the heart and the endocrine glands. Early introduction of a chelating agent to prevent iron overload in vulnerable organs leads to improved life expectancy (54).

TM patients often present with multiple endocrine dysfunctions including growth failure, hypogonadism, diabetes, hypothyroidism, hypoparathyroidism and, less frequently, hypoadrenalism (5,55,56,57). Several authors demonstrated that these abnormalities were closely related to iron overload, as shown by histological findings in different endocrine glands (58). Shamshirsaz et al (5), analysing 220 TM patients, found significant differences in mean serum ferritin levels between TM patients affected by primary amenorrhea and hypogonadism and TM patients without endocrinopathies. Moreover, the authors observed that impaired puberty was the most common endocrine abnormality (over 70% of the participants). The prevalence of other endocrinopathies was much lower with 17.5% hypogonadism, 8.7% diabetes mellitus, 7.7% primary hypothyroidism and 7.6% hypoparathyroidism. De Sanctis et al (55), analysed 1861 patients and reported slightly different data. In particular, failure of puberty was the major clinical endocrine defect and was present in 51% of boys and 47% of girls, all over the age of 15 years. Secondary amenorrhoea was recorded in 23% of patients, primary hypothyroidism in 6.2%, insulin dependent diabetes mellitus in 4.9% and hypoparathyroidism in 3.6% of the patients.

Hypogonadism

Although data on prevalence are discordant, as reported above, TM patients often show gonadal impairment (6). Haemosiderosis of the pituitary gonadotrophic cells and iron deposition in the testes and ovaries are involved in the pathogenesis of hypogonadism in TM (59,60). In addition hypogonadism is a well-recognised cause of osteoporosis and osteopenia, not only in TM, but also in the general population (61,62,63).

In our previous study (22), in accordance with Anapliotou et al (6) and Jensen et al (8), we showed that hypogonadism produces more severe bone loss in TM. Our group had already shown that TM patients complained of various degrees of osteopenia due to their hormonal status. In fact, we observed that in TM patients without evidence of hypogonadism because of hormone replacement therapy, bone status was less compromised and osteoporosis was observed only at the lumbar site, where the influence of bone marrow expansion is prominent, as described above. However, in hypogonadic patients, osteoporosis may be more severe and may also affect the femoral site. Furthermore, we found a significant positive correlation between BMD values and hormonal treatment duration.

GH-IGF-1 Axis

Several studies showed that the GH-IGF-1 axis is altered in TM patients (11,12). These patients have significantly lower circulating levels of IGF-1 and the corresponding binding protein (IGFBP-3) than normal individuals (11,12). IGF-1 plays an important role in bone remodelling. Low serum IGF levels decrease osteoblast proliferation and bone matrix formation and reduce the activation of osteoclasts (64). A positive correlation between BMD at the lumbar spine and IGF-1 concentration has been reported (48,65). In our previous work (12), we found lower serum levels of IGF-l and IGFBP-3 in TM patients than in age-matched, healthy controls and a significant correlation between IGF-1, osteocalcin which is a marker of bone formation, and BMD values. Similarly, low concentrations of IGF-1 in TM adults and their correlation with BMD have been reported by Dresner Pollak et al (37).

The mechanisms responsible for the reduced action of the IGF-1/IGFBP-3 axis in TM are still being debated. Danesi et al (66) found an impairment of GH secretion in a considerable proportion of TM patients, compatible with hypothalamic and/or pituitary damage. It is unclear whether the IGF-1 level decreases before or after GH secretion dysfunction (67,68,69). Chrysis et al (70) suggested that impaired GH secretion, rather than GH insensitivity, is the cause of growth retardation in TM patients.

Iron Deposition in Bone

Iron deposition in bone damages osteoid maturation and inhibits mineralisation, resulting in focal osteomalacia. This is due to the incorporation of iron into crystals of calcium hydroxyapatite, which consequently affects the growth of hydroxyapatite crystals and reduces basic multicellular unit tensile strength (71). Mahachoklertwattana et al (23) observed increased osteoid thickness, osteoid maturation time and mineralisation lag time in TM patients.

Deferoxamine

Subcutaneously administered deferoxamine was for a long time the treatment of choice for iron overload in TM. Its chelating action is not solely specific for iron. Deferoxamine also inhibits DNA synthesis, collagen formation and osteoblast precursor differentiation and enhances osteoblast apoptosis (16,17). Data on bone safety of new oral chelating agents are still limited.

Vitamin D

Vitamin D deficiency is involved in the pathogenesis of osteoporosis in TM patients due to its regulatory effects on bone cells and calcium homeostasis. Lower 25-hydroxyvitamin D levels, in comparison to healthy controls, are a common finding and are inversely correlated with ferritin levels and age. Lower sun exposure due to reduced physical activity and defective skin synthesis associated with jaundice are probably responsible for this deficiency (72).

Genetic Factors

Genetic factors also have an important role in determining BMD in TM patients, although the genes responsible are poorly defined in this population. Some studies provide partially support for an association between BMD and specific *COL1A1* (73) and *TGF-β1* (74) gene polymorphism in TM. Vitamin D receptor (VDR) polymorphisms could also represent a risk factor for low BMD in adult TM patients (37,75). In our thalassemic population, we found that VDR (FokI, BsmI) and *COL1A1* (Sp1) gene polymorphisms had no influence on BMD, but BsmI was found to display beneficial effects on patient response to alendronate therapy (76). It has recently been reported that the f allele of the *Fok-I* gene polymorphism, when found in homozygosity, confers protection on the BMD values of young thalassemic patients (77).

Conclusion

Multiple acquired factors, together with genetic variants that predispose individuals to reduced BMD, contribute to bone fragility in TM. Bone marrow expansion, hypogonadism, a defective GH-IGF-1 axis and imbalanced cytokine profiles play major roles in the development of osteoporosis. Iron overload, deferoxamine toxicity and other endocrine dysfunctions could be additional factors. Figures 1 and 2 summarise potential factors contributing to the imbalanced bone turnover in TM patients. To date, in spite of adequate hormone replacement therapy, acceptable haemoglobin levels and chelating therapy, TM patients unexpectedly display impaired BMD and imbalanced bone turnover, indicating that the puzzle of the pathogenesis of TM-related osteoporosis is still far from being fully solved.

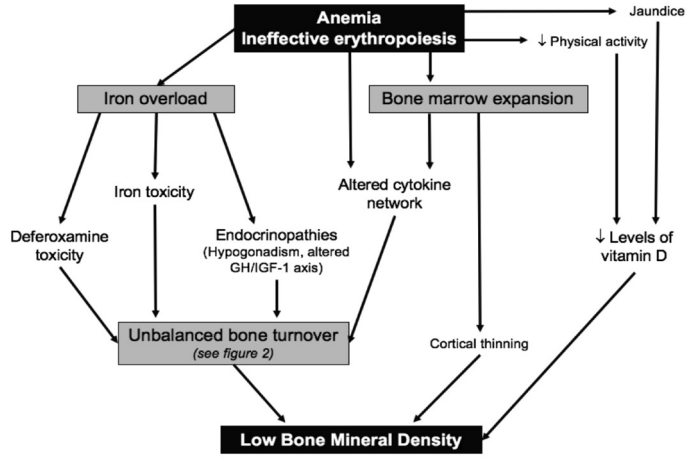

Figure 1. Pathogenesis of low bone mineral density in thalassemic patients

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

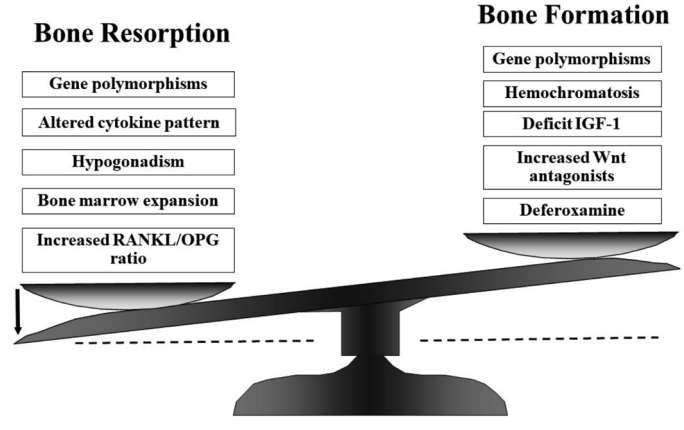

Figure 2. Possible causes for uncoupling bone turnover in thalassemic patients

IGF-1: insulin-like growth factor-1, RANKL/OPG: receptor activator of nuclear factor kappa-ß/osteoprotegerin

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Agostino Gaudio, Nancy Morabito, Design: Nancy Morabito, Antonino Lasco, Data Collection and Processing: Antonino Catalano, Anastasia Xourafa, Analysis and Interpretation: Agostino Gaudio, Nancy Morabito, Literature Research: Agostino Gaudio, Antonino Catalano, Rosario Rapisarda, Writing: Agostino Gaudio, Nancy Morabito, Antonino Catalano.

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The Glucose Control Resistance Scale

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What is already known on this topic?

Adolescents with diabetes have more problems with adherence than any other pediatric age group. Previous research has shown that multiple factors, including family conflict, disordered eating and anxiety, are related to adherence.

What this study adds?

The Glucose Control Resistance Scale (GCRS) is a measure of adolescent adherence to treatment that may allow diabetic care teams to better understand the origin of family conflict perceptions and the motivational beliefs that modify behavior and contribute to independent self-management and glucose control. Each question was designed to be meaningful in interventions by addressing common items of resistance to adherence and impulsive management decisions. The GCRS may be used by providers as an initial short screening survey on an annual or semi-annual basis.

Abstract

Objective: While past research found family conflict, disordered eating, body image concerns and anxious self-doubts may affect adolescent diabetic glucose control, available measures of adherence mainly focus on management tasks. The current study aimed to combine measures of emotional distress and beliefs with decisions concerning management in a new measure of resistance to treatment adherence: the 12-item Glucose Control Resistance Scale (GCRS).

Methods: Participants included 135 adolescents and their parents from a pediatric diabetes clinic. Family conflict, body image concerns, anxious self-doubts and glucose control resistance were assessed.

Results: Factor analysis identified 12 items, with loadings of ≥0.40, which were used to form the GCRS. The scale had adequate reliability and there was a significant correlation between child and parent GCRS scores. One factor, family conflict, was significantly related to hemoglobin A1c (HbA1c) levels, but a set of four factors explained a total of 12% of the variance in HbA1c levels. Of the demographic variables considered (gender, number of parents at home, age, body mass index z-score), only gender was significantly associated with adolescent perceptions of family conflict.

Conclusion: The GCRS may allow diabetic care teams to better understand the origin of family conflict perceptions and the motivational beliefs that modify behavior and contribute to independent self-management and glucose control. Each question was designed to be meaningful in interventions by addressing common items of resistance to adherence and impulsive management decisions. The GCRS may be used by providers as an initial short screening survey on an annual or semi-annual basis.

Keywords: Adolescent beliefs, type 1 diabetes, family conflict, resistance, non-compliance

Introduction

It is widely accepted that uncontrolled diabetes is associated with increased risk for morbidity and mortality. Current management, based on compliance with treatment recommendations, is often frustrating for patients, their families and providers (1,2). Glucose control can be especially difficult for adolescents due to typical adolescent challenges such as defiance or resistance and the effect this behavior has on their decisions and beliefs regarding diabetes and its treatment. Available screening questionnaires focus on management tasks but fail to link and adapt these

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tasks to adolescent psychological challenges. Combining attitudes of defiance or resistance with specific decisions on management can be useful in patient care because, for example, lack of motivation may influence compliance as much as the large number of burdensome management tasks.

Challenges regarding diabetic management may be related to developmental changes. Adolescents are becoming more independent, yet often require parental intervention with diabetic care. In some cases. this leads to increased family conflict which has been found to be associated with poorer glycemic control as measured by higher hemoglobin A1c (HbA1c) levels (3,4,5). Increased sexual interest can influence both weight and body image concerns which have been found to result in higher HbA1c (6,7). Additionally, adolescents often report anxiety and self-doubt which have been associated with worse glucose control (3,8). Other beliefs prevalent in adolescents are a sense of physical "invincibility," identifying with being independent, being in control and defying or being resistant to authority figures, usually the parents (9). These beliefs, combined with a physiologic lag in cognitive processing, can lead to difficulty with focusing on the future or with accepting the adverse consequences of poor metabolic control. These emotional beliefs may also lead to a feeling that there are no problems with resisting medically-recommended guidelines for glucose control. In many cases of uncontrolled diabetes, disordered beliefs in one of four areas of competence (family conflict, disordered eating, anxiety or resistance) are the cause of non-adherence to treatment recommendations (1,2,7).

Commonly used screening tests of adherence measure management skills directly correlated with glucose control (10,11). These measures have largely removed psychological parameters from the management tasks, despite that the psychological factors may limit adherence and require intervention.

Studies have explored issues related to motivation in adolescents with diabetes. For example, self-efficacy and outcome expectations were found to be related to diabetes self-management adherence and glycemic control (12). Motivation has also been integrated into treatment as a growing number of providers advocate the use of motivational interviewing (13). Some surveys contain distress items generated by the burden of management tasks, such as "feeling overwhelmed by my diabetes regime" (14). Other questionnaires list management tasks. However, intervention in one of these areas may not generalize to the other. Attention to both distress and task is needed for improvement of diabetic self-management.

In our experience, discussing both distress and management in the diabetes outpatient visit with both the patient and their family is useful. This discussion can be initiated by exploring whether the patient not only values the required management tasks, but can also accomplish these tasks. This information can help the provider determine whether the adolescent understands these tasks and how the family can be sufficiently supportive to accomplish these tasks. The intervention process begins with identifying a distressing problem as the starting point from which to develop new problem-solving skills related to the specific stress in question. For example, "It's too hard to calculate my insulin dose" would lead to exploring easier ways for the individual patient to determine a safe dose.

The purpose of the present study was to develop a scale measuring resistance and impulsive decisions, thus enabling providers to be more effective in dealing with distress and fostering management tasks.

Methods

The primary sample included adolescents with type 1 diabetes from a hospital-based pediatric diabetes program. All patients had been referred to the diabetes program by primary care providers. To examine parents' perception of their adolescents' resistance, parents of a sub-group of the patients were asked to answer the same questions presented to the adolescents.

Item Development

We observed clinically and hypothesized that patients focus on modifying their behavior much more quickly when a perceived burden or distress is discussed in terms of a specific impulsive management decision as these impulsive behaviors may not be admitted by the patient (5). For example, the patient who makes a statement such as "I fear hypoglycemia, so when I feel low I just eat rather than checking my blood sugar" was not likely to admit that he does not check his blood glucose if he were not emotionally engaged by the memory of fear. The questions also explore the burden of managing diabetes which includes the large number of daily self-control tasks required. In aggregate, this burden may lead to distress and maladaptive management decisions. Patient phrases such as "It's too hard to calculate my insulin dose, so I guess how much to take" are examples of this type of behavior. All questions in this study addressed adolescents with uncontrolled diabetes despite having completed diabetic education. The questions covered areas of diabetic care including diet, insulin administration, blood testing and hypoglycemia management. Each question

contained both a burden and impulsive maladaptive management decision, such as "If I think my blood sugar is high, I decide not to take it". Most questions contained explicit burden and management decision components. For example in one question distress may be implied by the endorsed decision, such as "I do not eat breakfast". All questions were reviewed and edited by a team of two pediatric endocrinologists, five diabetes educators (three pediatric diabetic nurses and two pediatric dietitians) and a pediatric psychologist.

Measurements

Research procedures were approved by the Institutional Review Board at Penn State Hershey (protocol no. 37210EP). Informed consent was obtained from both adolescents and their parents/caregivers prior to completion of the surveys. The primary sample of adolescents with diabetes completed surveys to develop the new Glucose Control Resistance Scale (GCRS), and to compare which of four adolescent beliefs best explained variance in the adolescents' HbA1c levels (see Table 1 for descriptive statistics). In addition to the initial adolescent and parent surveys, a sub-group of the participants completed items from the GCRS on a second occasion, two to four weeks after the initial completion, for assessment of test-retest reliability.

Adolescents and parents completed a survey to report whether or not (0 = no, 1 = yes) they agreed with 19 possible beliefs about glucose control resistance. Factor analysis identified a final set of 12 questions included in the new GCRS and the score was calculated as the total number of agreements.

The adolescent's perception of family conflict was measured with the Diabetes Family Conflict Scale (4). The score for family conflict was calculated as the mean three-point rating for the 19 items, and internal reliability for this measure was adequate as measured by Cronbach's alpha ($\alpha = 0.86$).

The adolescent's perception of weight and body image concern was measured with the 16-item Diabetes Eating Problem Survey (15). The score for weight concern was calculated as the mean six-point rating for the 16 items and internal reliability for this measure was adequate ($\alpha = 0.84$).

The adolescents' anxious self-doubts were measured with the 11-item Anxiety Sensitivity Index (16). The score was calculated as the mean five-point rating for the 11 items and internal reliability for this measure was adequate ($\alpha = 0.85$).

Table 1. Descriptive demographic data of the 135 participants

Variable	α	Mean (SD)	(Range)
Age (years)		15.05 ± 2.35	10-22
BMI z-score		0.73 ± 0.97	-1.90-2.95
HbA1c		9.03 ± 1.84 (75 mmol/mol)	5.4-14.0% (36-130 mmol/mol)
Family conflict; Hood et al (4)	0.86	0.25 (0.26)	0-1.47
Weight concern; Markowitz et al (15)	0.83	0.67 (0.56)	0-3.56
Anxious self-doubt; Blais et al (16)	0.85	0.69 (0.62)	0-2.64
GCRS	0.80	4.92 (3.11)	0-12

BMI: body mass index, HbA1c: hemoglobin A1c, GCRS: Glucose Control Resistance Scale, SD: standard deviation

Statistical Analysis

Exploratory factor analysis was conducted for the 19 beliefs using a principle components approach with the requirement that each item showed a factor loading of 0.40 or higher (see Table 2). Internal reliability in the form of Cronbach's alpha (α) was calculated for the remaining 12 items. Test-retest reliability was calculated as the Pearson correlation coefficient (r) in a sub-group of adolescents across two occasions, two to four weeks apart.

To examine how well parents perceived beliefs of their adolescents, a Pearson correlation coefficient (r) was calculated between the GCRS score of the adolescent and that of the parent.

Because the four adolescent beliefs (Family Conflict, Weight Concerns, Self Doubt and Resistance) are likely to be inter-correlated, their association with HbA1c must be analyzed with more than bivariate correlations. To examine which beliefs held by adolescents were associated with HbA1c, a multiple regression analysis of the whole cohort of adolescents was conducted with HbA1c values serving as the criterion variable and with their four belief scores serving as possible predictor variables: perceptions of family conflict (DFCS), weight and body image concerns (DEPS), anxious self-doubts (ASI), and glucose control resistance (the new GCRS).

To determine which demographic characteristics [gender, number of parents at home, age, body mass index (BMI) z-score] were associated with beliefs found in the above analyses and were associated with HbA1c, a 2 x 2 ANCOVA examined the belief score as the dependent variable compared across two adolescent genders (male, female) and across two parent conditions (both parents at home, single parent at home), with adolescent age and BMI z-score considered as covariates.

Results

Patient Demographics

The patient group consisted of 135 adolescents who had been diagnosed with type 1 diabetes. Of these 51.9% were males and 77.4% lived at home with both parents. Mean age was 15.05 ± 2.35 years; mean BMI z-score was 0.73 ± 0.97 and mean HbA1c was 9.03 ± 1.84%.

The subgroup of patients whose parents (n = 127) also answered GCRS questions had the following demographic characteristics: 52.0% of male adolescents; 77.4% with both parents at home. Mean age of the sample was 14.94 ± 2.2 years. Mean BMI z-score was 0.72 ± 0.98 and mean HbA1c 9.04% ± 1.80.

The subgroup of patients who completed the repeat questions 2-4 weeks later for test-retest reliability assessment had the following characteristics: 29/135 (21.5%) took part, 41.4% were male; 72.4% had both parents at home; mean age was 15.15 ± 1.93 years, mean BMI z-score was 0.60 ± 0.76 and mean HbA1c was 9.39 ± 1.68%. Thirty-five adolescents also completed a follow-up survey following a mean interval of 1.8 years (6 months-4 years).

Psychometrics of the New GCRS

Exploratory factor analysis revealed a principal component of 12 items showing factor loadings of 0.40 or higher which would be selected to comprise the new GCRS (Table 2). Internal reliability was adequate for both the adolescents' beliefs ($\alpha = 0.80$) and for their parents' perceptions of their beliefs ($\alpha = 0.81$). Test-retest reliability was 0.68, near and only slightly lower than the traditionally recommended 0.70 value ($r = 0.68$, $p < 0.001$). Additionally, see Table 3 for

Table 2. Exploratory factor analysis results for the Glucose Control Resistance Scale

#	Item	Factor loading
1.	It's too hard to calculate my insulin dose, so I guess how much to take.	0.677
2.	If I think my blood sugar is high, I decide not to check it.	0.617
3.	It takes too much time to weigh foods, so I do not weigh foods, even if it is important for me.	0.607
4.	I like to get or buy extra food at school, more than on my meal plan.	0.596
5.	I fear hypoglycemia, so when I feel low I just eat, rather than checking my blood sugar.	0.590
6.	I eat extra snacks and decide not to take additional (coverage) insulin.	0.581
7.	I am not going to count carbs-I am lazy, it is who I am.	0.555
8.	The meal plan is too complicated to follow.	0.503
9.	I am not going to take time to log (write down) my blood sugars every day.	0.498
10.	I do not eat breakfast.	0.474
11.	I like to eat snacks any time during the day	0.466
12.	I do not write down the correct blood sugar from my meter.	0.431
	Excluded Items	
13	When I check my urine, I like to find ketones.	
14	When my sugar is high, I exercise instead of taking recommended insulin.	
15	I take less insulin than instructed.	
16	I decide to skip meals or snacks needed for diabetic control.	
17	I want to eat less than the dietician suggested I need.	
18	I cannot see the syringe (pen) well enough to be sure of the dose I take.	
19	I cannot focus (concentrate) long enough to check my blood sugar.	

Table 3. Bivariate correlations between study variables

	HbA1c	Family conflict	Weight concern	Anxious self-doubt	GCRS
HbA1c		0.339***	0.247**	0.203*	0.211*
Family conflict			0.718***	0.401***	0.546***
Weight concern				0.477***	0.595***
Anxious self-doubt					0.273**
GCRS					

*p < 0.05, **p < 0.01, ***p < 0.001, HbA1c: hemoglobin A1c, GCRS: Glucose Control Resistance Scale

bivariate correlations for each pair of the four adolescent beliefs examined in the present study: glucose control resistance, family conflict, weight concern, anxious self-doubt.

How well Do Parents Understand Their Adolescents' Glucose Control Resistance?

The Pearson correlation coefficient of the the adolescents' and parents' GCRS scores suggests parents had moderately high understanding of their adolescents' glucose control resistance beliefs ($r = 0.50$, $p < 0.001$).

Which of Four Adolescent Beliefs are Associated with Glucose Control (HbA1c)?

The multiple regression analysis revealed that of the four adolescent beliefs considered (family conflict, weight concerns, self-doubts, and glucose control resistance), only family conflict was significantly associated with worse (higher) HbA1c levels ($r = 0.31$), with the set of four beliefs explaining a total of 12% of the variance in HbA1c levels ($R^2 = 0.12$; see Table 4). Although not associated directly with HbA1c, GCRS was highly correlated with family conflict ($r = 0.54$) and with weight/eating concerns ($r = 0.59$). Also associated with family conflict were weight/eating concerns ($r = 0.71$), and anxiety ($r = 0.40$).

Table 4. Multiple regression analysis of four adolescent beliefs for their association with glucose control (HbA1c)

Adolescent belief	beta	t	p
Family conflict (DFCS)	0.312	2.56	0.012
Weight concern (DEPS)	-0.043	0.33	0.745
Anxious self-doubt (ASI)	0.084	0.89	0.374
GCRS	0.044	0.42	0.672

$R^2 = 0.12$

$F_{(4,129)} = 4.46$

$p = 0.002$

GCRS: Glucose Control Resistance Scale, HbA1c: hemoglobin A1c

Table 5. 2 x 2 ANCOVA to examine demographics associated with family conflict beliefs in adolescents with type 1 diabetes, comparing cross two genders, two parents-at-home conditions with adolescent age and body mass index z-score as covariates

Partial eta² effect	F	df	p	Effect size
Gender	4.53	(1, 118)	0.035	0.037
Parents-at-home	0.60	(1, 118)	0.439	
Gender x Parents-at-home	1.96	(1, 118)	0.164	
Age	0.16	(1, 118)	0.692	
BMI z-score	0.01	(1, 118)	0.944	

BMI: body mass index

Which Adolescent Demographics are Associated with Their Family Conflict Beliefs?

The 2 x 2 ANCOVA revealed that of the adolescent demographic variables considered (gender, number of parents at home, age, BMI z-score), only gender was significantly associated with adolescent perceptions of family conflict with females reporting more than did males (female mean ± SD = 0.30 ± 0.30; male mean ± SD = 0.21 ± 0.22; see Table 5).

Discussion

A new GCRS showed strong psychometric characteristics of internal reliability and adequate test-retest reliability, both for the adolescents themselves and their parents. The psychological factor of defiance or "resistance" to recommended glucose control practices has frequently not been the focus of screening surveys. A correlation of GCRS was found with a measure of anxiety and a high correlation with weight concern and family conflict, providing convergent validity with measures important for diabetic self-management. The unique feature of "resistance" and the convergent validity with other scales of diabetic distress suggest that GCRS may be useful as an initial short screening measure in the diabetic follow-up routine on an annual or biannual basis. Items could be addressed in real time at the outpatient visit and, if needed, changes in management could be made, such as referral to a psychologist or other provider, further education, or more intensive follow-up. The items may also present an opportunity for providers to utilize the protective processes of benefit finding, optimism, or adaptive coping with a specific diabetic management task. This approach has been shown to ameliorate family conflict (17).

Another new feature of the present study is comparison of the four areas of competence for their association with HbA1c levels. The four beliefs included perceptions of family conflict, weight and body image concerns, anxious self-doubts, and the concept of glucose control resistance as measured by the newly developed scale, the GCRS described herein. Taken together, these four adolescent beliefs explained 12% of the variance in A1c levels. The scales of individual psychologic parameters (GCRS, weight concern, anxiety) were all moderately or highly correlated with family conflict (Table 3) and family conflict was correlated with HbA1c, explaining 9% of the variability. This suggests reducing family conflict is a critical step in glucose control, and that in working on the reduction of family conflict, it may be important to address the beliefs

and decisions of GCRS, weight concerns or anxiety in the adolescent.

Our findings support the reported correlation of family conflict and HbA1c levels (4,18). Poor control by the adolescent generates conflict and any resultant strict authoritarian style of parents may lead to anger or anxiety in the adolescent with worsening glucose control and threatened self-motivation (19).

Both diabetic management and family therapy with the adolescent are challenging but the GCRS may be used for problem-solving in the office or to monitor a patient after referral to a mental health care provider. The most productive focus is how family can help the adolescent improve self-management which then improves feelings of self-sufficiency. The key is to be helpful when needed (authoritative) but not controlling (authoritarian) (20) and the tone of communication is more important than the frequency of talking (20,21). Continued family involvement as the teen shares greater responsibility is recommended (7).

Anxiety which may involve needle phobia or fear of hypoglycemia (22) was found to predict HbA1c one year later (3). Depression was correlated with HbA1c but knowledge of diabetic management was not (23). Symptoms of anxiety and depression are frequently found in the same patient, with anxiety reported even more frequently than depression. Screening for anxiety has been proposed to be an adequate measure for identifying those at risk of depression (24). Diabetic distress was correlated with anxiety and family conflict (25) and supported our findings that GCRS questions of distress and decision were correlated with anxiety and highly correlated with family conflict.

Use of the GCRS may uncover previously unmentioned information that can be utilized in treatment. The set of 12 questions contains several areas of impulsive or maladaptive behavior decisions. One type of behavior in several questions is a lack of action, possibly based on a belief that it is acceptable to deny the problem, defy parents or feel invincible. For example, "If I think my blood sugar is high, I decide not to check it". Another type of behavior is endorsing "liking" as a justification for not adhering to recommendations, such as "I like to get or buy extra food at school, more than on my meal plan". This new measure may allow adolescents to become more comfortable in discussion with health care providers, help both patient and provider better understand concerns of the adolescent and thus enable behavioral change. For example, in response to the question "It's too hard to calculate my dose, so I just guess how much to take," discussion with the adolescent might include how this decision makes him feel and the benefits he perceives from the belief. Discussion might unveil underlying needs and feelings that, when expressed, can facilitate the discovery of alternative approaches in management that are workable and acceptable to the family.

Study Limitations and Directions for Future Research

One limitation of the present study was the relatively small sample size of 135 adolescents diagnosed with type 1 diabetes. Also, participants came exclusively from a diabetes clinic in the northeastern region of the United States and demographic information did not include their ethnic identity, sexual orientation or religious affiliation. Future research should include larger and more diverse samples of adolescents to conduct confirmatory factor analyses for the new GCRS and to determine whether family conflict continued to be the adolescent belief most strongly associated with poor glucose control. Future research might focus more specifically on the use of the GCRS in management of family conflict and on which parenting styles (such as permissive, authoritative, authoritarian) improve the adolescent's motivation for diabetic self-management, internalizing self-sufficiency and quality of life.

Conclusion

The findings from the present study show that the GCRS is correlated with HbA1c. However the comparison of the four areas of belief demonstrates that family conflict is the most significant predictor of HbA1c. These results may alert clinicians to the importance of addressing family conflict as part of their overall diabetes intervention.

Ethics

Ethics Committee Approval: Research procedures were approved by the Institutional Review Board at Penn State Hershey (protocol no. 37210EP).

Informed Consent: Informed consent was obtained from both adolescents and their parents/caregivers prior to completion of the surveys.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Maria-Eleni Nikita, Paul L. Mueller, Concept: Paul L. Mueller, Maria-Eleni Nikita, Design: Helen M. Hendy, Paul L. Mueller, Data Collection or Processing: Paul L. Mueller, Keith E. Williams, Analysis or Interpretation: Helen M. Hendy, Literature Search: Paul L.

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Vaspin, a Compensatory Mechanism Against High Glucose Levels Since Birth?

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What is already known on this topic?

Vaspin, is expressed in visceral adipose tissue and has insulin-sensitizing effects. Elevated vaspin expression could represent a compensatory mechanism of insulin resistance secondary to the metabolic complications of obesity.

What this study adds?

The results showed negative association between glucose and vaspin levels in umbilical cord blood. The predictive nature of glucose levels on vaspin levels support the idea that elevated vaspin levels protect against insulin resistance.

Abstract

Objective: Hormones produced by fat tissue, adipokines, produced during intrauterine life have recently been implicated in fetal growth. Vaspin is an adipokine expressed in visceral adipose tissue and has insulin-sensitizing effects. Elevated serum vaspin concentrations are associated with alterations in insulin sensitivity. We aimed to determine if vaspin concentrations in cord blood from healthy, term newborns differ among those born small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA). A secondary objective was to determine whether an association existed between vaspin and anthropometric measurements, glucose and insulin levels in the newborn.

Methods: The study population included healthy term newborns, 30 subjects in the SGA, 12 in the AGA, and 34 in the LGA group. Anthropometry was documented in all subjects. Blood was taken from the umbilical cord vein from each child for later analysis for vaspin, insulin and glucose concentrations.

Results: Cord blood vaspin, insulin and glucose concentrations were not different between the three study groups. A negative correlation between vaspin and glucose concentrations was demonstrated in the whole cohort ($r = -0.364$, $p = 0.001$). This correlation was also observed in the LGA group ($r = -0.482$, $p = 0.004$). Glucose concentrations significantly predicted vaspin concentrations ($r^2 = 0.132$, $p = 0.001$).

Conclusion: We found a negative association between glucose and vaspin concentrations in umbilical cord blood. In addition there was a predictive association between blood glucose and resulting vaspin concentration, suggesting that vaspin can be used as a predictor of alterations in the insulin-glucose metabolism from birth.

Keywords: Vaspin, insulin, glucose, birth weight, cord blood

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Introduction

The belief that adipose tissue was only an energy reservoir began to change with the discovery of hormones, known as adipokines, produced by fat tissue, conferring an endocrine function on fat deposits (1).

Adipokines, which are produced during intrauterine life, have recently been implicated in fetal growth. Thus there is a growing interest in exploring their physiology in early life (2). Vaspin, identified as a member of the serine protease inhibitor family, is specifically expressed in visceral adipose tissue and has insulin-sensitizing effects. Elevated vaspin concentrations in serum are associated with obesity and alterations in insulin sensitivity in humans (3), even in infancy (4).

The administration of vaspin improved glucose tolerance and insulin sensitivity in rodents. Moreover, the administration of insulin significantly upregulated vaspin mRNA in subcutaneous adipose tissue, and to a lesser extent, reduced expression in visceral adipose tissue. These authors concluded that elevated vaspin expression could represent a compensatory mechanism of insulin resistance, secondary to the metabolic complications of obesity (3,5). Therefore, better understanding of the biology of vaspin may lead to the development of new treatment strategies for obesity, diabetes and insulin resistance (6).

Alterations in fetal nutrition can result in development adaptations that permanently change the physiology and metabolism of the progeny, specifically, insulin–glucose metabolism (7). This is supported by the recognition of an increased risk of developing type 2 diabetes, hypertension and metabolic syndrome later in life in infants that are either small for gestational age (SGA) or large for gestational age (LGA) (8,9).

The objective of this study was to determine if vaspin concentrations in cord blood of healthy, term newborns differed among SGA, appropriate for gestational age (AGA) and LGA newborns. A secondary objective was to determine whether there is an association between vaspin and anthropometry and glucose and insulin concentrations in the newborn.

Methods

The study was approved by the Ethics Committee in Research of the Hospital Universitario "Dr. Jose Eleuterio Gonzalez" with the code PE16-00013. A written informed consent before enrollment was obtained from all mothers.

Seventy-six newborns (>37 weeks gestation) evaluated from December 2012 to January 2015 at the Universidad Autonoma de Nuevo Leon Medical School and the "Dr. Jose E. Gonzalez" University Hospital in Monterrey, Mexico were included in the study.

The study population consisted of healthy term newborns. Exclusion criteria were newborns of mothers with gestational diabetes, pregestational diabetes mellitus, pre-eclampsia, hypertension or thyroid disease. Elimination criteria were any disease that required inpatient management or intercurrent disease affecting nutritional status.

A blood sample was taken from the umbilical cord vein from each infant immediately after birth and centrifuged at 1600 g and 4 °C. Aliquots of serum were separated, frozen, and stored at -70 °C for later analysis for vaspin, insulin and glucose. Birth weight was documented prospectively using a Torrey scale (Torrey, S.A. de C.V., Monterrey, Mexico) and length was obtained using a SECA 210 infantometer (SECA North America, Chino, CA., USA) at birth. The total sample was classified into three groups, SGA (less than 10th percentile), AGA (percentile between 10 and 90) or LGA (greater than 90th percentile), according to birth weight for gestational age (10).

Serum vaspin concentrations were analyzed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Biovendor Human Vaspin ELISA, Brno, Czech Republic) according to the manufacturer's instructions. Sensitivity of the assay was 0.01 ng/mL, the intra- and interassay coefficients of variation were 7.6% and 7.7%, respectively. The values reported below the sensitivity of the assay reported as <0.010 ng/nL were scaled to 0.010 ng/mL.

The determination of insulin concentrations was performed by electrochemiluminescence immunoassay using a commercial kit (Roche Diagnostics, Indianapolis, IN., USA). Sensitivity of the assay was 0.2 μU/mL; the intra- and interassay coefficient of variation were 3.6% and 3.4%, respectively. Glucose concentrations were determined by the glucose oxidase method using a comercial kit (Pointe Scientific Inc., Canton, MI., USA) according to the manufacturer's instructions.

Statistical Analysis

Measures of central tendency are presented as medians (range) and means ± standard deviation, according to the distribution of the variables.

The Kolmogorov-Smirnov test was applied to check the normality of the variables. A non-Gaussian distribution was shown for the data of vaspin and insulin, while glucose data were normally distributed.

The chi-square test was used to compare proportions. For comparison of dimensional continuous variables, a non-parametric Mann-Whitney U test and Kruskal-Wallis test were performed. Univariate analysis of variance was used for normally distributed data.

Pearson's correlation coefficient was applied to detect any positive or negative correlations. For multiple regression analysis, the stepwise forward model was used. A $p \leq 0.05$ was considered statistically significant. Statistical Package for the Social Sciences for Macintosh, v.22.0 (IBM Corp., Armonk, NY., USA) was used for analysis.

Results

The study population (n = 76) included 30 subjects in the SGA group, 12 in the AGA group and 34 in the LGA group. Demographic and anthropometric characteristics are presented in Table 1. None of the groups showed significant differences in terms of gender, delivery route, gestational age or mother's age.

Cord blood-derived serum vaspin, insulin and glucose concentrations were not different among the three study groups; concentrations and comparisons are shown in Table 2. A significant correlation between vaspin concentration and birth length (cm) was found (r = 0.277, p = 0.016), but not with birth weight or cephalic perimeter (see Table 3).

Median (range) cord blood vaspin concentrations were significantly higher in males, 0.054 (0.010-5.64) ng/mL compared to females, 0.017 (0.010-1.14) ng/mL across the whole cohort (p = 0.034) (Figure 1). Insulin correlated with birthweight in the total population (r = 0.328, p = 0.004; see Table 3). However when this was analyzed by study group, the correlation was only present in the LGA group (r = 0.507, p = 0.002). Likewise, a positive correlation was found between insulin and glucose only in the SGA group (r = 0.400, p = 0.028). A negative correlation between vaspin and glucose concentrations was demonstrated in the total population (r = -0.364, p = 0.001; see Table 3). In the analysis by study group, this correlation was only observed in the LGA group (r = -0.482, p = 0.004).

Birth length weakly, but significantly, predicted vaspin cord blood concentrations for the total population ($r^2 = 0.077$, p = 0.016). In the multivariate analysis, including either a stepwise or an all-at-once approach, the anthropometric variables (birth weight and cephalic perimeter) did not increase prediction of vaspin levels (Table 4).

Table 1. Demographic and anthropometric characteristics of the study groups

Characteristic	SGA n (%)	AGA n (%)	LGA n (%)	p
Gender				0.766
Male	17 (56.7)	6 (50)	21 (61.8)	
Female	13 (43.3)	6 (50)	13 (38.2)	
Delivery route				0.055
Vaginal	16 (53.3)	3 (25)	9 (26.5)	
Cesarean section	14 (46.7)	9 (75)	25 (73.4)	
Gestational age (weeks)	39.10 ± 0.82	38.52 ± 1.37	39.18 ± 1.12	0.181
Mother's age (years)	23.43 ± 5.23	26.83 ± 7.06	24.82 ± 4.95	0.184
Birth weight (g)	2608 ± 241.94	3185 ± 519.99	4262.65 ± 324.62	< 0.005
Birth length (cm)	48.07 ± 1.85	49.29 ± 2.26	52.91 ± 1.71	< 0.005
Cephalic perimeter (cm)	33.33 ± 1.10	34.41 ± 1.29	36.10 ± 1.24	< 0.005

SGA: small for gestational age, AGA: appropriate for gestational age, LGA: large for gestational age. Data are given as mean ± standard deviation.

Table 2. Vaspin, insulin and glucose concentrations by study groups

	SGA (n = 30)	p (SGA *vs* AGA)	AGA (n = 12)	p (AGA *vs* LGA)	LGA (n = 34)	p (LGA *vs* SGA)	p value
Vaspin ng/mL	0.021 (0.010-1.890)	0.631*	0.010 (0.010-1.520)	0.174*	0.051 (0.010-5.64)	0.208*	0.266**
Insulin µU/mL	4.51 (0.57-20.34)	0.022*	12.20 (1.36-22.41)	0.368*	7 (0.95-114.1)	0.091*	0.055**
Glucose mg/dL	79.50 ± 22.38	0.781	82 ± 34.17	0.147	69.67 ± 20.90	0.074	0.161***

*Mann-Whitney U test. **Kruskal-Wallis test. ***Univariate analysis of variance. SGA: small for gestational age, AGA: appropriate for gestational age, LGA: large for gestational age

Glucose levels significantly predicted vaspin levels ($r^2 = 0.132$, $p = 0.001$). In the multivariate analysis, including either a stepwise or an all-at-once approach, including insulin in the model did not increase the prediction of vaspin levels (Table 4).

Table 3. Correlations of vaspin, glucose and insulin with one another and with anthropometric variables in the total population

Vaspin ng/mL	**r**	**p**
Birth weight	0.190	0.100
Birth length	0.277	0.016*
Cephalic perimeter	0.014	0.906
Glucose	-0.364	0.001*
Insulin	-0.136	0.241
Glucose mg/dL	**r**	**p**
Birth weight	-0.138	0.235
Birth length	-0.237	0.039
Cephalic perimeter	-0.143	0.222
Insulin	0.129	0.267
Insulin µU/mL	**r**	**p**
Birth weight	0.328	0.004*
Birth length	0.171	0.139
Cephalic perimeter	0.216	0.062

*p≤0.05 is statistically significant

Discussion

Vaspin, which has been recently discovered, and with promising beneficial effects on obesity and diseases related to insulin resistance, could be the basis for future pharmacological treatment (11). However the biology and metabolism of vaspin has not yet been extensively studied. To understand the role of vaspin in metabolically important periods, such as fetal life, is of great importance.

This study included a sample of healthy term newborns of mothers without any diagnosed comorbidities that

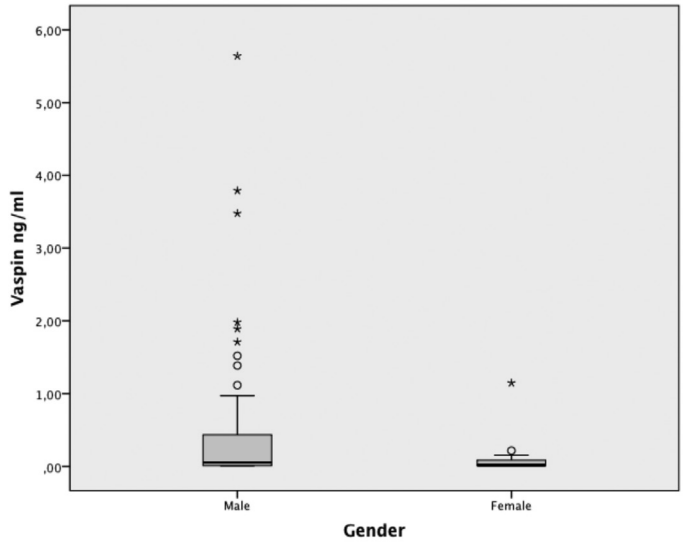

Figure 1. Serum vaspin concentrations in male (n = 44) and female (n = 32) newborns

Table 4. Predictor variables and multivariate analysis using vaspin as a dependent variable

Step	**Parameter**	**β ± s.e.m.**	**β ± s.e.m.**	**p**
Predictor variable: $r^2 = 0.077$				
	Birth length	0.277 ± 2.47	0.089 ± 0.036	0.016*
Multivariate analysis ($r^2 = 0.132$, $p = 0.018$)				
Predictor variables: birth weight, birth length, cephalic perimeter				
1	Birth weight	0.099 ± 0.393	0.000 ± 0.000	0.695
2	Birth length	0.408 ± 1.939	0.132 ± 0.068	0.056
3	Cephalic perimeter	-0.323 ± -1.871	-0.175 ± 0.094	0.065
Predictor variable: $r^2 = 0.132$				
	Glucose	-0.364 ± -3.358	-0.014 ± 0.004	0.001*
Multivariate analysis ($r^2 = 0.140$, $p = 0.003$)				
Predictor variables: glucose, insulin				
1	Glucose	-0.352 ± -3.216	-0.014 ± 0.004	0.002*
2	Insulin	-0.091 ± -0.829	-0.006 ± 0.007	0.410

*p≤0.05 is statistically significant

could have interfered with normal weight gain during intrauterine life. No differences in vaspin concentrations in umbilical cord serum were found between the SGA, AGA and LGA study groups. Vaspin concentrations were significantly higher in males than females. It was also found that length at birth and glucose concentration were independent variables that predicted vaspin concentration in umbilical cord blood.

In a previous study, Akcay et al (12) reported higher vaspin levels in the SGA group when compared to the AGA and LGA groups, concluding that this finding may be the result of differences in energy homeostasis in intrauterine life, since the SGA group had a reduced fat mass, an altered development of adipose tissue and relatively higher visceral fat deposits. The authors suggested that greater visceral fat deposits may be the source of higher vaspin concentrations in SGA neonates (9,13).

Human vaspin concentrations have been reported to be associated with obesity, insulin resistance and type 2 diabetes mellitus type 2 (14,15). Kafalidis et al (16) reported higher vaspin levels in an LGA group when compared with AGA infants, attributing these differences to altered fat accumulation and hyperinsulinemia in the LGA group.

A previous study compared vaspin concentrations in umbilical cord blood of newborns with intrauterine growth restriction (IUGR) and AGA without reporting statistically significant differences (17). Cekmez et al (18) studied vaspin levels in LGA and AGA and reported no statistically significant difference between the two study groups. The absence of differences in the previous reports, similar to this report, might be due to differences in race, or differences in the definition of SGA or LGA.

Although it has been previously described that vaspin can play a major role in fetal development (19), published studies do not report an association between cord blood vaspin concentrations and length at birth, as was found in this study. It is known that fetal macrosomia is related to hyperinsulinemia during fetal development, which is a result of elevated maternal glucose concentrations which allows glucose to be transferred across the placenta, and further concentrations produced by the fetal pancreas during the second trimester, when insulin is secreted autonomously and independently of maternal glucose stimulation (Pedersen's hypothesis) (20). It is possible that this mechanism leads to increased vaspin production to improve utilization of insulin, thus reducing glucose levels, to achieve an optimal intrauterine environment. However, this mechanism needs to be studied more extensively, since in our study vaspin levels were positively correlated with length at birth.

Körner et al (21) reported significantly higher serum vaspin concentrations in girls than in boys at ages seven to 18 years. They showed an increase in female vaspin levels at puberty while a non-dynamic increase was found in vaspin at puberty in boys. Consequently, it appears that the greatest difference between girls and boys occurs in the adolescent age group, despite a lack of correlation between sex steroids (estradiol and testosterone) and vaspin levels.

Briana et al (17) also reported higher vaspin levels in females than in males in a IUGR sample on postnatal day 1, while no difference was observed in umbilical cord blood. In contrast Akcay et al (12) did not find a difference by gender in vaspin levels in umbilical cord blood, despite differences in adipose tissue distribution and mass. In our study, in contrast to these previous studies, we found significantly elevated cord blood vaspin levels in males compared to females. Gender dependent behavior has been demonstrated for adiponectin (22) and leptin (23). It is not known if the same is true for vaspin and this remains to be elucidated as current evidence is contradictory.

Klöting et al (14) have reported that a single intracerebroventricular injection of vaspin was sufficient to cause a sustained and significant improvement in glucose concentration over at least six days in *db/db* mice, which are a rodent model of insulin resistance, but not in C57BL/6 mice. The authors suggest that these results indicate that vaspin reduces plasma glucose only in the presence of elevated blood glucose concentrations and go on to suggest that treatment with vaspin would not have the potential to cause hypoglycemia. In reference to our results, this mechanism may explain the negative correlation between vaspin and glucose concentrations which were only observed in the LGA group. It can be hypothesized that this group developed in an abnormal intrauterine environment, reflecting mild maternal hyperglycemia below the diagnostic threshold. Evidence to support this hypothesis comes from Chiesa et al (24) who reported that even a limited degree of maternal hyperglycemia, even within the normal range, may affect fetal weight. This finding is also supported by Hida et al (3) who administered vaspin to diet-induced obese mice, which significantly improved insulin sensitivity and glucose tolerance, while administration of vaspin to *in vivo* lean mice did not alter glucose tolerance. The authors concluded that the upregulation of vaspin may be a protective mechanism for insulin resistance.

The predictive nature of cord blood glucose concentration for vaspin cord blood concentration reflects their

interdependence. Fetal vaspin concentration is increased in response to elevated glucose, possibly from maternal circulation. As vaspin improves insulin resistance an increase in concentration will have the effect of improved fetal insulin utilization. This appears to be a compensatory mechanism for reducing fetal glucose, possibly derived from maternal sources, in order to achieve an optimal intrauterine environment.

Study Limitations

The main limitation of the study is the characteristics of the sample obtained for convenience, also, we did not have another study group of mothers with gestational diabetes, because it would allow comparison of the LGA group of mothers without co-morbidities and those with a clear alteration of the insulin-glucose metabolism. In addition, we did not evaluate the maternal vaspin and glucose concentrations and thus we can not have direct evidence of the relationship between maternal hyperglycemia resulting in LGA newborns with elevated vaspin levels.

However, the fact of having a sample without comorbidities allows us to conclude that birth weight determines alterations in the insulin-glucose metabolism where vaspin can serve as a marker.

Conclusion

We found a negative association between glucose and vaspin levels in umbilical cord blood. In addition glucose levels were found to be predictive of vaspin levels, supporting the idea that elevated vaspin levels may have a protective action against insulin resistance in the intrauterine period and suggesting that vaspin may be used as a predictor of alterations in insulin-glucose metabolism. This may be especially true in target populations, such as the LGA group. Further studies are needed to investigate the role of vaspin in newborns of mothers with a history of insulin resistance to confirm its involvement in pathological processes.

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Ethics

Ethics Committee Approval: Ethics Committee in Research of the Hospital Universitario "Dr. Jose Eleuterio Gonzalez" (Code PE16-00013).

Informed Consent: A written informed consent before enrollment was obtained from all mothers.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

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Evaluation of Normal Thyroid Tissue and Autoimmune Thyroiditis in Children Using Shear Wave Elastography

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What is already known on this topic?

Shear wave elastography (SWE) provides real-time quantitative information about tissue elasticity by measuring and displaying local tissue elasticity. This non-invasive technique has the advantages of operator independence, reproducibility, high spatial resolution and quantitative evaluation without compression artifacts. There have been many studies about the advantages of SWE in adullts however studies in children are few.

What this study adds?

The aim of this study was to measure the elasticity of thyroid tissue in children and adolescents using SWE and to investigate the role of SWE in the diagnosis of autoimmune thyroiditis in childhood. Quantitative elastographic analysis evaluated by SWE in autoimmune thyroiditis patients (3.7 ± 1.2 m/s) was significantly higher compared with normal pediatric patients (1.8 ± 0.3 m/s) and the optimal cut-off value was 2.39 m/s.

Abstract

Objective: Shear wave elastography (SWE) is a user-independent ultrasonographic technique that evaluates tissue elasticity. It is used especially in the evaluation of thyroiditis and thyroid nodules when it is capable of distinguishing malignant from benign thyroiditis in adults. To date, no studies have evaluated SWE in pediatric thyroid patients. The aim of this study was to measure the elasticity of normal thyroid tissue in children and adolescents using SWE and to investigate its role in the diagnosis of pediatric autoimmune thyroiditis.

Methods: In total, 113 healthy children of whom 66 (58.4%) were girls and 57 children with autoimmune thyroiditis of whom 45 (78.9%) were girls were evaluated by SWE after B-mode ultrasound. The quantitative evaluation of normal thyroid tissue in healthy children and those with autoimmune thyroiditis was performed using shear wave velocity (SWV) values (m/s). Thyroid antibodies were consistent with autoimmune thyroiditis. Data were compared using descriptive and analytical statistics and receiver-operating characteristic curves.

Results: The mean ± standard deviation (range) of SWV value in thyroid parenchyma of the healthy children was 1.82 ± 0.3 m/s (1.32-2.37) m/s. There was a significant positive correlation between age and SWV values which increased with age. The average SWV value of thyroid parenchyma in children with autoimmune thyroiditis was 3.7 ± 1.2 (2.59-6.25) m/s which was statistically significantly greater than in healthy children ($p = 0.00$). The cut-off value for elasticity with the highest diagnostic accuracy was 2.39 m/s; sensitivity and specificity were 97.4% and 100% respectively. There was no correlation between elasticity, thyroid function tests and autoantibody concentrations ($p > 0.05$).

Conclusion: SWE is a useful imaging method that can be used with routine ultrasonography in evaluation of the thyroid in children.

Keywords: Shear wave elastography, children, thyroid, autoimmune thyroiditis

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Introduction

Shear wave elastography (SWE) is a non-invasive method for measuring tissue elasticity whereby a quantitative estimate is provided of the elasticity of various soft tissues. It is a real time, quantitative, repeateable and user-independent imaging technique (1,2,3,4). With this technique, a short-time high-frequency acoustic repulsive force is applied using an ultrasonic probe, which causes small fluctuations in the tissues and the rate of advance of the formed waves through the tissue can be measured. The values of normal thyroid tissue elasticity in healthy adults are expressed as kilopascals (kPa) and shear wave velocity (SWV) in metres per second (m/s) in many studies, SWV of normal thyroid tissue of adults have been reported but few studies have been performed to evaluate the normal elasticity of thyroid tissue in children and adolescents (4,5,6).

Some pathologic conditions such as tumour and inflammation can change the normal tissue elasticity of thyroid. The elasticity measurement of thyroid tissue using SWE can be a useful, non-invasive test for the diagnosis of various thyroid diseases and this method has been performed successfully in the evaluation of thyroid tumours and autoimmune thyroiditis in adults (1,2,3).

Autoimmune thyroiditis is the most common thyroid pathology in childhood and adolescence (7,8). It is characterized by lymphocytic infiltration and fibrosis which may affect SWV. In studies performed in adults, SWV values were reported to be significantly higher in autoimmune thyroiditis than in normal thyroid parenchyma and its use as a dignostic method has been suggested. There are a few studies evaluating the elasticity of thyroid tissue with SWE in children with autoimmune thyroiditis (7,9,10,11). The aim of this study was to measure the elasticity of normal thyroid tissue with SWE in children and adolescents as well as to investigate the role of SWE in the diagnosis of autoimmune thyroiditis in childhood.

Methods

Healthy Control Group

One hundred and thirteen healthy children and adolescents of whom 66 were girls (58.4%) were evaluated with B-mode ultrasound and SWE. These children were recruited from patients referred for neck ultrasonography for non-thyroid pathologies (lymphadenopathy, thyroglossal cyst, brachial cyst, etc) between 4-14 years of age. Of these volunteers, those with a history of thyroid disease or a history of thyroid disease in their family were excluded. In all controls both left and right lobes were evaluated (226 lobes).

Autoimmune Thyroiditis Patients

Fifty seven children and adolescents between 7-17 years of age of whom 45 (78.9%) were girls who were being followed up for a diagnosis of autoimmune thyroiditis in the pediatric endocrinology department of our hospital were evaluated with B-mode ultrasound, followed by SWE. The diagnosis of autoimmune thyroiditis was based on the presence of high levels of antithyroid antibodies including anti-thyroid peroxidase (TPOAb) and/or anti-thyroglobulin (TGAb), normal or low thyroid function assessed by measurement of thyroxine (T4) and thyroid stimulating hormone (TSH), together with assessment of heterogenity and hypoechogenity of thyroid parenchyma at ultrasound examination. Some of the patients were receiving antithyroid treatment during ultrasonography. All autoimmune thyroid patients had both lobes evaluated (114 lobes).

Ultrasound Assessment

SWE measurements were performed using a linear transducer probe (7.5-10 MHz) with a Toshiba Applio 500 ultrasound machine (Toshiba, Japan). The evaluation of each thyroid gland was obtained with the children in the supine position and the neck in hyperextension, eased by positioning a pillow behind the neck. The measurements were performed in the longutidinal plane with sampling deeper than 1 cm and obtained during normal breathing. Measurement of SWV was made after checking the intensity of the signal. In healthy children, the region of interest (ROI) was 5×6 mm and the probe was placed perpendicular to homogenous parenchyma that did not include vessels or surrounding structures. The color-coded image showed soft tissue in blue and hard tissue in red. The quantitative evaluation of normal thyroid tissue in healthy children was performed by SWV (m/s). An average of five measurements were performed by an experienced pediatric radiologist and general radiologist in the healthy children at the same time in each of the two thyroid lobes (Figure 1). In healthy children, normal SWV values by age and averages were determined. Differences due to interobserver variability in the measurements were evaluated.

In autoimmune thyroiditis patients, SWE evaluation was done by one experienced pediatric radiologist, using the same technique (Figure 2). SWV values detected in patients with autoimmune thyroiditis were compared with SWV values of healthy children.

The mean examination time was 5.0 ± 1.5 minutes (range 4-8 minutes).

Ethical Aspects

This prospective study was approved by the local ethics committee (University of Health Sciences Bakırköy Dr. Sadi Konuk Training and Research Hospital Ethics Committee/2016/107). Written consent was obtained from patients and/or their parents.

Statistical Analysis

This statistical analyses were performed with SPSS 22.0 software (IBM Inc., Chicago, IL, USA). Descriptive statistics of the data included mean, standard deviation, median, minimum, maximum, frequency and ratio values. The Kolmogorov-Smirnov test was used to analyze a range of variables. The Mann-Whitney U test, Wilcoxon and chi-square test were used in the analysis of quantitative data. The analysis of correlation was evaluated with the Spearman correlation test. Receiver-operating characteristic (ROC) curves were plotted for elasticity values and the optimum elasticity cut-off value that distinguished autoimmune thyroiditis from normal thyroid parenchyma was determined. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated.

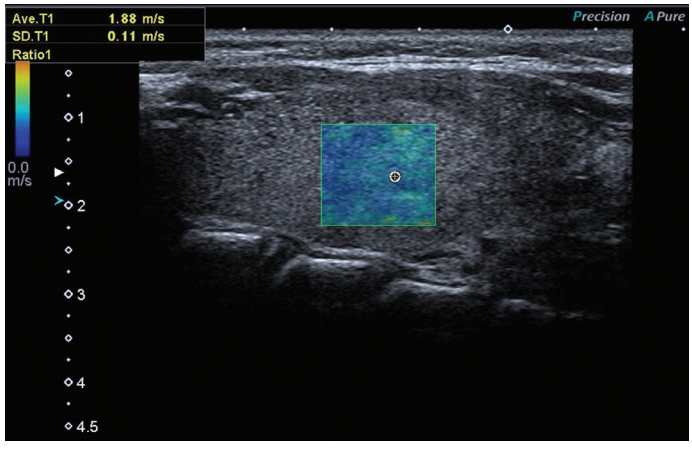

Figure 1. The evaluation of normal thyroid tissue with shear wave elastography

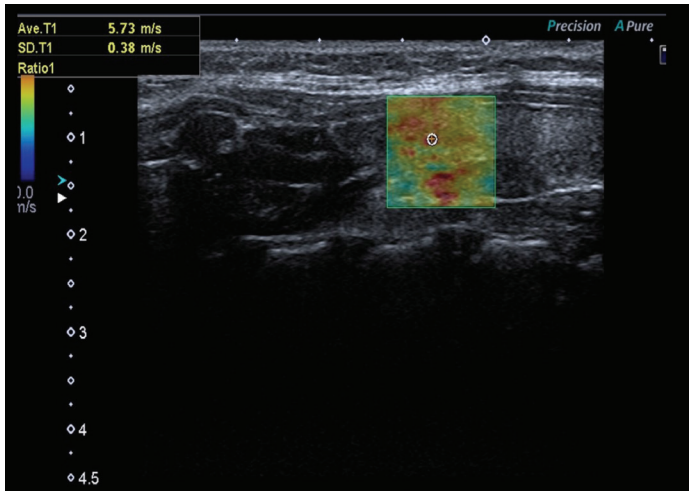

Figure 2. The evaluation of autoimmune thyroiditis with shear wave elastography

Results

The mean ± standard deviation (SD) age of healthy children and adolescents was 9.7 ± 2.9 years with a median of 10 year (range: 4-14 years). The mean ± SD SWV value of normal thyroid parenchyma was 1.8 ± 0.3 m/s with a median of 1.85 m/s (range: 1.32-2.37 m/s). There was no significant difference between right and left thyroid lobes ($p > 0.05$), nor between girls and boys ($p > 0.05$) (Figure 3). There was a significant positive correlation between age and SWV values ($r = 0.390$, $p < 0.001$) (Figure 4).

Comparison of the two observers in the assessment of normal thyroid parenchyma SWV values was made. There was no significant difference for intra-observer results ($p = 0.624$) and comparison showed a significant positive correlation ($r = 0.95$, $p < 0.001$) between the two (see Figure 5).

The mean age of children with autoimmune thyroiditis was 12.6 ± 2.7 years with a median of 13 (7-17) years. The mean ± SD SWV values of thyroid parenchyma was 3.7 ± 1.2 m/s with a median of 4.34 m/s in the patients (range: 2.59-6.25 m/s). There was no significant difference between right and left thyroid lobes ($p > 0.05$) nor between girls and boys ($p > 0.05$). In children with autoimmune thyroiditis, thyroid

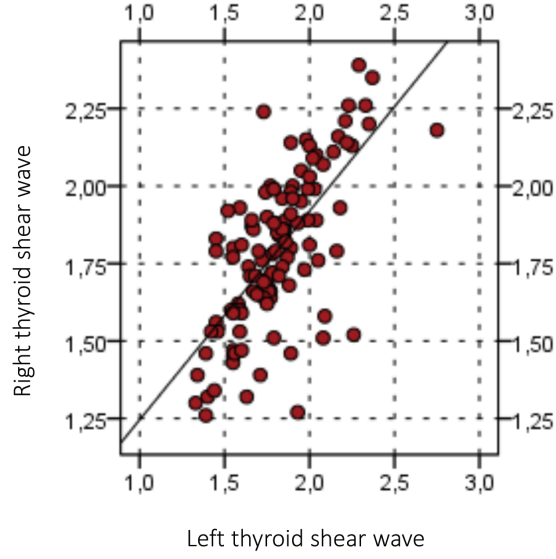

Figure 3. No significant differences are detected with shear wave velocity between the right and left thyroid lobes in healthy children

SWV values were significantly greater than those of healthy children (p≤0.05) (Table 1).

ROC curves were plotted for elasticity values based on presence of autoimmune thyroiditis and area under the curves (AUC) was calculated. The maximum AUC for mean elasticity value of both lobes was 0.996 (AUC, 0.996; 95% confidence interval 0.968-1.0). The cut-off value with the highest diagnostic accuracy for elasticity value was 2.39 m/s; sensitivity, specificity, PPV and NPV were 97.4%, 100%, 100% and 99.1%, respectively (Figure 6).

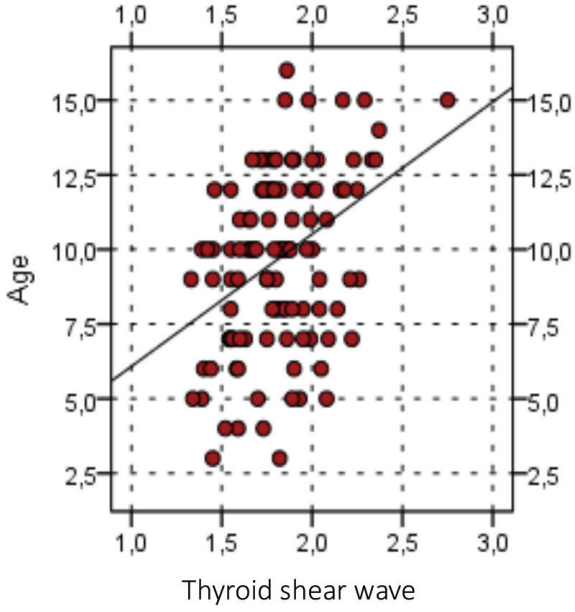

Figure 4. Positive correlation between age and shear wave velocity values

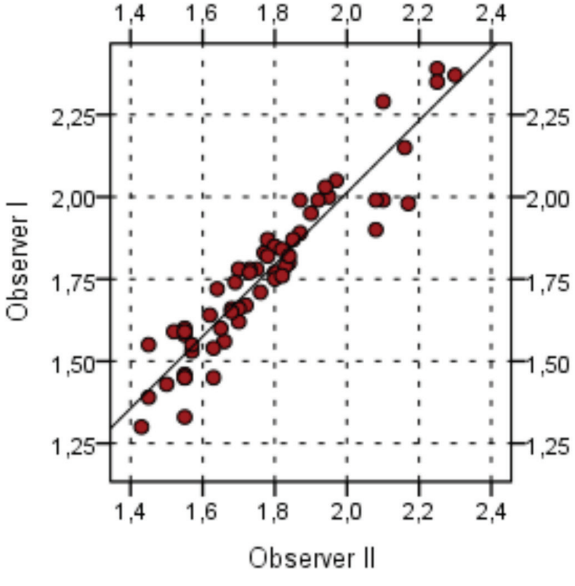

Figure 5. No significant differences were detected in intra-observer variability

SWV values and thyroid function tests showed no correlation with autoantibody concentrations in patients with autoimmune thyroiditis (p > 0.05) (Table 2).

Seventeen of the patients were receiving antithyroid treatment during SWE. The mean treatment duration was five months (range 2-24 months). There was no significant correlation between SWV values and antithyroid treatment duration (p > 0.05).

Discussion

Many studies on evaluation of normal thyroid tissue and its pathologies by SWE have been reported in adult populations (5,6,12,13,14,15). However there are few studies using this imaging method for the assessment of thyroid tissue in children. In this study we standardized a protocol for normal thyroid SWV measurements with regard to frequency, measurement depth and position, size of ROI and acquisition number in a pediatric population. We used high linear frequency probes and measured the SWV at a depth of more than one centimetre below the front edge of each thyroid lobe. Children who were able to cooperate were asked to hold their breath for a short time. In younger children, the study was performed with free-breathing. The operator applied similar amounts of transducer pressure only necessary to create a gray scale image thus avoiding preload. ROI was placed perpendicular to a homogeneous

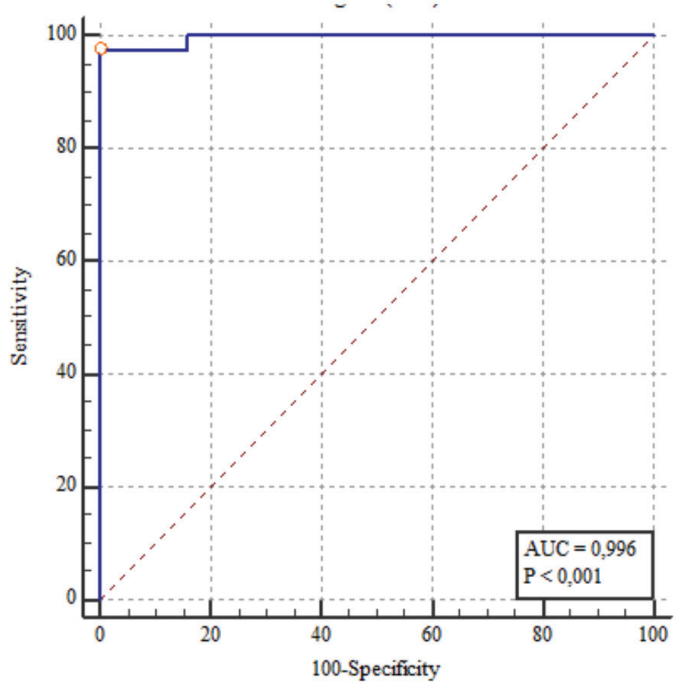

Figure 6. Receiver-operating charestics showing the optimal shear wave velocity cut-off value for autoimmune thyroiditis

parenchyma that did not include vessels or surrounding structures. This results in the thyroid tissue appearing perfectly homogeneous with a colour code corresponding to a soft tissue (blue colour code). The ROI size was small and it allowed for more accurate measurements. The size of ROI was determined as 5x6 mm, similar to that used for adults. In our study, five valid SWVs for each thyroid lobe were obtained because most children could not tolerate a prolonged examination. The mean examination time was 5 ± 1.5 minutes (range 4-8 minutes) in our study.

The SWE measurement technique for thyroid in adults has been reported by the World Federation for Ultrasound in Medicine and Biology in 2017 (9). The recommended measurement technique for adults is similar to the technique we used in children. However, there were some notable differences in technique. Young children cannot cooperate, thus cannot be required to hold their breath during SWE. The ROI used is standardized as 5x6 mm because of the small size of the thyroid in children. In addition, the measurement depth recommended for adults is 4-5 centimetres but SWE measurement of thyroid tissue in children, due to age dependent thyroid size, was evaluated as any depth above one centimetre. The measurements of thyroid tissue in adults is recommended to be from 5-6 different areas for each thyroid lobe. Sporea et al (13) reported that SWE of the thyroid is feasible with linear and convex probes and five measurements in every lobe are sufficient for an accurate assessment of thyroid tissue. Sporea et al (13) reported no significant difference between five or 10 measurements for thyroid stiffness in adults with acoustic radiation force impulse elastography. Vlad et al (14) performed three measurements on each thyroid lobe and calculated a mean elasticity in healthy adults. In our study, five SWV measurements were made in the children and the mean values were calculated as previously described by Ceyhan Bilgici et al (10).

We found no significant intra-observer variability for SWV measurements of thyroid tissue. Bhatia et al (16) reported that inter- and intra-operator reproducibility in SWE is acceptable with correlations ranging from 0.78 to 0.85 for intra-observer variability and between 0.97 and 0.98 for inter-observer variability. Bilgici et al (10) only assessed inter-observer variability and they found a value of 0.70 for the right lobe and 0.69 for the left lobe.

SWV values for normal adult thyroid tissue have been reported in various studies. These values were obtained from healthy control groups formed during SWE evaluation of diffuse thyroid diseases and thyroid nodules. Arda et al (4) reported, in an adult populations, a mean elasticity value of 10.97 + -3.1 kPa (approximately 1.89 m/s) for the thyroid. Fukuhara et al (17) found that the SWV value for normal thyroid tissue was 1.60 ± 0.18 m/s in adults. In healthy adults, Friedrich-Rust et al (6) reported a mean SWV value of 1.98 m/s, while Hekimoglu et al (15) reported an SWV value of 1.63 ± 0.12 m/s and that the range of SWV was between 1.59 and 1.98 m/s in adults.

Table 1. Comparison of thyroid shear wave velocity values in healthy children and children with autoimmune thyroiditis

Mean ± SD		Healthy children		Autoimmune thyroiditis		p	
		Median	Mean ± SD	Median			
Age		9.7 ± 3.0	10.0	12.6 ± 2.7	13.0	< 0.001	m
Gender	Girl n (%)	66 (58.4)	-	45 (78.9)	-	**0.008**	χ^2
	Boy n (%)	47 (41.6)	-	12 (21.1)	-		
SWV							
Right m/s		1.8 ± 0.3	1.8	3.6 ± 0.8	3.6	**0.000**	m
Left m/s		1.8 ± 0.3	1.8	3.8 ± 1.7	3.6	**0.000**	m

SD: standard deviation, SWV: shear wave velocity.

m: Mann-Whitney U test, χ^2: chi-square test

Table 2. Correlation between thyroid function tests and autoantibody titers and thyroid tissue elasticity values

	TSH	T4	TPOAb	TGAb	
Right lobe	-0.056	-0.080	0.120	0.089	r
	0.735	0.629	0.467	0.588	p
Left lobe	0.206	-0.102	-0.067	-0.047	r
	0.209	0.535	0.684	0.777	p

TSH: thyroid stimulating hormone, T4: thyroxine, TPOAb: anti-thyroid peroxidase, TGAb: anti-thyroglobulin

In our study, the mean SWV values of normal thyroid parenchyma was 1.82 ± 0.3 m/s with a range of between 1.32 and 2.37 m/s in healthy children. There was no significant difference between girls and boys. However, there was a significant positive correlation between age and SWV values, which increased with age. Studies have shown that changes in thyroid function occur with age and that the size of the thyroid gland shows a significant increase with puberty. Therefore, the increase in SWV values with age appear to follow, in the first years the elevation of TSH and the decrease in free T4 and free T3 hormones and the increase of thyroid volume with age (18,19). Ceyhan Bilgici et al (10), who conducted the first studies in this area in children, reported a mean SWE value of 1.22 ± 0.20 m/s for the thyroid gland at a mean age 10.5 ± 3.1 years. In addition, in contrast to our study, they did not find any correlation between age, thyroid gland volume and body mass index. Arioz Habibi et al (20) reported that SWV values of the thyroid gland were significantly higher in the 13 to 17 years age group and that there was a significant positive correlation between age and SWV values. These authors explained this finding as a function of age elasticity values of the thyroid which do not show a significant difference up to 12 years of age. The low SWE values may be explained by differences in age groups and thyroid hormone differences. In our study, a significant number of patients were at late childhood and adolescent ages.

The number of studies on the use of SWE in diffuse thyroid pathologies in adults is limited. Most of these studies concern chronic autoimmune thyroiditis (CAT). This is the subgroup of autoimmune thyroiditis which causes fibrosis in the thyroid gland. Autoimmune thyroiditis is also the most common thyroid pathology in childhood and adolescence (7). There are few studies evaluating the elasticity of thyroid tissue with SWE in children with autoimmune thyroiditis. Autoimmune thyroid diseases in children are usually diagnosed on the basis of clinical and laboratory findings, supported by ultrasound. The pathological features of autoimmune thyroiditis are interstitial infiltration by lymphocytes and a variable degree of fibrosis in tissue. It is thought that fibrosis leads to high SWV values as stiffness of a tissue is correlated with increased values of SWV.

Fukuhara et al (17) found that the SWV value in CAT (2.47 ± 0.57 m/s) was significantly higher than in healthy adults (1.59 ± 0.41 m/s) and that the SWV cut-off value was 1.96 m/s. Hekimoglu et al (15) reported a mean SWV value of 1.63 ± 0.12 m/s in normal adults and 2.56 ± 0.30 m/s in adults with CAT. They found that the optimal cut-off value for CAT prediction was 2.42 m/s (77% sensitivity, 71% specificity, 92% PPV, 81% NPV and 87% accuracy).

Sporea et al (21) found significant difference in SWV in autoimmune thyroid disease with a value of 2.07 ± 0.44 m/s. They found 2.68 ± 0.50 m/s in Graves disease and 2.34 ± 0.61 m/s in CAT in adults. They reported a cut-off value > 2.53 m/s for differentition between normal thyroid tissue and diffuse thyroid diseases with a PPV > 90%. Kim et al (22) found a cut-off value of 27.6 kPa (about 3.96 m/s), with a sensitivity of 40.9% and specificity of 82.9%. Vlad et al (14) stated that SWE may predict the presence of autoimmune thyroid disease. They found the best cut-off value for predicting thyroid pathology by SWE as 22.3 kPa (about 3.20 m/s) with a sensitivity of 59.6% and a specificity of 76.9%. Yucel et al (11) reported an SWV value of 1.67 ± 0.63 m/s in Hashimoto patients, a significantly higher value compared to healthy children. They reported an optimal cut-off value of 1.41 m/s with 73.1% sensitivity, 80.8% specificity, 79.2% PPV and 75% NPV. Kandemirli et al (12) determined 14.9 kPa in pediatric patients with Hashimoto's thyroiditis and reported significantly higher elasticity values than healthy subjects. The elasticity value cut-off with the highest diagnostic accuracy was 12.3 kPa and 1.968 m/s; sensitivity, specifity, PPV, NPV, and diagnostic accuracy of this cut-off were 86.4%, 96.3%, 98.1%, 76.5%, and 89.5%, respectively.

Our data is similar those published by Vlad et al (14). We found that normal thyroid parenchyma appears homogeneous, with low elasticity colored in blue, and autoimmune thyroiditis appears heterogeneous with areas of yellow and red scattered among the blue. The average SWV value of thyroid parenchyma in pediatric autoimmune thyroiditis was 3.7 ± 1.2 m/s and ranged from 2.59 to 6.25 m/s. In our study, the mean SWV measurement was higher than that of adults and also higher than values reported by Yucel et al (11) and Kandemirli et al (12). Fukuhara et al (17) found that SWV values are significantly affected by fibrosis but seldom by cellular density. This finding suggests that fibrosis may have an effect in the pathology of autoimmune thyroid diseases in children. Kandemirli et al (12) reported that SWV values increased as the degree of fibrosis increased in CAT.

In our study, when the correlations between SWV values and autoantibody levels and thyroid function tests were analyzed in patients autoimmune thyroiditis, no correlation was found. However, Magri et al (23) reported a positive correlation between tissue stiffness and serum TPOAb and concluded that TPOAb values of the patients could affect SWV measurements. In contrast, Liu et al (24) found that thyroid stiffness was weakly related to TSH and TGAb and was not correlated with T3, T4 or TPOAb. Kandemirli et al (12) reported a moderate but significant

correlation between elasticity values and TPOAb but no significant correlation between SWV values and TGAb levels. They explained this by suggesting that the presence of TPOAb might be characteristic of a late adaptive immune response whereas TGAb might reflect an early immune response. Yucel et al (11) found a correlation between TPOAb values and SWV values in patients but no correlation between SWV and either TGAb or thyroid function test results. Thyroid elasticity cannot currently be used to predict thyroid functions.

Ruchala et al (25) concluded that SWE might be useful in the diagnosis and differentiation between various types of thyroiditis but was not the optimal tool to differentiate Graves disease and CAT. Liu et al (24) found that SWE is helpful for distinguishing Graves disease from subacute thyroiditis but that it is unsuitable for differentiating CAT and Graves disease (24). All types of thyroiditis are characterised by increased thyroid stiffness and in CAT patients the degree of stiffness increases with fibrosis progression. Studies have shown that medical treatment in the presence of autoimmune thyroiditis has no effect on the elasticity of the thyroid tissue in adults (26). Some of our patients were using medical treatment during SWE. There was no significant correlation between elasticity values and antithyroid treatment with treatment duration in our study.

Study Limitations

The first of our limitations was that the mean age of our autoimmune thyroiditis cases was higher than our normal control cases. The reason is that these pathologies are more common in late childhood and adolescence. The second limitation was that only autoimmune thyroiditis patients were evaluated in the study. There is a need for studies on SWV measurements to demonstrate how this measurement is affected in other diffuse thyroid pathologies in children.

Conclusion

In this study, which evaluated the elasticity values of normal thyroid tissue, the measured values are presented. We also showed that the SWV values increased with age. In addition, it was demonstrated that SWV values in autoimmune thyroiditis patients were significantly higher compared to those of healthy children. There is a need for further studies to establish normal values for each age group. Also, further studies with larger series of children and adolescents are needed to compare the elasticity values of normal and pathologic tissues, such as diffuse thyroid diseases, thyroid nodules, etc, to determine the diagnostic role of this imaging technique in children. SWE is a useful imaging method complementing routine ultrasonography examination in pediatric patients in whom a diagnosis of autoimmune thyroid disease is considered.

Ethics

Ethics Committee Approval: This prospective study was approved by the Local Ethics Committee (University of Health Sciences Bakırköy Dr. Sadi Konuk Training and Research Hospital Ethics Committee/2016/107).

Informed Consent: Written consent was obtained from patients and/or their parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Esra Deniz Papatya Çakır, Concept: Figen Bakırtaş Palabıyık, Design: Figen Bakırtaş Palabıyık, Data Collection or Processing: Figen Bakırtaş Palabıyık, Analysis or Interpretation: Ercan İnci, Literature Search: Elif Hocaoğlu, Writing: Figen Bakırtaş Palabıyık.

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Comparison of Treatment Regimens in Management of Severe Hypercalcemia Due to Vitamin D Intoxication in Children

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What is already known on this topic?

There are various treatment options for hypercalcemia. Pamidronate treatment efficiently lowers serum calcium levels in children with hypercalcemia due to vitamin D intoxication.

What this study adds?

To our knowledge, this study is the first to compare first-line treatment options for hypercalcemia due to vitamin D intoxication. Children receiving prednisolone for severe hypercalcemia often require another type of drug treatment. Pamidronate treatment prevents recurrence of hypercalcemia.

Abstract

Objective: No large study has been conducted to date to compare the effectiveness of prednisolone, alendronate and pamidronate as first-line treatment in children with hypercalcemia due to vitamin D intoxication. The aim was to perform a multicenter, retrospective study assessing clinical characteristics and treatment results.

Methods: A standard questionnaire was uploaded to an online national database system to collect data on children with hypercalcemia (serum calcium level > 10.5 mg/dL) due to vitamin D intoxication [serum 25-hydroxyvitamin D (25(OH)D) level > 150 ng/mL] who were treated in pediatric endocrinology clinics.

Results: Seventy-four children [median (range) age 1.06 (0.65-1.60) years, 45 males (61%) from 11 centers] were included. High-dose vitamin D intake was evident in 77% of the cases. At diagnosis, serum calcium, phosphorus, alkaline phosphatase, 25(OH)D and parathyroid hormone concentrations were 15 ± 3.2 mg/dL, 5.2 ± 1.2 mg/dL, 268 ± 132 IU/L, 322 (236-454) ng/mL, and 5.5 (3-10.5) pg/mL, respectively. Calcium levels showed moderate correlation with 25(OH)D levels ($r_s = 0.402$, $p < 0.001$). Patients were designated into five groups according to the initial specific treatment regimens (hydration-only, prednisolone, alendronate, pamidronate, and

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combination). Need for another type of specific drug treatment was higher in children who initially received prednisolone ($p < 0.001$). Recurrence rate of hypercalcemia was significantly lower in children who were treated with pamidronate ($p = 0.02$).
Conclusion: Prednisolone is less effective in the treatment of children with severe hypercalcaemia secondary to vitamin D intoxication and timely implementation of other treatment regimens should be considered.
Keywords: Nutrition, rickets, stoss therapy, steroid, over-the-counter drugs

Introduction

Vitamin D exerts significant effects on intestinal absorption of calcium and phosphorus, renal reabsorption of calcium and mineralization of bone. The primary source of vitamin D in humans is its synthesis in the skin, which requires adequate sunlight exposure, since vitamin D content of most foods is low. Clinical problems associated with vitamin D metabolism are mostly due to its deficiency and, accordingly, several guidelines exist for evaluation and management of vitamin D deficiency (1,2,3). However, pediatricians are also encountering children with mild-to-severe consequences of vitamin D intoxication, often associated with hypercalcemia. Vitamin D intoxication is generally defined as serum levels of 25-hydroxyvitamin D [25(OH)D] above 100-150 ng/mL (250-375 nmol/L) (1,3,4,5). Possible causes include treatment of vitamin D-deficient rickets with single or daily high doses of vitamin D (6,7), manufacturing errors of over-the-counter drugs (8,9), parental dosing errors (10), over-fortification of milk (11) and prescription of vitamin D without prior measurement of its serum level or without a definite diagnosis of rickets (12,13,14,15).

Treatment options for vitamin D intoxication in children currently include discontinuation of vitamin D intake, intravenous hydration (IH) with normal saline, administration of F, glucocorticoids, calcitonin, alendronate, pamidronate and hemodialysis. These practises are mostly based on case reports and small studies (4,5,7,8,9,10,12,13,15,16,17,18,19,20,21).

We aimed to assess the clinical characteristics of children with vitamin D intoxication in a multicenter, retrospective study. A further aim was to compare the results of different first-line treatment schedules in a large sample.

Methods

A standard questionnaire was established in an online national database system (formerly www.favorsci.org, and currently http://cedd.saglik-network.org/) to collect clinical and laboratory data on children with hypercalcemia (serum calcium level >10.5 mg/dL) due to vitamin D intoxication [concurrent serum 25(OH)D level >150 ng/mL] who were treated in pediatric endocrinology clinics. The data were collected by a single nominated pediatric endocrinologist per center, who was responsible for registering patients onto the online database. The study protocol was approved by the Institutional Ethical Review Board University of Health Sciences Dr. Behçet Uz Children's Hospital, 2014-01). Informed consent was not taken from the parents of the patients, given the retrospective design of the study, for which the data were simply extracted from patient files.

Seventy-four patients from 11 tertiary referral centers were enrolled. Participating centers were located in five of the seven geographical regions of Turkey. Forty of the cases had been previously reported elsewhere (8,12,14,19,20). All biochemical evaluations were performed in a standard laboratory setting. Hypercalcemia was classified according to the following serum calcium levels as: mild (10.5-11.9 mg/dL); moderate (12-14 mg/dL); and (severe >14 mg/dL) (22). Hypercalciuria was defined when spot urine calcium/creatinine ratio exceeded the upper limits of normal calcium excretion for different age groups: ≤6 months of age, >0.8; 7-12 months of age, >0.6; 1-3 years of age, >0.53; 3-5 years of age, >0.39; 5-7 years of age, >0.28; >7 years of age, >0.21 (23).

Firstly, the patients were assessed by their clinical characteristics of vitamin D intoxication. Secondly, patients were designated into groups according to the specific treatment type they had received in the first 48 hours, as shown below:

Group 1 (n = 25): Oral hydration (OH) or IH ± furosemide (F)

Group 2 (n = 9): IH ± F + prednisolone

Group 3 (n = 11): IH + F + alendronate

Group 4 (n = 21): IH + F + pamidronate

Group 5 (n = 8): IH + F + prednisolone + pamidronate ± alendronate

Primary outcome measures related to treatment efficacy included: a) need for another specific drug treatment; b) elapsed time to achieve normocalcemia (8.5-10.5 mg/dL); and c) recurrence of hypercalcemia (elevation of calcium levels above >10.5 mg/dL after achievement of normocalcemia).

Secondary outcome measures were clinical features of, and factors associated with, hypercalcemia in children with vitamin D intoxication.

Statistical Analysis

The data were statistically analyzed using Statistical Package for the Social Sciences Software, version 15.0 (IBM Inc., Chicago, Illinois, USA). Descriptive analyses were performed for all data sets. Depending on the distribution type of the variables, Pearson or Spearman correlation analysis was performed to detect the factors associated with serum calcium levels at the time of admission. Subsequently, variables associated with serum calcium levels at the time of admission were entered into a multiple linear regression analysis. The least explanatory covariates were consecutively removed from the model in a backward stepwise elimination method. Two separate Kruskal-Wallis tests were performed for comparison of non-parametric numerical data between groups 1-5 and 2-4. Chi-square or Fisher's exact test (if expected count was below 5 in any of the cells) was used to compare categorical variables. All data were presented as n (%), mean ± standard deviation or median and interquartile range (IQR) minimum-maximum (range), where appropriate. Figures were prepared using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Results

The study group included 74 children who were treated for vitamin D intoxication between the years 2002 and 2014. The median age of the subjects was 1.06 (IQR 0.65-1.60; range 0.04-7.38) years and 45 were male (60.8%) (Table 1). The median number of patients enrolled per center was 4 (IQR 2-7; range 1-27). Nearly half of the patients were younger than one year of age (n=33, 44.6%). Twenty-one cases were between 1-2 years of age (39.2%) and 12 (16.2%) subjects were older than two years of age. Only seven (9.5%) of the cases had chronic illnesses (hypotonic infant, n=2; developmental dysplasia of the hip, n=2; meningomyelocele, n=1; wheezy infant, n=1; cerebral palsy and epilepsy, n=1). The most common presenting symptoms were vomiting (n=47, 63.5%), loss of appetite (n=35, 47.3%), and constipation (n=27, 36.5%). Five of the patients were asymptomatic and were incidentally found to have mild-to-moderate hypercalcemia (serum calcium levels, 10.8-13 mg/dL).

Approximately three-quarters of the patients (n=57, 77%) had a clear history of high-dose vitamin D intake [median dose, 600,000 (IQR 600,000-900,000; range 300,000-5,400,000) units. The majority of the patients (n=40, 70.2%) had received multiple doses of vitamin D on separate days due to accidental overdose by parents or overdose secondary to faulty dose. The median time from first dose of vitamin D to admission was 6.2 (IQR 3.6-9.4; range, 1.5-67.1) weeks (Table 1). The most common reason for vitamin D use was presumptive diagnosis of vitamin D deficiency, based on non-specific complaints including delay in walking or eruption of teeth without proper evaluation (n=41, 71.9%). Active rickets was the reason for vitamin D treatment in only three cases (5.3%, serum calcium levels, 10.8, 10.8, and 15 mg/dL, with the latter being due to parental dosing error).

Serum calcium, phosphorus, alkaline phosphatase (ALP), 25(OH)D and parathyroid hormone (PTH) concentrations of the study group were as follows: 15±3.2 mg/dL (range, 10.8-23.5), 5.2±1.2 mg/dL (range, 2.48-7.7), 268±132 IU/L (range, 89-652), 322 (IQR 236-454, range 150-1978) ng/mL, 5.5 (IQR 3.0-10.5, range 0.5-38.0) pg/mL, respectively (see Table 1). The majority of the patients (n=43, 58.1%) had severe hypercalcemia (>14 mg/dL), normal phosphate values in 55/69 (79.7%) cases with available data and suppressed PTH in 51/65 (78.4%) cases with available data. At the time of admission, hypercalciuria and nephrocalcinosis and/or nephrolithiasis were found in 46/57 (81%) of cases with available data and 33/68 patients (48.5%) of cases with available data, respectively.

Serum calcium concentrations at onset showed a moderate negative correlation with serum PTH (n=65, r_s=-0.588, p<0.001) and weak or moderate correlations with serum concentrations of 25(OH)D (n=74, r_s=0.402, p<0.001), phosphorus (n=69, r=-0.379, p=0.001), ALP (n=66, r=-0.416, p=0.001), vitamin D dose (n=57, r_s=0.383,

Table 1. Characteristics of the total group of subjects at admission

Children with vitamin D intoxication (n=74)	
Age (years)	1.06 (0.65-1.60)
Male gender n (%)	45 (60.8)
Vitamin D intake (units)[a]	600,000 (600,000-900,000)
Vitamin D intake (units/kg)[a]	77,900 (63,800-126,700)
Time to admission (weeks)[a]	6.2 (3.6-9.4)
Calcium (mg/dL)	15±3.2
Phosphorus (mg/dL)[b]	5.2±1.2
Alkaline phosphatase (IU/L)[c]	268±132
25(OH)D (ng/mL)	322 (236-454)
Parathyroid hormone (pg/mL)[d]	5.5 (3-10.5)

Data were presented as median (25^{th}-75^{th} percentile), n (%) and mean ± standard deviation.

[a]n=57 (77%), [b]n=69 (93.2%), [c]n=66 (89.2%), [d]n=65 (87.8%).

Normal ranges: calcium (mg/dL), 8.5-10.5; phosphorus (mg/dL), 4.3-8.7 (newborns), 3.8-6.5 (1-3 years), and 3.7-5.6 (4-11 years); alkaline phosphatase (U/L), 48-406 (newborns), 82-383 (1 month-2 years), 69-325 (2-8 years); 25(OH)D (ng/mL), 20-100; parathyroid hormone (pg/mL), 15-88

p = 0.004), and vitamin D dose per kilogram of body weight (n = 57, r_s = 0.483, p < 0.001) (Figure 1). However, spot urine calcium/creatinine ratio (n = 57, r = -0.095, p = 0.484) and time to admission from first dose of vitamin D (n = 57, r = -0.169, p = 0.235) showed no correlation with serum calcium levels. In the multiple linear regression analysis including age, vitamin D dose, vitamin D dose per kilogram of body weight, time to admission from first dose of vitamin D and serum levels of 25(OH)D, the final model contained two baseline variables which were independently associated with serum calcium levels. These were serum levels of 25(OH)D [B = 0.005 (95% CI 0.02, 0.008), p = 0.001] and vitamin D dose (per 100,000 IU) [B = 0.089 (95% CI 0.023, 0.155), p = 0.009]. These two variables together explained 22.6% of the variance of serum calcium levels [R2 = 0.226, F(4) = 9.327, p < 0.001].

Patients were designated into five groups according to their specific treatment regimens in the first 48 hours (Table 2). None of the patients had renal failure or required hemodialysis. Vitamin D intake and serum levels of calcium, phosphorus, ALP, 25(OH)D and PTH were significantly different among groups 1-5 (Table 2). We hypothesized that calcium and 25(OH)D levels at the time of admission should be similar among groups to make a reliable comparison regarding treatment efficiency. Figure 2 shows that only groups 2, 3, and 4 met this criterion.

The data regarding treatments and outcomes are shown in Table 3. Type and volume of hydration fluid, dose and duration of F treatment were similar among groups 2, 3, and 4. Six subjects (66.7%) in group 2 required another specific drug treatment after the first 48 hours of admission (one patient, pamidronate and calcitonin on day 10; two patients, pamidronate on days 3 and 4; three patients, calcitonin) while this was the case for one patient (4.8%) in group 4 (prednisolone, starting from day 6 of treatment) and none in group 3 (p < 0.001). The time to achieve normocalcemia was comparable (p = 0.099) among groups 2, 3, and 4. However, recurrence rate of hypercalcemia

Table 2. Characteristics of the patients among the groups at admission

	Group 1 (n = 25)	Group 2 (n = 9)	Group 3 (n = 11)	Group 4 (n = 21)	Group 5 (n = 8)	p (groups 1-5)	p (groups 2-4)
Age (years)	1.06 (0.72-1.39)	0.96 (0.25-2.10)	0.85 (0.54-1.80)	1.2 (0.9-1.8)	0.9 (0.5-1.9)	0.285	0.272
Male gender	15 (60%)	5 (55.6%)	8 (72.7%)	14 (66.7%)	3 (37.5%)	0.576	0.662
Vitamin D intake (units)	600,000 (525,000-600,000)	600,000 (300,000-4,275,000)	600,000 (525,000-975,000)	900,000 (600,000-1,200,000)	1,950,000 (750,000-4,125,000)	0.005	0.693
Vitamin D intake (units/kg)	65,200 (49,400-76,000)	71,700 (64,700-622,500)	115,800 (19,000-140,000)	98,100 (73,900-129,400)	329,700 (109,100-441,200)	0.002	0.497
Time from treatment to admission (weeks)	8.3 (3.9-17.2)	8.5 (2.9-19.4)	6.5 (2.1-8.3)	5.7 (3.6-8.7)	4.2 (2.1-9.4)	0.992	0.977
Calcium (mg/dL)	11.6 (11.1-12.4)	17.1 (14.2-18.8)	14.5 (14.2-16.8)	16.1 (14.8-17.6)	19.5 (17.1-22)	< 0.001	0.248
Phosphorus (mg/dL)	6.1 (5.8-6.3)	4.5 (3.3-5.1)	4.6 (4.1-5.0)	5.1 (4.2-6.3)	3.8 (3.1-4.5)	< 0.001	0.103
Alkaline phosphatase (IU/L)	345 (290-390)	192 (112-307)	195 (139-243)	186 (134-343)	174 (116-219)	< 0.001	0.909
25-hydroxyvitamin D (ng/mL)	245 (186-300)	361 (193-760)	348 (240-422)	450 (327-714)	312 (198-418)	< 0.001	0.893
Parathormone (pg/mL)	11 (7.1-19)	3 (1.9-6.7)	3 (2.5-3.1)	6 (3-9)	1.7 (0.5-3)	< 0.001	0.005
Number of subjects with nephrocalcinosis and/or nephrolithiasis[a]	3 (12.5%)	6 (66.7%)	8 (80%)	12 (66.7%)	4 (57.1%)	< 0.001	0.733

Group 1: Oral hydration (OH) or intravenous hydration (IH) ± furosemide (F); group 2: IH ± F + prednisolone, group 3: IH + F + alendronate, group 4: IH + F + pamidronate, group 5: IH + F + prednisolone + pamidronate ± alendronate; [a]Data are lacking for one case in each of groups 1, 3, and 5 and for three cases in group 4. Data are presented as median (25^{th}-75^{th} percentile) and n (%)

was significantly lower in group 4 compared to groups 2 and 3 [0 (0%), 2 (25%), and 3 (30%), respectively, p = 0.02]. Sixty-four of 68 subjects with initial renal sonograms were reassessed during follow-up and the ratio of nephrocalcinosis and/or nephrolithiasis was found to have decreased to 28.1% (n = 18) after a follow-up duration of 1 ± 0.9 years. The distribution was not significantly different among groups 2, 3, and 4 (p = 0.268).

Discussion

The majority of the children in our study group were younger than two years of age and did not have a pre-existing chronic health condition. Their pretreatment serum 25(OH) D levels were unknown. The upper limit of daily oral intake of vitamin D for healthy children aged < 1 and 1-3 years are reported as 1000-1500 IU and 2000-2500 IU, respectively (1). In the present study, minimum and mean doses of vitamin D intake that led to hypercalcemia were 300,000 IU and 1,020,000 IU, respectively. In one study, treatment with 300,000 IU of vitamin D in 3 to 36-month-old subjects with nutritional vitamin D deficiency rickets (n = 20) was reported to cause hypercalcemia in two patients (10%) (24). On the other hand, it was reported that calcium levels did not exceed the upper limit after treatment with the same vitamin D dose in 32 children aged between 3-17 years with vitamin D deficiency/insufficiency (25). In addition, vitamin D dose (both total and per kg of body weight) and serum 25(OH)D levels in our study were only moderately correlated with the degree of hypercalcemia. Dietary calcium intake and existence of conditions leading to vitamin D hypersensitivity might contribute to development and severity of hypercalcemia associated with vitamin D intoxication (1,3,26).

Treatment is warranted in vitamin D intoxication, as resulting hypercalcemia is associated with mild-to-severe gastrointestinal, renal, central nervous system,

Table 3. Treatment characteristics of the patients among the groups

	Group 1 (n = 25)	Group 2 (n = 9)	Group 3 (n = 11)	Group 4 (n = 21)	Group 5 (n = 8)	p (groups 1-5)	p (groups 2-4)
IH with isotonic saline[a]	20 (80%)*	4 (44.4%)	5 (45.5%)	13 (61.9%)	3 (37.5%)	0.089	0.544
Volume of hydration fluid (lt/m^2/day)	2.5 (2-2.5)*	3 (2.3-3)	2.5 (2-3)	2.5 (2-3)	2.8 (2.1-3)	0.238	0.400
Duration of hydration (days)	4 (3-5)	6 (3-10)	6 (3-9)	4 (3-4.8)	10 (4.5-15.5)	0.005	0.047
F dose (mg/kg/day)	2 (2-2)**	2 (2-2)***	2 (2-2)	3 (2-4)	4 (3.4-4)	< 0.001	0.221
Duration of F Rx (days)	4 (3-5)	6 (5-7)	5 (4-9)	7 (3-9)	4.5 (2.8-9.3)	0.118	0.916
Specific drug treatment in the first 48 hours	N/A	Pr, 1 (1-2) mg/kg/day, 5 (3-10) days	A, 6.7 (5-10) mg/dose, 3 (1-10) times	P, 1 (0.8-1) mg/kg/dose, 2 (1-3) times	Pr, n = 8, 1 (1-2) mg/kg/day, 4 (2-14) days P, n = 7, 1 (1-1) mg/kg/dose, 2 (2-3) times A, n = 2, 5 (5-5) mg/dose, 13 (9-13) times	N/A	N/A
Need for another type of specific drug treatment after 48 hours of therapy	0 (0%)	6 (66.7%)	0 (0%)	1 (4.8%)	0 (0%)	< 0.001	< 0.001
Days to normocalcemia	3 (2-4.5)	6 (3.5-11.5)	5 (4-12)	4 (3-6)	6.3 (4.3-11)	0.001	0.099
Duration of follow-up (years)	1 (0.6-1.4)	1.2 (0.2-2.4)	0.15 (0.1-0.2)	1 (0.4-2.2)	0.4 (0.1-0.8)	0.010	0.006
Recurrence rate[b]	0 (0%)	2 (25%)	3 (30%)	0 (0%)	1 (12.5%)	0.012	0.02
Nephrocalcinosis and/or nephrolithiasis[c]	1 (4.2%)	2 (33.3%)	5 (50%)	5 (27.8%)	5 (83.3%)	0.018	0.268

Group 1: Oral hydration (OH) or intravenous hydration (IH) ± furosemide (F); group 2: IH ± F + prednisolone, group 3: IH + F + alendronate, group 4: IH + F + pamidronate, group 5: IH + F + prednisolone + pamidronate ± alendronate: Pr: prednisolone, A: alendronate, P: pamidronate, C: calcitonin, N/A: not applicable, [a]All patients, except 5 cases in group 1 received IH with various fluid types, [b]Data are lacking for 1 case in each of groups 1-4. *5 cases (20%) received OH; **Seventeen cases (68%) received F, none of them were treated with OH; ***Eight cases (88.9%) received F; [c]Data are lacking for 1 case in each of groups 1,3, for 3 cases in each of groups 2 and 4, and for 2 cases in group 5. Data were presented as median (25th-75th percentile) and n (%)

cardiovascular, musculoskeletal, ophthalmological, and skin complications (4). The most common symptoms in our series were related with the gastrointestinal system including vomiting (63.5%), loss of appetite (47.3%) and constipation (36.5%). The most common clinical finding was nephrocalcinosis and/or nephrolithiasis (48.5%). Various studies have demonstrated that the majority of symptoms associated with vitamin D-induced nephrocalcinosis persist for years (27,28). In the present study, nephrocalcinosis and/or nephrolithiasis disappeared in nearly half of the affected cases.

Currently, there are various treatment regimens for vitamin D intoxication. A report including 11 adults from 1948 indicates that the only available methods at that time were elimination of vitamin D, low calcium diet and OH. Howard and Meyer (29) reported that the shortest time to achieve normocalcemia was 3-12 weeks in four subjects (36.3%, calcium levels 12.4-14.9 mg/dL) while it took over a year in three cases (27.2%, calcium levels 13.7-14.9 mg/dL). In the present study, a similar treatment was applied in group 1 [median (range) calcium level 11.6 mg/dL (11.1-12.4)]. In addition, intravenous fluids and F were also

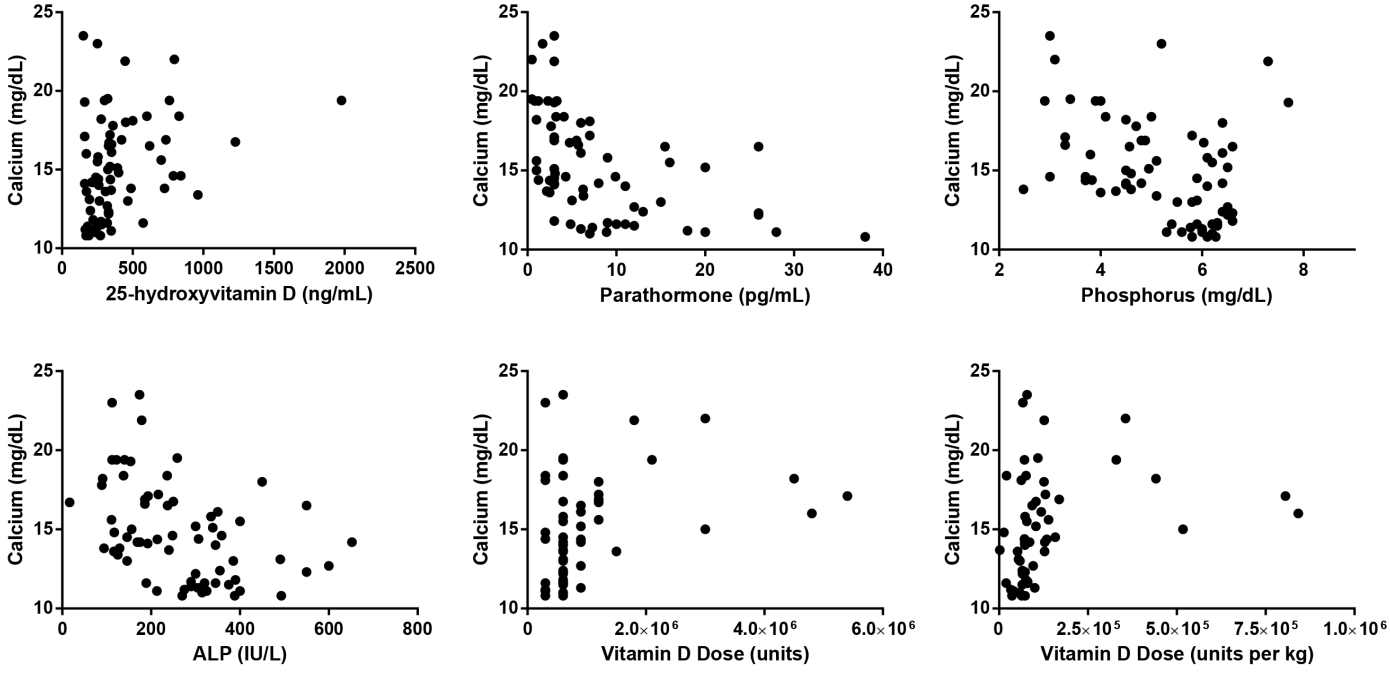

Figure 1. Correlation analyses of various variables with calcium and 25-hydroxyvitamin D levels at the time of admission
ALP: alkaline phosphatase

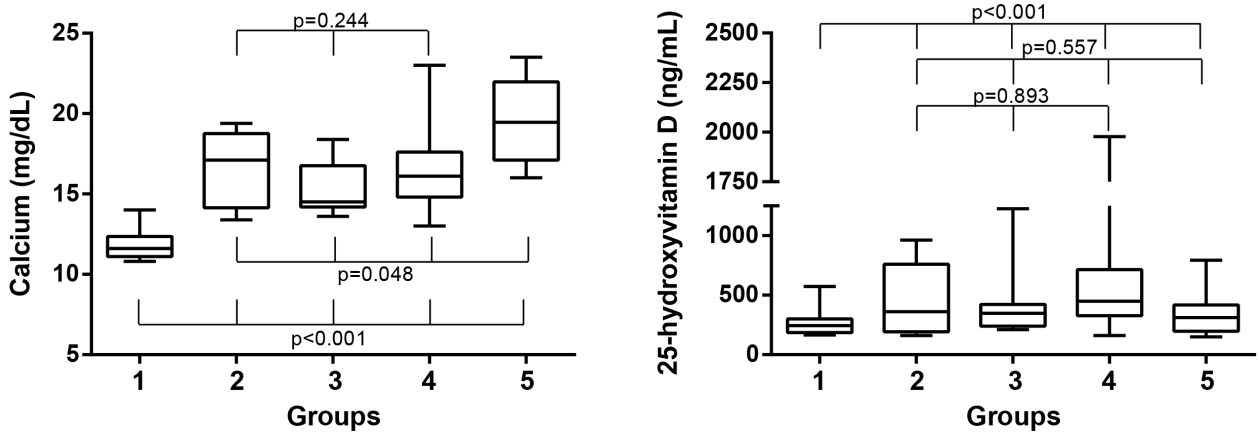

Figure 2. Box-whisker graphs of serum calcium and 25-hydroxyvitamin D levels among the groups (the horizontal lines within the boxes indicate the median, boundaries of the boxes indicate the 25th and 75th percentiles, and the whiskers indicate the highest and lowest values of the results)

used. Both additional therapies as well as milder degree of hypercalcemia at presentation resulted in a much shorter time to reach normocalcemia.

Other treatment regimens for vitamin D intoxication include calcitonin, prednisolone, alendronate, pamidronate and hemodialysis (3,4). Glucocorticoids decrease both renal reabsorption and intestinal absorption of calcium. However, their onset of action may take up to three days (3). Hatun and Cizmecioğlu (16) noted that normocalcemia could not be achieved and bisphosphonates were needed after over one month of glucocorticoid treatment in two infants with vitamin D intoxication (calcium levels at the time of admission, 14.9 and 18 mg/dL). Sezer et al (13) reported that four infants with vitamin D intoxication were given prednisolone (2 mg/kg/d) initially and two of them (calcium levels at the time of admission 16.5 and 19.1 mg/dL) required further alendronate treatment due to persistence of hypercalcemia after 15 and 23 days. Kara et al (15) reported that three children who were given prednisolone (1 mg/kg/d) for vitamin D intoxication (calcium levels at the time of admission: 16.0, 16.7, and, 19.7 mg/dL) reached normocalcemia after 12-26 days but that the hypercalcemia recurred in all of them after discontinuation of treatment. In the present study, nine patients with median calcium and 25(OH)D levels of 17.1 mg/dL and 361 ng/mL, respectively, were started on prednisolone [group 2, median (range) dose of 1 (1-2) mg/kg/day for 5 (3-10) days] as first-line treatment. However, two-thirds of these patients required another specific drug treatment due to persistence of the hypercalcemia and recurrence rate in this group was 25%. These data, together with the published evidence, indicate that prednisolone treatment has a low efficiency in "severe" hypercalcemia.

Bisphosphonates can lower calcium levels in subjects with vitamin D intoxication via their antiresorptive effect on bones (3). Alendronate as a first-choice treatment for vitamin D intoxication was first reported by Bereket and Erdogan (17) in 2003 in a 3-month-old infant with a serum calcium level of 18.5 mg/dL. A total of 30 mg of alendronate was given between the second and sixth days of treatment, resulting in normocalcemia. Orbak et al (21) reported a 7-year-old male child who was given 4,500,000 units of vitamin D for suspected vitamin D deficiency. Alendronate treatment was started at a serum calcium level of 14.8 mg/dL and normocalcemia was achieved by the 15th day after a cumulative dose of 45 mg that was given in five doses, 2-7 days apart. Sezer et al (13) described two subjects (serum calcium levels at the time of admission, 15.2 and 17 mg/dL) who were given a single dose of 10 mg of aledronate (13). Calcium levels returned to normal after five days and did not increase afterwards. Kara et al (15) reported two cases (serum calcium levels at the time of admission, 13.7 and 16.9 mg/dL) for whom alendronate (10 mg/d for seven consecutive days) was used directly. Normocalcemia was achieved after 3 and 4 days and no recurrence was reported. In the present study eleven patients in group 3, with a median (range) calcium concentration of 14.5 (14.2-16.8) mg/dL, received alendronate at a median dose of 6.7 mg [median (range) number of administrations, 3 (1-10)]. Although none of the cases required another specific drug treatment, hypercalcemia recurred in three patients.

The first experience with pamidronate, an intravenously given bisphosphonate, for vitamin D intoxication in children was reported by Ezgu et al (18) in 2004. The patient was a 3-month-old infant and was first treated with prednisolone. Four doses of pamidronate (0.2 mg/dose) were needed to achieve normocalcemia. Kara et al (15) reported pamidronate use as the first-line treatment in 13 children with vitamin D intoxication in whom median (range) calcium level at the time of admission was 16.5 (13.6-18.8) mg/dL. The first dose of pamidronate was 1 mg/kg when serum calcium levels were between 12-15 mg/dL and 2 mg/kg for levels above 15 mg/dL. Only two cases required a second pamidronate dose and none of them required prednisolone or alendronate with no recurrence being observed. In the present study, 21 children in group 4 with a median (range) calcium level 16.1 (14.8-17.6) mg/dL received pamidronate (median dose 1 mg/kg) as first-choice treatment. Similarly, none of the subjects required an alternative drug treatment or experienced recurrent hypercalcemia.

There exist two studies comparing the consequences of different treatments. Sezer et al (13) noted the superiority of alendronate (n = 4) compared to prednisolone (n = 4) and Kara et al (15) reported superiority of pamidronate (n = 18) to prednisolone (n = 6) and alendronate (n = 3). However, in both studies, some of the children had received other treatment regimens previously. In the present study which comprised a larger cohort, we were able to group the subjects according to the first-line treatments only. In mild hypercalcemia (group 1), oral or IH and F were sufficient to achieve normocalcemia. For very severe hypercalcemia, physicians tended to use combination therapies as first-line treatment (group 5). Groups 2, 3, and 4 had similar patient characteristics and serum calcium and 25(OH)D levels enabling us to compare the consequences of prednisolone (group 2), alendronate (group 3) and pamidronate (group 4) treatments. Pamidronate as a first-line treatment resulted in shorter duration of IH and no recurrence of hypercalcemia. Prednisolone treatment was not as effective as other regimens in lowering serum calcium levels and the majority

of children who were given prednisolone subsequently required another specific drug treatment in order to achieve normocalcemia.

Study Limitations

There were some limitations associated with our study. Numbers of reported patients were not similar in the different centers which contributed to the study. Over representation of one center in a particular treatment group might have influenced other unmeasured factors including variability in laboratory measurements that could affect outcomes. In addition, lower number of cases in group 2 (n = 9) compared to group 4 (n = 21) might have affected our judgements regarding the efficiency of prednisolone. However, as discussed above, there are many case reports in the medical literature supporting our findings.

Conclusion

In conclusion, evaluation of this largest cohort of pediatric vitamin D intoxication resulting in hypercalcemia suggests that cases with serum calcium levels below 12 mg/dL can be treated without prednisolone and bisphosphonates. Prednisolone treatment is less effective in the treatment of children with "severe" hypercalcemia (serum calcium levels above 14 mg/dL) and prompt implementation of pamidronate should be considered.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Dr. Behçet Uz Children's Hospital Clinical Research Ethical Committee (2014-01).

Informed Consent: Informed consent was not taken from the parents of the patients, given the retrospective design of the study, for which the data were simply extracted from patient files.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Korcan Demir, Hakan Döneray, Cengiz Kara, Zeynep Atay, Semra Çetinkaya, Atilla Çayır, Ahmet Anık, Erdal Eren, Ahmet Uçaktürk, Gülay Can Yılmaz, Ayça Törel Ergür, Mustafa Kendirci, Zehra Aycan, Abdullah Bereket, Murat Aydın, Zerrin Orbak, Behzat Özkan, Concept: Behzat Özkan, Design: Korcan Demir, Behzat Özkan, Data Collection or Processing: Korcan Demir, Hakan Döneray, Cengiz Kara, Zeynep Atay, Semra Çetinkaya, Atilla Çayır, Ahmet Anık, Erdal Eren, Ahmet Uçaktürk, Gülay Can Yılmaz, Ayça Törel Ergür, Mustafa Kendirci, Zehra Aycan, Abdullah Bereket, Murat Aydın, Zerrin Orbak, Behzat Özkan, Analysis or Interpretation: Korcan Demir, Cengiz Kara, Behzat Özkan, Literature Search: Korcan Demir, Behzat Özkan, Writing: Korcan Demir, Cengiz Kara, Abdullah Bereket, Behzat Özkan.

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ORIGINAL ARTICLE

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Clinical and Laboratory Characteristics of Hyperprolactinemia in Children and Adolescents: National Survey

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What is already known on this topic?

Hyperprolactinemia affects gonadal function in the adolescent. Cabergoline is a useful treatment model for pituitary adenomas. Pituitary surgery for macroadenomas may be needed in some patients. Some drugs increase the prolactin level. Macroprolactinemia is one of the causes of hyperprolactinemia.

What this study adds?

Cabergoline is an effective treatment in the adolescent. There is no difference in terms of age between micro- and macroadenomas. Physicians should review the indications for surgery in macroadenomas. Macroprolactinemia is a neglected cause of hyperprolactinemia in cases with unexplained etiology.

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Abstract

Objective: We aimed to report the characteristics at admission, diagnosis, treatment, and follow-up of cases of pediatric hyperprolactinemia in a large multicenter study.

Methods: We reviewed the records of 233 hyperprolactinemic patients, under 18 years of age, who were followed by different centers. The patients were divided as having microadenomas, macroadenomas, drug-induced hyperprolactinemia and idiopathic hyperprolactinemia. Complaints of the patients, their mode of treatment (medication and/or surgery) and outcomes were evaluated in detail.

Results: The mean age of the patients with hyperprolactinemia was 14.5 years, and 88.4% were females. In terms of etiology, microadenomas were observed in 32.6%, macroadenomas in 27%, idiopathic hyperprolactinemia in 22.7% and drug-induced hyperprolactinemia in 6.4%. Other causes of hyperprolactinemia were defined in 11.3%. Common complaints in females (n = 206) were sorted into menstrual irregularities, headaches, galactorrhea, primary or secondary amenorrhea and weight gain, whereas headache, gynecomastia, short stature and blurred vision were common in males (n = 27). Median prolactin levels were 93.15 ng/mL, 241.8 ng/mL, 74.5 ng/mL, 93.2 ng/mL, and 69 ng/mL for microadenomas, macroadenomas, idiopathic hyperprolactinemia, drug-induced hyperprolactinemia, and other causes of hyperprolactinemia, respectively. Of 172 patients with hyperprolactinemia, 77.3% were treated with cabergoline and 13.4% with bromocriptine. 20.1% of the patients with pituitary adenomas underwent pituitary surgery.

Conclusion: We present the largest cohort of children and adolescents with hyperprolactinemia in the literature to date. Hyperprolactinemia is more common in females and cabergoline is highly effective and practical to use in adolescents, due to its biweekly dosing. Indications for surgery in pediatric cases need to be revised.

Keywords: Pituitary, prolactin, children, microadenomas, macroadenomas, cabergoline, surgery

Introduction

Prolactin (PRL) is a luteotropic and pleiotropic hormone involved in many physiological functions, such as angiogenesis, the immune response, osmoregulation, reproductive behavior and lactogenesis. It is needed for the regulation of gonadal luteinizing hormone receptors in both genders, and it is necessary for lactation in females (1,2). Elevated PRL levels lead to various problems such as pubertal, mentrual and neurological. Stress, the use of drugs affecting the dopaminergic system and macroprolactinemia increase PRL to moderate levels, but pituitary adenomas increase PRL levels significantly (3,4,5). Signs and symptoms related to increased PRL such as oligomenorrhea, amenorrhea, and galactorrhea are more common in women in the adolescent period (4). In women, menstrual irregularity is the most common reason for referral, while males tend to experience intracranial pressure symptoms due to tumor growth. Medical treatment with a dopamine agonist is the first line treatment option for prolactinoma (5). Surgical intervention is considered in some cases (6).

Hyperprolactinemia is a common problem in adults and its etiology is different from that in children. Hyperprolactinemia is less frequently diagnosed in children. Accordingly, reports of pediatric hyperprolactinemia are less common.

In the present retrospective study, we aimed to investigate the differences between children and adults with hyperprolactinemia including etiology, treatment modality and treatment outcome in a large national cohort.

Methods

Patient Analysis

We reviewed 233 hyperprolactinemic patients under 18 years of age who were followed at 32 centers. Some of these cases have been reported previously (7,8,9). Hyperprolactinemia was diagnosed when repeated PRL concentrations were above 20 ng/mL. A microadenoma was defined as a pituitary tumor of less than 1 cm in diameter and a macroadenoma was defined as a tumor above 1 cm in diameter. The maximal diameter of the adenoma was evaluated using cranial magnetic resonance imaging (MRI). Drug-induced hyperprolactinemia was diagnosed if the patient had a history of medications such as antipsychotic, antidepressant or antidopaminergic agents. In this group PRL levels decreased to normal when the drug was withdrawn. If there was no mass evident on a pituitary MRI, no drug exposure and thyroid, kidney and liver dysfunction were excluded, the patient was accepted as a case of idiopathic hyperprolactinemia. Serum macroprolactin concentrations were sought in the group with idiopathic hyperprolactinemia and the complaints of these patients and their responses to treatment (medication and/or surgery) were evaluated in detail. The age, sex, and auxological evaluation results including height, weight and body mass index (BMI), and the respective standard deviation (SD) scores (SDS) of the patients were evaluated according the Turkish standards (10).

Data Collection

This retrospective, multicenter, nationwide, web-based study was conducted using an electronic recording form

(ERF) designed by two physicians (EE, OT) competent in PRL disorders as well as in ERF preparation. The ERF was used to collect the demographic data and clinical and laboratory findings of the patients with hyperprolactinemia. The ERF was uploaded to the CEDD Net Web Registry System website (www.cedd.saglik-network.org). Informed consent was obtained from the parents of the patients. The study protocol was approved by the Uludağ University Ethics Committee (number: 2015-19/10).

Statistical Analysis

Statistical analyses were performed using SPSS v.23 for Windows (IBM Inc., Chicago, IL, USA). Normality was tested using the Shapiro-Wilk test. Data are presented as mean ± SD for parametric data and median (range) for non-parametric data. Student's t-test was used for comparison of parametric variables, and Mann-Whitney U test was used for non-parametric data. Chi-square tests were used to determine significant differences in proportions among categorical variables. Spearman rank test was used for analysis of correlation among parameters. A p value of less than 0.05 was considered statistically significant.

Results

The median (range) age of the patients with hyperprolactinemia was 15.3 (0.12-17.7) years, and 88.4% (n = 203) were females. In terms of etiology, pituitary microadenoma was observed in 32.6% (n = 76), macroadenoma in 27% (n = 63), idiopathic hyperprolactinemia in 22.7% (n = 53) and drug-induced hyperprolactinemia in 6.4% (n = 15) cases. Other causes of hyperprolactinemia were defined in 11.3% (n = 26) (Table 1, Figure 1). Common complaints in females (n = 206) were menstrual irregularity, headache, galactorrhea, primary or secondary amenorrhea and weight gain, whereas headache, gynecomastia, short stature and blurred vision were common in males (n = 27) (Table 2). A family history of high PRL levels was detected in only seven cases. However mutation analysis of the *MEN* or *AIR* genes were not performed in that group. Patients with idiopathic hyperprolactinemia (n = 53) complained of menstrual irregularity (49%), headache (22.6%), weight gain (22.6%), pubertal delay (20.7%) and galactorrhea (17%). Hyperprolactinemia was coincidentally detected in 13.2%. Other causes of hyperprolactinemia were sorted into non-pituitary masses (n = 6), craniopharyngioma (n = 5), macroprolactinemia (n = 5), hypothyroidism (n = 3), polycystic ovary syndrome (n = 2), pituitary stalk interruption syndrome (PSIS) (n = 2), rapid-onset obesity with hypothalamic dysfunction, hypoventilation, autonomic dysfunction syndrome (n = 2) and tuberous sclerosis (n = 1). In the group with drug-induced hyperprolactinemia, risperidone was used in nine of 15 (60%) cases and various antipsychotics or antidepressants were used in the other cases. Serum macroprolactin was measured in 48 cases with idiopathic hyperprolactinemia and detected in 5 (10.4%), with a median PRL level of 127 (63.5-200) ng/mL. The median PRL levels were 93.15 ng/mL, 241.8 ng/mL, 74.5 ng/mL, 93.2 ng/mL, and 69

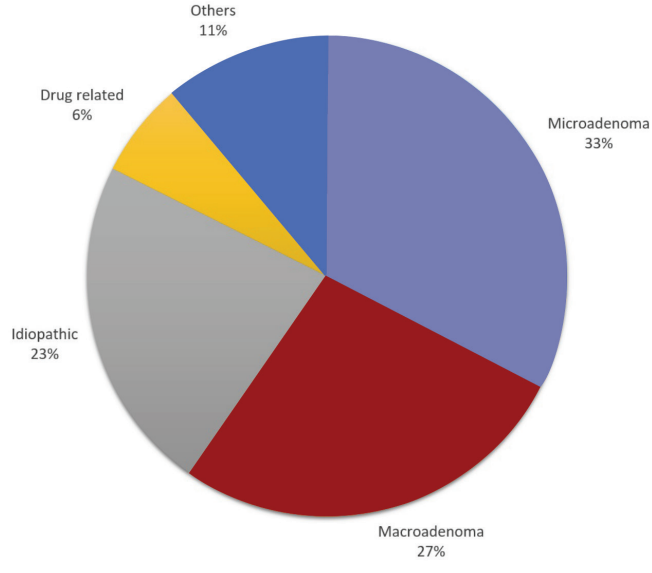

Figure 1. Diagnostic distribution of hyperprolactinemia patients

Table 1. Age, gender distribution and serum prolactin level according to diagnosis

			Prolactin (ng/mL)	
	Age (years)	n (M/F)	Median	Range
Microadenomas	15.08 ± 1.97	76 (71/5)	93.15	31.5-929
Macroadenomas	14.77 ± 1.86	63 (49/14)	241.8	52.7-5097
Idiopathic	14.65 ± 3.03	53 (50/3)	74.5	27.2-288
Drug induced	13.72 ± 4.49	15 (14/1)	93.2	50.6-200
Others	12.27 ± 4.72	26 (22/4)	69.0	35-200
Total	**14.49 ± 2.93**	**233 (206/27)**	**99.2**	**27.2-5097**

M: male, F: female

ng/mL for microadenomas, macroadenomas, idiopathic hyperprolactinemia, drug-induced hyperprolactinemia and other causes of hyperprolactinemia, respectively (see Table 1).

When the cases with a prolactinoma (n = 139) were divided into two groups, micro- and macroadenoma, there were no statistically significant differences in terms of age. In terms of gender distribution, 93.4% of microadenoma cases were female (71 female, 5 male) and 77.7% of macroadenoma cases were female (49 female, 14 male) ($p < 0.05$). There was no significant difference in height, weight, height SDS, weight SDS, BMI, and BMI SDS between the two groups. However, BMI and BMI SDS tended to be greater in the macroadenoma group (Table 3). The maximal diameter of the adenomas was 5.9 ± 2.1 mm in microadenomas and 17.3 ± 7.4 mm in macroadenomas. There was a significant correlation between adenoma size and PRL level ($p < 0.05$, $r = 0.494$; see Figure 2).

Of 172 patients with hyperprolactinemia (micro- and macroadenomas plus some patients with idiopathic hyperprolactinemia), 77.3% were treated with cabergoline and 13.4% with bromocriptine. The remaining 9.3% were switched from bromocriptine to cabergoline because of treatment failure. The median (range) initial doses of cabergoline and bromocriptine were 0.5 (0.25-2.5) mg/week and 2.5 (0.5-7.5) mg/day, respectively, and the normalization period for PRL was 2 (0.5-47) months for cabergoline and 3 (1-17) months for bromocriptine, showing a statistically significant difference ($p < 0.170$). There were no serious side effects for either drug. In total, 20.1% (28/139) of the patients with pituitary adenomas

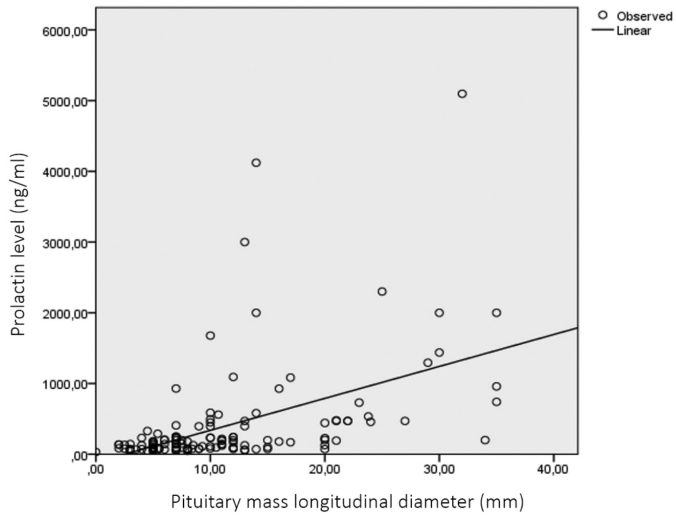

Figure 2. The correlation between serum prolactin levels and pituitary mass longitudinal diameter

Table 2. Common complaints and symptoms at diagnosis by gender in hyperprolactinemia patients

Females	n = 206 (%)	Males	n = 27 (%)
Menstrual irregularities	83 (40.2)	Headache	13 (48.1)
Headache	58 (28.1)	Gynecomastia	4 (14.8)
Galactorrhea	54 (26.2)	Short stature	4 (14.8)
Secondary amenorrhea	27 (13.1)	Blurred vision	4 (14.8)
Weight gain	20 (9.7)	Convulsions	4 (14.8)
Incidental finding	18 (8.7)	Visual field defect	3 (11.1)
Primary amenorrhea	13 (6.3)	Weight gain	2 (7.4)
Blurred vision	10 (4.8)	Incidental finding	2 (7.4)
Hirsutism	6 (2.9)	Delayed puberty	2 (7.4)
Infantile spasm	6 (2.9)	Infantile spasm	1 (3.7)

Table 3. Age, gender, body mass index (BMI), BMI standard deviation scores, and pituitary adenoma diameter values in pituitary microadenoma and macroadenoma patients

	Microadenomas	Macroadenomas	p
Age (years)	15.08 ± 1.97	14.77 ± 1.86	0.348*
n (F/M)	76 (71/5)	63 (49/14)	0.023**
BMI	23.11 ± 4.57	24.30 ± 4.73	0.133*
BMI SDS	0.65 ± 1.40	0.98 ± 1.34	0.164*
Prolactin (ng/mL) [median (min-max)]	93.15 (31.5-929)	241.8 (52.7-5097)	< 0.001***
Maximal diameter of the adenoma (mm)	6 (1.3-10)	14 (10-35)	< 0.001***

*Student's t-test, **Chi-square test, ***Mann-Whitney U test. BMI: body mass index, SDS: standard deviation scores, M: male, F: female, min: minimum, max: maximum

underwent pituitary surgery. Surgical option was the treatment of choice of the neurosurgeon or the patients who was not seen by an endocrinologist prior to consulting the surgeon. Transcranial surgery was performed in only two cases, while transsphenoidal surgery was performed in the remaining cases. In addition, 86.2% of these cases required a dopamine agonist after the operation. Only four cases received radiotherapy.

Discussion

In this large, retrospective, multicenter cohort study, children and adolescents with hyperprolactinemia were evaluated and prolactinoma was detected in 60%. While some of the cases (23%) were idiopathic, others were due to various medications or other causes of hyperprolactinemia. Pituitary adenomas, most common in adults, are rare in children, and few studies have described the clinical signs and treatment outcomes of these adenomas in children. To date and to our knowledge, this is the largest cohort of pediatric patients with hyperprolactinemia in the pertinent literature. We therefore believe that this study will shed light on all aspects of this disease in the pediatric age group and reveal the differences from the adult population.

The ratio of macroadenoma appears to be very high in both girls (7%) and boys (28.5%) compared to adults. Adults are reported to have a higher prevalence of macroadenomas in males (11,12). A similar disproportion has also been reported in other pediatric cohorts (5,13).

The mean age of the two groups (micro-/macroadenomas) was not different significantly. This finding is contrary to the assumption that neglect of symptoms for many years leads to diagnosis of macroadenomas in men. The predominance of large tumors in men may be related to the biologic behavior of the prolactinomas.

Macroprolactinemia can be present in some cases with idiopathic PRL elevation. Macroprolactin is a big-PRL, accounting for 1% of total PRL (14). In some cases, this ratio increases and leads to a false diagnosis of hyperprolactinemia. Diagnosis is made with chromatography or polyethylene glycol analysis and there is no need for treatment. Macroprolactinemia is detected in 15-46% of hyperprolactinemic cases (14,15). In our study, macroprolactinemia was investigated in 48 cases and found in only five (10.5%). Some cases of idiopathic hyperprolactinemia are likely to have received unnecessary treatment because macroprolactinemia was not excluded. Macroprolactinemia should be considered in cases having non-specific symptoms and no abnormal features on pituitary imaging. Another cause of idiopathic hyperprolactinemia may be a PRL receptor mutation. Familial hyperprolactinemia has been described in some of these cases (14,16,17). In familial cases, *AIP* and *MEN1* should be included in the genetic analysis. In a study, patients with macroprolactinoma were found to have *AIP* (9%) and *MEN* (5%) variants, and dopamine agonist resistance was found in *MEN1* mutations (18). In our study, neither *MEN1* nor *AIP* were investigated.

Another cause of hyperprolactinemia is drug use. Many antipsychotic agents increase PRL by affecting the dopaminergic system (19,20). In total, 6% of our cases had increased PRL due to use of antipsychotic drugs. In this group the mean level of PRL was 100 ng/dL, with a maximum level of 200 ng/dL. The treatment of drug-induced hyperprolactinemia consists of a reduction of the drug dose or a transition to another drug. Pituitary imaging should be performed in cases with clinical symptoms. Other causes of hyperprolactinemia include craniopharyngioma affecting the pituitary gland, non-pituitary tumors and PSIS that may affect the tuberoinfundibular pathway and increase PRL.

Hyperprolactinemia is most frequently observed after the onset of puberty and in the female gender. Menstrual irregularity, galactorrhea and gynecomastia are commonly seen in these patients. In a study in which 27 pediatric cases were evaluated, 17 were female (63%) and had a mean age of 15.6 years (3). In our study, the mean age of the participants was 14.49 years, and the mean age of the patients with prolactinoma was 15 years.

In patients with macroprolactinoma, headache and visual problems are the first signs in males whereas primary or secondary amenorrhea is seen in all females (13). Oligomenorrhea and galactorrhea were the most common symptoms of macroadenoma in a study of 13 cases (10 female) (21). In another study, 80% of females with hyperprolactinemia presented with menstrual problems, galactorrhea and headache, while males presented with headache, visual problems and gynecomastia (18). In our study, 60% of girls had menstrual problems, 25% had headache and 25% had galactorrhea, whereas half of boys complained of headaches. To summarize, half of the males had headache, while half of the females presented with menstrual problems. It is possible that a larger pituitary adenoma could cause headaches due to delayed diagnosis. Other complaints in men were not specific. Hyperprolactinemia should be considered in the differential diagnosis of women with menstrual problems during puberty and cranial and pituitary imaging should be performed to elucidate the etiology.

In our series, interestingly, about 10% of cases experienced weight gain and 30.9% (n = 72) of our cases were overweight or obese. The increase in BMI was encountered more frequently in macroprolactinoma. However, there was no correlation between BMI and PRL levels. In a study of 11 cases, six of whom were female, with hyperprolactinemia, who presented with short stature or growth deceleration, four had problems with weight gain and three had pubertal problems (4). In another study, 23% of the cases were referred to a physician with weight gain (18). In a study in which non-functional pituitary adenomas and prolactinoma were evaluated, BMI was reported to be significantly higher in the prolactinoma group. That group also had diminished growth hormone and insulin-like growth factor-1 levels (22). It has been shown that the modulatory effect of PRL may influence fat tissue and PRL changes body weight and composition. In 44 patients with prolactinoma, waist and hip circumference increased significantly, while fasting insulin and triglyceride were found to be elevated and fasting glucose and high-density lipoprotein cholesterol were normal (23). The relationship between PRL and obesity is unclear and remains to be elucidated. The PRL-releasing peptide (PrRP) is secreted by the hypothalamus, and it increases pituitary PRL production. It has also been found that PrRP is associated with nutrient and energy balance and that PrRP reduces weight gain and has an anorexigenic effect (24). The discovery of the relationship between PrRP and PRL could help in explaining weight gain in hyperprolactinemic individuals.

Dopamine agonists are first choice drugs to treat prolactinoma. Cabergoline has been used for many years as a highly effective and tolerable treatment. It was first used about 30 years ago in treating a patient who developed bromocriptine resistance (25,26). Cabergoline shrinks tumor cells and performs best with weekly dosing. It has been shown to be effective even in pituitary adenomas with no function (27) and has been used for years even in giant macroadenomas (28,29). In a study of 26 prolactinomas, bromocriptine was initiated in all cases, and conversion to quinagolide or cabergoline was made due to development of intolerance or resistance to bromocryptine (13). In our study, most of the centers preferred cabergoline as first line medication, and some had switched to cabergoline due to drug resistance. This study has shown that cabergoline can be used safely and effectively in children and adolescents.

Due to the high efficacy of dopamine agonists, surgery is rarely needed in prolactinomas. The rate of surgical treatment in our series was 20%, and this rate is likely to be higher than that reported in the literature on adult cases. Since adult studies generally show outcomes of cases who underwent surgery, it is not worthwhile to compare adult and child data for surgery ratio. In a pediatric study, seven of 27 patients (25.9%) were treated surgically, while 37.5% of adult patients with macroprolactinomas underwent surgery and 33% of these developed hypopituitarism (5,30). In another study evaluating nine surgically treated patients, transient complications, such as electrolyte disturbance, were observed postoperatively and no long-term sequelae were observed (31). Surgically, the transsphenoidal procedure was not associated with mortality, and no serious complications were observed (32).

It is unclear when surgical treatment of prolactinoma should be considered in children. It has been stated that transsphenoidal surgery can be used in patients who develop dopaminergic agonist intolerance or resistance or side effects from a drug. In addition, large adenomas causing visual problems and cerebrospinal fluid leakage due to pressure on the base of the skull are also candidates for surgery (33,34). In our cases, dopaminergic drugs had to be started or continued in 86.2% of the patients who underwent surgery. The surgical option should be avoided in children with prolactinoma because of recurrence after surgery and also because of development of various complications, including hypopituitarism, and the need to restart dopamine agonistic therapy.

Study Limitations and Strength

The limitations of our study include the use of different methodology in laboratory data in different centers and also the use of different treatment modes and different approaches. *AIP* and *MEN* genes were not investigated. The strength of the study lies in its large case series, the inclusion of most pediatric endocrinology centers in the country and also its detailed data content.

Conclusion

Hyperprolactinemia, which is more common in girls, is mostly caused by pituitary adenomas. Macroprolactinemia should be investigated in cases of unexplained hyperprolactinemia. Cabergoline is an effective treatment in children because of its weekly usage and the absence of significant side effects. The surgical option should not be considered in children, even in giant adenomas, because dopaminergic agonistic therapy is highly effective. Surgical indications need to be carefully considered by all relevant clinicians including endocrinologists and surgeons.

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Ethics

Ethics Committee Approval: The study protocol was approved by the Uludağ University Ethics Committee (number 2015-19/10).

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Erdal Eren, Ömer Tarım, Concept: Erdal Eren, Ömer Tarım, Design: Erdal Eren, Data Collection or Processing: Ayça Törel Ergür, Şükriye Pınar İşgüven, Eda Çelebi Bitkin, Merih Berberoğlu, Zeynep Şıklar, Firdevs Baş, Servet Yel, Serpil Baş, Elif Söbü, Abdullah Bereket, Serap Turan, Halil Sağlam, Zeynep Atay, Oya Ercan, Tülay Güran, Mehmet Emre Atabek, Hüseyin Anıl Korkmaz, Aylin Kılınç Uğurlu, Ayşehan Akıncı, Esra Döğer, Enver Şimşek, Emine Demet Akbaş, Ayhan Abacı, Ülkü Gül, Sezer Acar, Eda Mengen Uçaktürk, Melek Yıldız, Edip Ünal, Analysis or Interpretation: Erdal Eren, Ömer Tarım, Literature Search: Erdal Eren, Writing: Erdal Eren, Ömer Tarım.

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ORIGINAL ARTICLE

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Urine Levels of Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinases in Children with Type 1 Diabetes Mellitus

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What is already known on this topic?

It has been demonstrated that mesangial expansion in diabetic nephropathy begins before microalbuminuria occurs. Only a few studies have reported alterations in urine levels of matrix metalloproteinases and tissue inhibitor of metalloproteinases in patients with type 1 diabetes mellitus and these studies have conflicting results.

What this study adds?

The indicators of fibrosis in urine do not increase in the early stage of type 1 diabetes mellitus. This finding suggests that the chronic changes in the kidney evolve at a later stage of the condition.

Abstract

Objective: Histopathological changes in the kidney in type 1 diabetes mellitus (T1DM) begin before detection of microalbuminuria. Therefore, there is interest in finding a better biomarker for the early detection of diabetic kidney injury. The aim of this present study was to determine whether urinary indicators of fibrosis are detectable early in the development of T1DM in children and if they may predict progressive renal injury.

Methods: Urinary matrix metalloproteinase 2 and 9 (MMP2 and MMP9), tissue inhibitor of metalloproteinase 1 and 2 (TIMP1 and TIMP2) and transforming growth factor-β1 (TGF-β1) were assessed in 33 patients with T1DM with normal renal functions and in 24 healthy controls. Microalbuminuria was not present in the patient group with the exception of three patients. The results were adjusted to urine creatinine (Cr) and the differences between patients and controls were evaluated. These measurements were repeated after one year and the results were compared with the first year results.

Results: Urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr, TGF-β1/Cr were not different between the patient and control groups ($p > 0.05$). There were also no significant differences between the first and second year results for these biomarkers ($p > 0.05$). None of these parameters were correlated with hemoglobin A1c, body mass index and duration of T1DM. Interestingly, all parameters were negatively correlated to age of onset of T1DM ($p < 0.05$)

Conclusion: Our findings suggest that urinary biomarkers of fibrosis do not show an increase in diabetic children without microalbuminuria. The results also indicate that the risk of early fibrosis may increase as age of onset of T1DM decreases.

Keywords: Type 1 diabetes mellitus, diabetic nephropathy, children, biomarker, MMP, TIMP

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Introduction

Type 1 diabetes mellitus (T1DM) is one of the most common chronic diseases of childhood (1,2). T1DM causes many macro- and microvascular complications. Diabetic nephropathy (DN) is one of the microvascular complications of T1DM (3,4). If T1DM is not well controlled, it eventually leads to end-stage renal disease (ESRD) due to renal fibrosis (5,6,7). It is known that increased production and decreased degradation of matrix leads to excessive accumulation of extracellular matrix (ECM) components and consequently to development of renal fibrosis (8). Matrix components are regulated by matrix metalloproteinases (MMPs) such as MMP2, MMP9 (9). They cleave denatured collagens, laminin and some cell adhesion molecules and growth factors such as transforming growth factor-β (TGF-β). Tissue inhibitors of metalloproteinases (TIMPs) are known as regulators of MMPs. TIMPs are usually inhibitory, although sometimes stimulate, MMP activity (10).

The prominent characteristic of DN is ECM accumulation and consequent development of mesangial expansion (8). These changes begin in the second stage of DN and become more prominent in later stages (11). Since MMPs regulate remodeling of ECM, they are important for tissue development (9). MMP2 and MMP9 have a crucial role on the degradation and regulation of ECM in the glomeruli (8). Therefore, MMPs may be involved in the pathophysiology of DN (8). TGF-β1 is an important growth factor also involved in kidney fibrosis and DN, via a number of pathways.

It has been suggested that DN usually manifests in adulthood and microalbuminuria is considered as the first laboratory sign of nephropathy (11). Usually, microalbuminuria occurs 6-15 years after diagnosis of T1DM. It would be clinically useful to identify earlier biomarkers than urinary microalbumin for predicting DN thus allowing more effective management and possibly delaying or preventing ESRD.

We hypothesized that the biomarkers of renal fibrosis may increase before microalbuminuria becomes manifest, since microalbuminuria is not the first finding of the disease, but a result of ongoing renal damage in DN (11). The aim of this study was to determine whether urine levels of MMP2, MMP9, TIMP1, TIMP2 and TGF-β1 increase in children with T1DM and serve to predict a progressive renal injury.

Methods

Thirty-three consecutive patients (18 male, 15 female) with T1DM who attended the outpatient clinic of the Pediatric Endocrinology Department of İstanbul University Faculty of Medicine were enrolled in the study. Demographic and clinical characteristics of the patients are given in Table 1. To our knowledge, there are no standard normative data for urine levels of MMP2, MMP9, TIMP1, TIMP2, TGF-β1 in children by age group. For this reason, 24 healthy children (15 male, 9 female) were enrolled in the study as a control group. This study was approved by the İstanbul University of Local Ethics Committee (No: 2013/108) and written informed consent was obtained from the childrens' parents.

A standard physical examination was performed in all patients and blood samples were drawn for biochemical examination. Height and weight measurements of the patients were taken by the same auxologist according to standard methods. Body mass index (BMI) in kg/m^2 was evaluated according to the percentile curves of Turkish children and patients with a BMI above 95th percentile were considered obese (12). Standard deviation (SD) score (SDS) of BMI was calculated according to national data (12). Hypertension was defined as a systolic and/or diastolic blood pressure higher than the 95th percentile for age and gender (13).

Hemoglobin A1c (HbA1c) levels collected within the previous three months were collected from the patient

Table 1. Demographic and clinical characteristics of the patients

	Mean ± SD (range)
Age (years)	11.73 ± 3.82 (4.5-17.8)
Gender (female/male)	15/18
DM duration (months)	40.60 ± 25.5 (6.4-93.9)
HbA1c (%)	9.11 ± 2.17 (5.7-15.5)
Microalbuminuria (mg/g creatinine)	20.17 ± 47.51 (1.28-239.41)
Body mass index	19.32 ± 3.49 (13.72-26.65)
Standard deviation score of body mass index	0.08 ± 1.01 (-1.15-2.32)
Estimated glomerular filtration rate (mL/min/1.73 m^2)	157.46 ± 34.61 (107.25-303.32)
	n
Gender (female/male)	15/18
Pubertal status at first year (pubertal/prepubertal)	22/11
The status of metabolic control	
- Good (HbA1c 6.5-7.5%)	88
- Moderate (HbA1c 7.5-9%)	14
- Poor (HbA1c >9%)	11

HbA1c: hemoglobin A1c, SD: standard deviation, DM: diabetes mellitus

files. Estimated glomerular filtration rate (GFR) values were calculated by using the Schwartz formula (14). Urinary assessment and urine culture were performed to exclude urinary tract infection for each patient. None of the patients had urinary tract infection. In addition no patient had a record of urinary tract infection, urolithiasis or nephrotoxic drug usage in the past three months. Patients with a urine microalbumin to creatinine (uMA/Cr) ratio greater than 30 mg/g in at least two of the three urine specimens were considered microalbuminuric (15).

Urine samples were obtained to measure urine levels of MMP2, MMP9, TIMP1, TIMP2, TGF-β1, microalbumin and creatinine. The samples were centrifuged at 4 °C for 15 minutes at 4,000 x g. Until analyzed, the supernatants were stored at -80 °C. All processes were performed under uniform conditions in all children. The Abbott Architect c16000 (Illinois, USA) analyzer with original kits was used to measure uCr and uMA, with uMA expressed in mg/L and uMA/Cr expressed in mg/g. Urine levels of MMP2, MMP9, TIMP-1, TIMP-2, TGF-β1 were assessed by enzyme-linked immunosorbent assay (ELISA) technique. Urine MMP2, MMP9, TIMP1 and TIMP2 levels were analysed following the manufacturer's instructions, using Human MMP2 ELISA Kit (Cat no: YHB1973Hu), Human MMP9 ELISA Kit (Cat no:YHB1982Hu), Human TIMP-1 ELISA Kit (Cat no: YHB3003Hu), Human TIMP-2 ELISA Kit (Cat no: YHB3004Hu) and Human TGF-β1 ELISA Kit (Cat no: YHB3051Hu) purchased from YH Biosearch Laboratory (Pudong District, Shanghail, China). The intra-assay and the inter-assay coefficients of variation for MMP2, MMP9, TIMP1, TIMP2 and TGF-β were < 10% and < 12%, respectively. MMP2 and TIMP2 levels were expressed as ng/mL, MMP9 and TGF-β1 levels as ng/L. TIMP1 levels were expressed as pg/mL. The results were adjusted per unit of urine/Cr. Results of TGF-β1/Cr, MMP2/Cr, MMP9/Cr and TIMP2/Cr were expressed as ng/mg, and TIMP1/Cr as pg/mg. The same measurements were repeated after one year to determine whether urine levels of these markers altered in diabetic children with time.

Statistical Analysis

Statistical calculations were performed with IBM SPSS Statistics for Windows, Version 22.0. (IBM Inc., Armonk, NY, USA). Besides standard descriptive statistical calculations (mean, standard deviation, median and interquartile range), a t-test was employed in the comparison of two groups and in the assessment of first and second year values. Kruskal-Wallis test was used to compare subgroups of diabetic control and diabetes duration. Pearson correlation test was used in the correlations between variables. Statistical significance level was established at $p < 0.05$.

Results

Mean ± SD age was 11.73 ± 3.82 (range 4.5-17.8) years in the T1DM group and 11.6 ± 3.0 years in the controls. There was no statistical difference between the two groups regarding age and gender distribution ($p > 0.05$). Mean ± SD follow-up duration was 40.6 ± 25.5 (range 6.4-93.9) months. All patients were on intensive insulin treatment. Mean ± SD BMI of the patients was 19.32 ± 3.49 (range 13.72-26.65) and mean ± SD BMI SDS was 0.08 ± 1.01 (-1.15-2.32). Normal blood pressure was observed in all patients. Mean ± SD estimated GFR was 157.46 ± 34.61 mL/min/1.73 m^2 (range 107.25-303.32). Mean ± SD HbA1c was 9.11 ± 2.17% (range 5.7-15.5). Mean ± SD uMA/Cr was 20.17 ± 47.51 (range 1.28-239.41) mg/g Cr. Urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr, TGF-β1/Cr were not different in the patient and control groups ($p > 0.05$) (Table 2). There was also no significant difference between the results of the first and second year samples of the diabetes patients in these biomarkers ($p > 0.05$). None of these parameters were correlated to age, HbA1c, BMI and duration of T1DM. Interestingly, all parameters were negatively correlated to the age of onset of T1DM ($p < 0.05$) (Table 3). A positive correlation was found among urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr and TGF-β1/Cr ($p < 0.05$). Microalbuminuria was present in only three patients. Among the three patients with microalbuminuria, only one had higher values of the urine biomarkers than the patients group mean values.

The patients were divided into two subgroups according to duration of diabetes: 0-5 years (n = 19) and over 5 years (n = 14). There was no difference between the two groups according to urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr, TGF-β1/Cr values (Table 4). Also, the patients were divided into three groups depending on diabetic control as measured by HbA1c: good (n = 8), moderate (n = 14) and poor glycemic control (n = 11) (see Table 5). The urine biomarkers did not differ between the groups with good, moderate or poor glycemic control (Table 5).

Discussion

Since changes in the ECM are a significant pathogenetic mechanism in DN, we hypothesized that the onset of alterations in urine MMP2, MMP9 and TIMP1, TIMP2 may occur prior to appearance of microalbuminuria. We also expected this change in markers of renal fibrosis to become more prominent with time because kidney injury in DN is a progressive process. However, our results did not support our hypothesis. Urine MMP2/Cr, MMP9/Cr, TIMP1/Cr, TIMP2/Cr values were essentially similar in the patients and controls,

and they did not change over one year in the T1DM patients. From these results it seems that chronic changes in DN do not begin in the early stages of the disease.

The role of MMPs in the pathogenesis of DN is not fully understood. Although it has been demonstrated that dysregulation of MMPs occurs in DN, the reported results are contradictory (8). Decreased expression of MMP2 and MMP9 was reported in several experimental studies of DN, while other studies reported increased expression of MMPs (9,16,17). Additionally, it has been noted that while MMP2 knock-out mice show an exacerbation of

Table 2. Urinary biomarkers in the patients in the first and second years of onset *vs* the controls

	Control group	T1DM group first year	T1DM group second year	Controls vs T1DM first year	Controls vs T1DM second year	T1DM first vs second year
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)	p	p	p
MMP2/Cr ng/mg	0.403 ± 0.321	0.737 ± 1.125	0.539 ± 0.367	0.123	0.152	0.250
MMP9/Cr ng/mg	1.386 ± 1.041	2.418 ± 3.698	1.911 ± 1.317	0.147	0.110	0.340
TIMP1/Cr pg/mg	0.286 ± 0.237	0.478 ± 0.688	0.359 ± 0.252	0.182	0.276	0.245
TIPMP2/Cr ng/mg	0.035 ± 0.029	0.066 ± 0.097	0.072 ± 0.132	0.098	0.167	0.872
TGF-β1/Cr ng/mg	0.795 ± 0.608	1.145 ± 1.705	0.893 ± 0.628	0.324	0.561	0.309

MMP2/Cr: matrix metalloproteinase 2/creatinine, MMP9/Cr: matrix metalloproteinase 9/creatinine, TIMP1/Cr: tissue inhibitor of metalloproteinase 1/creatinine, TIMP2/Cr: tissue inhibitor of metalloproteinase 2/creatinine, TGF-β1/Cr: transforming growth factor-β1/creatinine, T1DM: type 1 diabetes mellitus, SD: Standard deviation

Table 3. Correlations of urine matrix metalloproteinase/creatinine and tissue inhibitor of metalloproteinases/creatinine with age of onset of the diabetes, with hemoglobin A1c, body mass index and diabetes duration

		Age of onset of diabetes	HbA1c	BMI	Diabetes duration
MMP2/Cr (ng/mg)	r	-0.461	-0.063	0.219	0.199
	p	**0.012**	0.749	0.254	0.300
MMP9/Cr (ng/mg)	r	-0.461	-0.043	0.245	0.222
	p	**0.012**	0.826	0.199	0.246
TIMP1/Cr (pg/mg)	r	-0.484	-0.076	0.214	0.205
	p	**0.008**	0.699	0.266	0.287
TIPMP2/Cr (ng/mg)	r	-0.422	-0.070	0.262	0.211
	p	**0.023**	0.724	0.170	0.272
TGF-β1/Cr (ng/mg)	r	-0.462	-0.025	0.217	0.199
	p	**0.012**	0.898	0.258	0.301

MMP2/Cr: matrix metalloproteinase 2/creatinine; MMP9/Cr: matrix metalloproteinase 9/creatinine, TIMP1/Cr: tissue inhibitor of metalloproteinase 1/creatinine, TIMP2/Cr: tissue inhibitor of metalloproteinase 2/creatinine, TGF-β1/Cr: transforming growth factor-β1/creatinine, HbA1c: hemoglobin A1c, BMI: body mass index

Table 4. The relationships between urine biomarkers and diabetes duration

	Control group (n = 24) (median)	Diabetes duration 0-5 years (n = 19) (median)	Diabetes duration > 5 years (n = 14) (median)	p
MMP2/Cr (ng/mg)	0.34	0.39	0.40	0.193
MMP9/Cr (ng/mg)	1.12	1.17	1.45	0.147
TIMP1/Cr (pg/mg)	0.21	0.28	0.28	0.187
TIMP2/Cr (ng/mg)	0.03	0.04	0.03	0.120
TGF-β1/Cr (ng/mg)	0.63	0.61	0.71	0.315

MMP2/Cr: matrix metalloproteinase 2/creatinine, MMP9/Cr: matrix metalloproteinase 9/creatinine, TIMP1/Cr: tissue inhibitor of metalloproteinase 1/creatinine, TIMP2/Cr: tissue inhibitor of metalloproteinase 2/creatinine, TGF-β1/Cr: transforming growth factor-β1/creatinine

Table 5. The relationships between urine biomarkers and diabetic control

	Control group (n = 24) (median)	Good glycemic control HbA1c: 6.5-7.5% (n = 8) (median)	Moderate glycemic control HbA1c: 7.5-9% (n = 14) (median)	Poor glycemic control HbA1c: > 9% (n = 11) (median)	p
MMP2/Cr (pg/mg)	0.34	0.52	0.43	0.35	0.319
MMP9/Cr (pg/mg)	1.12	1.53	1.43	1.33	0.531
TIMP1/Cr (pg/mg)	0.21	0.34	0.31	0.25	0.458
TIPMP2/Cr (pg/mg)	0.03	0.04	0.04	0.03	0.275
TGF-β1/Cr (pg/mg)	0.63	0.75	0.65	0.62	0.934

MMP2/Cr: matrix metalloproteinase 2/creatinine, MMP9/Cr: matrix metalloproteinase 9/creatinine, TIMP1/Cr: tissue inhibitor of metalloproteinase 1/creatinine, TIMP2/Cr: tissue inhibitor of metalloproteinase 2/creatinine, TGF-β1/Cr: transforming growth factor-β1/creatinine, HbA1c: hemoglobin A1c

DN, MMP9 knock-out mice show an attenuation of DN (18,19). Expression of TIMP1 and TIPM2 are increased in DN (8,9,20,21).

There are only a few studies evaluating urinary MMPs in patients with diabetes. McKittrick et al (22) evaluated urine activities of MMP2 and MMP9 in the urine of patients with T1DM and they found that urinary MMP9 did not differ between the patients and controls; our results are in concordance with these earlier findings. However, unlike our results, they reported an increase in the activities of MMP2 (22). Lauhio et al (23) demonstrated elevation of the urinary activity of MMP9 in adult patients with type 2 DM. However, their study group was quite different from our group. Most of their patients had macroalbuminuria and a diabetes duration longer than 10 years. Tashiro et al (24) evaluated urinary MMP9 in adult patients with type 2 DM who are different our study population. They did not find any differences in urinary MMP9 between normo/microalbuminuric patients and healthy controls, findings similar to our results. However, in this study, urinary MMP9 were found to be higher in macroalbuminuric patients. van der Zijl et al (25) evaluated urinary MMP2 and MMP9 levels in adult patients with type 2 DM and reported that urinary MMP9 levels were higher in the microalbuminuric group than in the controls, while there was no difference in MMP2 activity. Elevation of urinary MMP9 activity was found to be related to older age, longer duration of diabetes, high levels of HbA1c and increased blood pressure. Thrailkill et al (26) evaluated MMP2 in T1DM patients and found that MMP2 increased in the plasma and urine although they did not find any differences between patients and controls in TIMP1 and TIMP2 concentrations. Similarly to our results, when they evaluated the younger groups (< 18 years) they did not find any differences according to urine MMP2/Cr and total urine MMP2 concentrations (26). In a later study from the same group Thrailkill et al (27) reported elevation of urinary MMP9 in normoalbuminuric patients with T1DM with duration of diabetes being nine years, a disease duration longer than that of our study group. These studies suggest that the role of the clinical use of urinary MMPs is not fully understood. These differences between the studies may be due to the fact that the patient groups as well as the evaluation method of urine MMPs are quite different from one another. According to these studies, diabetes duration has a significant role on the alteration of urinary MMP2 and MMP9. Also this alteration appears to become more prominent in the later stages of DN. The mean duration of diabetes was only 3.5 years in our patients. ECM accumulation and mesangial expansion begin in the second stage of DN (11). Also, with the exception of three patients, our patients did not have microalbuminuria. We could not demonstrate any differences according to these biomarkers, probably because of the short duration of the diabetic state and because our patients had not yet reached the second and/or later stages of DN. Based on a few previous studies which demonstrated higher values of urine MMP2/Cr and MMP9/Cr in adult diabetic patients, we thought that these markers may increase with time as diabetic injury progresses (24,25,26,27). We also did not find any difference in the values of urinary MMP2/Cr, MMP9/Cr, TIMP/Cr and TIMP2/Cr at initial measurement and when measured a year later. These results show that urine levels of these markers do not change in the early phases of DN and cannot predict early progression of DN.

Some comorbid conditions other than diabetes mellitus such as renal scars, nephrotic syndrome, focal segmental glomerulosclerosis, pancreatic cancer and chronic kidney failure may also affect urine MMP2 and MMP9, TIMP1 and TIMP2 and TGF-β1 concentrations (28,29,30,31,32,33). However, our diabetic patients did not have any known comorbid disorders.

TGF-β1 is considered as the most important cytokine in glomerular and tubulointerstitial fibrosis (34). Additionally, expression of TGF-β1 is increased with hyperglycemia, thus TGF-β1 is involved in various pathways having a role

in the pathogenesis of DN (34). Furthermore, MMPs not only cleave ECM proteins but also target some non-ECM proteins, including TGF-β1, and activation of the TGF-β/Smad signal pathway which is accompanied by MMP2 and MMP9 upregulation (9,10). Therefore, in addition to urinary MMPs, we evaluated urinary TGF-β1 in our patients. Again TGF-β1 was not increased in our patients. In fact, this result was consistent with our results for MMP2 and MMP9. These results suggest that chronic fibrotic changes may not become apparent and these markers do not increase in the urine in the early phases of diabetic kidney injury.

Poor metabolic control, higher BMI, longer duration of disease and onset of diabetes at puberty have been identified as risk factors for DN. Therefore, we evaluated the correlations between these biomarkers and HbA1c, BMI, duration of T1DM and age of onset of T1DM. Only age of onset was negatively correlated with all these biomarkers of renal fibrosis. This finding suggested that among the indicators of poor prognosis of T1DM in terms of renal damage, the most important determinant seems to be the age of onset of the diabetic state.

Study Limitations

The limitations of our study are the relatively small sample size with only three microalbuminuric patients. Thus, we were not able to compare microalbuminuric and normoalbuminuric patients for these markers. We did not perform kidney biopsies and thus we are not in a position to make any statements on the pathological DN stage of our patients. Despite these limitations our study has yielded important results. One of the most significant findings was that there were no difference between patients and controls according to these biomarkers and this finding did not change after one year of follow-up. These findings weaken the role of these biomarkers in the detection of early diabetic kidney injury. In this respect, future studies with longer follow-up and larges samples in a pediatric age group are needed to highlight this issue.

Conclusion

In conclusion, our findings suggest that urinary biomarkers of fibrosis are not increased in diabetic children without microalbuminuria even when disease duration is longer than five years.

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Ethics

Ethics Committee Approval: The study was approved by the İstanbul University of Local Ethics Committee (Protocol number: 2013/108).

Informed Consent: Written informed consent was obtained from the childrens' parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

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Management of Thyrotoxicosis in Children and Adolescents: A Turkish Multi-center Experience

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What is already known on this topic?

Graves' disease is the most common cause of thyrotoxicosis in children and adolescents, as in adults. Management of thyrotoxicosis in children and adolescents is controversial and often unsatisfactory. There are no published data on clinical features and treatment outcomes of Turkish children and adolescents with thyrotoxicosis.

What this study adds?

Using anti-thyroid drugs (ATDs) with the hope that the patients will enter a remission over time is the generally accepted first-line approach in Turkey. This study shows that this approach achieved low remission rates, a result which was consistent with previous studies. Surgery is preferred to radioactive iodine ablation for patients with poor disease control and those experiencing adverse events associated with ATDs in Turkish children and adolescents with Graves' disease.

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Abstract

Objective: To determine the demographic and biochemical features of childhood and juvenile thyrotoxicosis and treatment outcome.
Methods: We reviewed the records of children from 22 centers in Turkey who were diagnosed with thyrotoxicosis between 2007 to 2017.
Results: A total of 503 children had been diagnosed with thyrotoxicosis at the centers during the study period. Of these, 375 (74.6%) had been diagnosed with Graves' disease (GD), 75 (14.9%) with hashitoxicosis and 53 (10.5%) with other less common causes of thyrotoxicosis. The most common presenting features in children with GD or hashitoxicosis were tachycardia and/or palpitations, weight loss and excessive sweating. The cumulative remission rate was 17.6% in 370 patients with GD who had received anti-thyroid drugs (ATDs) for initial treatment. The median (range) treatment period was 22.8 (0.3-127) months. No variables predictive of achieving remission were identified. Twenty-seven received second-line treatment because of poor disease control and/or adverse events associated with ATDs. Total thyroidectomy was performed in 17 patients with no recurrence of thyrotoxicosis and all became hypothyroid. Ten patients received radioiodine and six became hypothyroid, one remained hyperthyroid and restarted ATDs and one patient achieved remission. Two patients were lost to follow up.
Conclusion: This study has demonstrated that using ATDs is the generally accepted first-line approach and there seems to be low remission rate with ATDs in pediatric GD patients in Turkey.
Keywords: Graves' disease, hashitoxicosis, thyrotoxicosis, antithyroid drug, radioactive iodine, total thyroidectomy

Introduction

Thyrotoxicosis is characterized by excess circulating thyroid hormones, irrespective of the source (1). Juvenile thyrotoxicosis can have various clinical manifestations, including adverse effects on growth and development and may cause pronounced neuropsychological manifestations (2). Graves' disease (GD) is the most common cause of thyrotoxicosis in childhood (2). Incidence of GD increases with age, but is less common in children than in adults. An increase in the incidence rate of childhood GD has been reported by several studies (3,4,5,6).

Although a few treatment guidelines have been published, management of juvenile thyrotoxicosis is controversial and often unsatisfactory (7,8,9). Treatment options include anti-thyroid drugs (ATDs), radioactive iodine therapy (RAI) and surgical interventions. Most patients diagnosed with thyrotoxicosis are initially treated with ATDs, as there is a chance of remission with ATD therapy, although optimal treatment duration is controversial. Patients with thyrotoxicosis who do not respond to medical therapy or who have adverse reactions to ATDs must be managed with a second line treatment, such as RI or thyroid surgery. However, all of the treatment options have distinct advantages and disadvantages (10). There are novel approaches such as using rituximab, thyroid stimulating hormone (TSH) receptor (TSHR) specific peptides or monoclonal TSHR-blocking antibodies with the aim of ameliorating the immune dysfunction seen in GD. At present, there are very few ongoing studies in this area and some completed studies have shown conflicting results (11,12,13).

Remission has been reported in approximately one third of children with GD and relapse occured in half of the patients after remission was achieved (7,14,15,16,17,18,19,20,21). Remission rates are lower and relapse rates are higher in children than in adults (22). However, the definitions of remission and relapse also differed among studies.

In this study, we aimed to assess the demographic and biochemical features of children and adolescents with thyrotoxicosis, the preference of physicians for treatment options in juvenile thyrotoxicosis and management outcome in these patients.

Methods

In December 2017, an online invitation was sent to all pediatric endocrinology departments across Turkey asking them, if they were willing, to review their patients under the age of 18 years who presented between 2007 and 2017 with elevated free thyroxine (fT4) concentrations, above the upper limit of the local reference range. Thus, the clinical and biochemical features, treatment preferences and outcome in relation to treatment were documented by analysis of these patient records returned by 22 pediatric endocrinology departments responding positively to the study invitation. Thyrotoxicosis was defined as an elevated fT4 and/or free tri-iodothyronine (fT3) concentration, above the upper limit of the local reference range, together with suppressed TSH levels, below the lower limit of the local reference range.

Data on the clinical features of the sample at first presentation included gender, age, clinical symptoms and anthropometric measurements which consisted of weight in kilograms to the nearest 0.1 kg, height in centimetres to the nearest 1 mm and body mass index (BMI) calculated by the formula weight in kilograms divided by the square of height in meters. Standard deviation scores (SDS) of weight, height and BMI of patients were calculated by using reference values for Turkish children (23).

J Clin Res Pediatr Endocrinol
2019;11(2):164-172

Biochemical data collected included: serum alanine transaminase (ALT); aspartate transaminase (AST); TSH; fT4; fT3; anti-thyroid peroxidase (anti-TPO) antibodies; anti-thyroglobulin (anti-Tg) antibodies and anti-TSH receptor antibodies (TRAb). Commercial kits were used by the participating centers to assay these hormones and antibodies. Because different commercial kits had been used to assay TRAb among clinics and sometimes in the same clinic, TRAb/upper limit for TRAb ratio (TRAb ratio) was used in the data analysis and a TRAb ratio >1.0 was accepted as an elevated TRAb.

GD was defined as thyrotoxicosis with either elevated TRAb or clinical signs or findings suggestive of GD such as thyroid ophthalmopathy or diffuse uptake of radioisotope on thyroid scan or persistent thyrotoxicosis of more than two years standing without any other cause. Presence of ophthalmopathy was based on clinical reports of physicians. The thyrotoxic phase of chronic lymphocytic thyroiditis (hashitoxicosis) was defined as thyrotoxicosis with the presence of at least one of the anti-TPO or anti-Tg antibodies (based on the reference range of locally used commercial kits) in patients without any other cause.

The preferred approach to ATD treatment (block-and-replace or dose reduction), type of ATD [methimazole (MMI) or propylthiouracil (PTU)], duration of therapy, side effects and date/age of stopping treatment and if the patient subsequently relapsed and underwent RAI treatment or surgery were also recorded. Remission was defined as biochemical euthyroidism at the time of collecting data after cessation of ATD for at least three months.

Patients were classified by their final diagnosis and the results were expressed in percentages. Patients with GD or hashitoxicosis have been analyzed in greater detail. Insufficient data, a diagnosis of subclinical hyperthyroidism (low serum TSH, but normal fT4 and fT3 concentration) and gestational thyrotoxicosis were considered as exclusion criteria.

Ethical approval for this study was obtained from Ethical Committee of the Fırat University Medical School (05.10.2017-0015). Informed consent was not obtained from patient's parents because this paper does not report on experimental protocol and all data analyzed were collected as part of routine diagnosis and treatment options.

Statistical Analysis

Continuous variables were described as medians and ranges or means ± SD. Intergroup comparisons were performed using the Mann-Whitney U or Student's t-test. χ^2 test was used for categorical variables. Multiple regression analysis was used to determine whether age, sex, weight SDS, height SDS, BMI SDS, fT4, fT3, TRAb ratio at diagnosis and duration of ATD had independent associations with occurring relapse in the patients' group in which ATD therapy was stopped for possible remission. Statistical significance was assumed if $p<0.05$ and all analyses were performed using IBM SPSS Statistical Software (version 22, SPSS Inc., Chicago, IL, USA).

Results

Case records of 514 patients were received and 11 patients were excluded due to: insufficient data (n = 2); duplication (n = 4); diagnosis of subclinical hyperthyroidism (n = 4); or gestational thyrotoxicosis (n = 1). Thus, between 2007 and 2017, the medical records of 503 eligible children from 22 institutions in 12 different cities were reviewed. Of the 503 patients who were included in the study, 375 (74.6%) were diagnosed with GD and 75 (14.9%) patients had hashitoxicosis. The diagnosis in the remaining patients were thyroid hormone receptor-beta mutation in 22 (4.4%), subacute thyroiditis in four (0.8%), toxic nodular goiter in four (0.8%), neonatal GD in three (0.6%), papillary thyroid carcinoma in two (0.4%) and 18 patients (3.6%) who were not assigned a specific diagnosis. Diagnosis of patients with GD was mostly based on positive results for TRAb (81.6%) or elevated radioactive iodine or technetium (^{99m}Tc) uptake or observed ophthalmopathy (9.6%). Only 32 patients (8.8%) who were negative for TRAb or had no TRAb measurement had been assigned a diagnosis of GD based on clinical findings during follow up.

The most common reported presenting complaints among patients with GD or hashitoxicosis were tachycardia and/or palpitations, weight loss and excessive sweating. Distribution and frequencies of complaints at presentation were similar between groups, except that goiter and ocular symptoms were more frequent in patients with GD (see Table 1).

Clinical features of GD and hashitoxicosis are shown in Table 2. There was no significant difference between the two groups with regard to gender distribution, age at diagnosis, weight SDS, height SDS and BMI SDS. Patients with GD had higher median fT4 and fT3 levels. The mean starting dose of ATDs was significantly higher and duration of ATD therapy was significantly longer in patients with GD ($p<0.05$). With respect to medical treatment options, the vast majority of the patients were treated with MMI as ATD (89.2% and 91.3% for GD and hashitoxicosis, respectively) and subjected to a dose reduction regimen. The mean starting dose of ATDs was significantly higher in the GD than hashitoxicosis groups (Table 2).

Table 1. The most frequently reported presenting complaints among patients with Graves' disease and hashitoxicosis. Data are given as n (%)

Complaints	Graves' disease n = 375	Hashitoxicosis n = 75
Tachycardia and/or palpitation	169 (45.1%)	29 (38.7%)
Weight loss or no weight gain	106 (28.3%)	15 (20.0%)
Excessive sweating	105 (28.0%)	18 (24.0%)
Swelling in the neck (goiter)	74 (19.7%)	9 (12.0%)
Hand tremor	65 (17.3%)	16 (21.0%)
Irritability or nervousness	64 (17.1%)	15 (20.0%)
Ocular symptoms	38 (10.1%)	1 (1.0%)
Weakness	36 (9.6%)	9 (12.0%)
Hair loss	19 (5.1%)	4 (5.3%)
Intolerance to heat	18 (4.8%)	4 (5.3%)
Sleep problems	15 (4.0%)	2 (2.7%)
Restlessness	10 (2.7%)	-
Headache	4 (1.1%)	2 (2.7%)
Decreased school performance	4 (1.1%)	-
Family history of Graves' disease	3 (0.8%)	-
Incidental	12 (3.2%)	14 (18.7%)

Outcome of patients with GD is shown in Figure 1.

Five of 375 (1.3%) patients with GD did not receive ATDs. These patients were managed only with beta-blockers. Approximately 62% (231/370) of patients who received ATDs initially were continued on ATD at the time of the last contact for data collection. The median (range) duration of ATD therapy in these patients was 16.1 (0.3-99.6) months. ATDs were stopped in approximately one-third of patients (112/370; 30.2%) for possible remission. In 58 patients, the trial of cessation of ATD was initiated in the first three years of treatment and 46 of these patients (79.3%) remained in remission. In 54 patients, requirement of ATD persisted and a trial of cessation of ATD attempted beyond three years of treatment. Of this group, 19 patients (35.2%) remained in remission. Forty-seven of 112 patients who relapsed after stopping ATD therapy for possible remission. Of these 34 remained on ATD, six underwent total thyroidectomy and all became hypothyroid. Seven of this group received radioiodine, four developing hypothyroidism and three remaining hyperthyroid. Two of three who remained hyperthyroid remained on ATD and the other received a second dose of radioiodine and thereafter became hypothyroid (Figure 1). The median (range) interval between ATD treatment cessation and relapse of hyperthyroidism was 6.0 (0.7-60.8) months. The cumulative remission rate was 17.6%

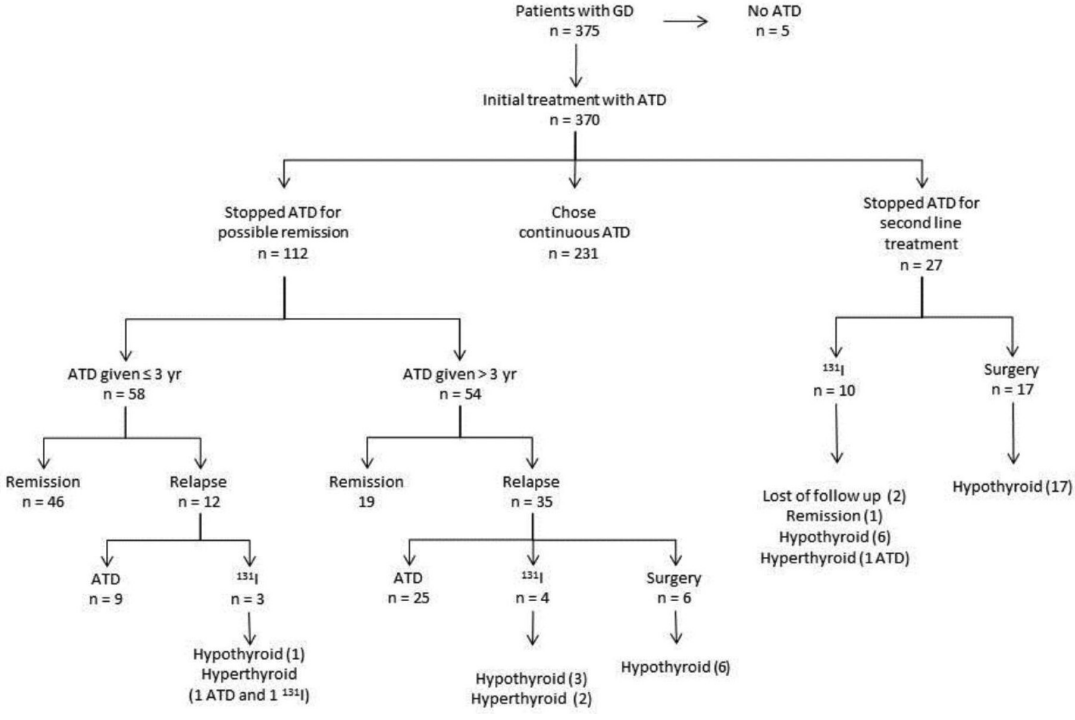

Figure 1. Outcome in 375 Turkish children and adolescents with Graves' disease followed between 2007-2017

ATD: anti-thyroid drugs, GD: Graves' disease

in 370 patients with GD who received ATDs for initial treatment and they were treated for a median (range) of 22.8 (0.3-127) months. Clinical and biochemical features of patients with GD who stopped ATDs for remission or relapsed afterwards are shown in detail in Table 2. Patients who did not achieve remission had a lower BMI SDS at diagnosis, higher initial fT4 and fT3 concentrations and longer duration of ATD therapy (Table 3). However, these variables were not identified as independent predictors of relapse by regression analysis (Table 4). Four patients who had high AST and/or ALT levels did not remain euthyroid and relapsed after discontinuation of ATD.

Twenty-seven patients with GD (7.3%) received second-line treatment (surgery or radioactive iodine ablation) for poor disease control and adverse events associated with ATD (Figure 1). Total thyroidectomy was performed in 17 patients with no recurrence and all these patients became hypothyroid without severe complications. Ten patients received radioiodine; six became hypothyroid, one remained hyperthyroid and started taking ATD again, one achieved remission and outcome is unknown in two patients due to loss of follow up. There was no significant difference in the age of patients at second-line treatment time between patients who received radioiodine [16.1 ± 2.8 years (range 10.8-19.4)] and patients who underwent surgery [15.2 ± 2.4 years (range 9.3-19.6)].

Six of 75 patients with hashitoxicosis did not receive ATDs and were managed solely with beta-blockers. ATDs were stopped after a mean period of 9.3 ± 6.3 months (range 0.7-22.5) in 32 patients with hashitoxicosis for possible remission and all of them achieved remission. The remaining 37 patients were continuing to use ATDs at their last visits with a mean duration of treatment of 8.0 ± 6.9 (range 0.3-34.0) months.

Discussion

This report is possibly one based on the second largest group of patients with childhood and juvenile thyrotoxicosis to date in the literature.

Table 2. Clinical and biochemical features of patients with Graves' disease and hashitoxicosis

	GD	Hashitoxicosis	p value
Number	375	75	-
Gender (F/M)	284/91	64/11	0.072
Age at diagnosis, mean ± SD median (range) (years)	12.6 ± 3.6 13.4 (1.2-17.9)	13.3 ± 3.6 13.6 (4.0-17.9)	0.130
Weight SDS (mean ± SD)	-0.29 ± 1.37	-0.16 ± 1.26	0.447
Height SDS (mean ± SD)	0.07 ± 1.24	-0.18 ± 1.30	0.117
BMI SDS (mean ± SD)	-0.44 ± 1.34	-0.11 ± 1.36	0.055
Ophthalmopathy (%)	117 (31.2)	N/A	-
Positive for TRAb (%)	306 (81.6)	N/A	-
TRAb/TRAb upper limit ratio, median (range)	4.1 (0.06 - >40.00)	N/A	-
fT4 (ng/dL), median (range)	3.65 (1.69 - >7.70)	2.50 (1.69 - >7.70)	-
fT3 (ng/dL), median (range)	13.60 (1.40 - >30.00)	8.07 (1.14 - >30.00)	-
Positive for anti-TPO and/or anti-Tg (%)	309 (82.4)	N/A	-
Elevated AST and/or ALT n (%)	27 (7.2)	4 (5.3)	-
Diagnosed by scintigraphy n (%)	87 (23.2)	8 (10.6)	-
Dose reduction/block and replace	290/88	65/5	<0.001
Preferred antithyroid drug, MMI/PTU	330/40	63/6	0.003
Starting dose of MMI (mg/kg/day) mean ± SD (range)	0.46 ± 0.22 (0.07-1.51)	0.39 ± 0.20 (0.08-1.05)	0.017
Starting dose of PTU (mg/kg/day) mean ± SD (range)	4.92 ± 1.80 (1.79-10.13)	2.83 ± 0.73 (1.90-3.45)	<0.001
Duration in months of ATD therapy mean ± SD median (range)	27.5 ± 22.8 22.8 (0.3-127.0)	9.0 ± 7.5 7.7 (0.3-34.0)	<0.001

GD: Graves' disease, F: female, M: male, SD: standard deviation, BMI: body mass index, SDS: standard deviation scores, TPO: thyroid peroxidase, Tg: thyroglobulin, TRAb: anti-thyroid stimulating hormone receptor antibodies, fT4: free thyroxine, fT3: free tri-iodothyronine, AST: aspartate transaminase, ALT: alanine aminotransferase, MMI: methimazole, PTU: propylthiouracil, ATD: anti-thyroid drugs, N/A: not applicable

Table 3. Clinical and biochemical features of patients with Graves' disease who stopped anti-thyroid drug treatment for possible remission resulting in achieved remission or relapse

	Remission	Relapse	p value
Number	65	47	-
Gender (F/M)	50/15	37/10	0.821
Age at diagnosis, mean ± SD, median (range) (years)	12.2 ± 3.6 12.9 (1.4-17.8)	12.4 ± 3.2 13.3 (3.1-17.1)	0.664
Weight SDS, mean ± SD	-0.09 ± 1.47	-0.50 ± 1.22	0.111
Height SDS, mean ± SD	-0.13 ± 1.32	-0.09 ± 1.07	0.340
BMI SDS, mean ± SD	-0.07 ± 1.35	-0.66 ± 1.11	0.013
Ophthalmopathy, n (%)	16 (24.6%)	12 (25.5%)	0.912
TRAb ratio, median (range)	2.67 (0.16 - 35.00)	3.84 (0.26 - > 40.00)	-
fT4 (ng/dL), median (range)	3.39 (1.70 - > 7.70)	4.50 (1.70 - > 7.70)	-
fT3 (ng/dL), median (range)	10.20 (2.90 - > 30.00)	14.74 (1.40 - > 30.00)	-
Positive for anti-TPO and/or anti-Tg, n (%)	56 (86.2%)	43 (91.5%)	0.384
High AST and/or ALT level, n (%)	0 (0.0%)	4 (8.5%)	0.029
Dose reduction/block and replace	45/20	33/14	0.911
Duration of ATD therapy (months), mean ± SD, median (range)	29.8 ± 18.4 25.5 (3.1-77.7)	51.8 ± 26.3 49.8 (10.4-127.0)	< 0.001

F: female, M: male, SD: standard deviation, SDS: standard deviation scores, BMI: body mass index, anti-TPO: anti-thyroid peroxidase, anti-Tg: anti-thyroglobulin, TRAb: anti-thyroid stimulating hormone receptor antibodies, fT4: free thyroxine, fT3: free tri-iodothyronine, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ATD: anti-thyroid drugs

Table 4. Multiple regression analysis in the patient group in which anti-thyroid drugs therapy was stopped for possible remission, with occurring relapse as the dependent variable

Independent variable	Beta	p
Age at diagnosis	0.057	0.629
Gender	0.021	0.823
Weight SDS	0.686	0.356
Height SDS	-0.162	0.630
BMI SDS	-0.866	0.162
fT4	-0.006	0.972
fT3	0.031	0.867
TRAb ratio	0.148	0.234
Duration of ATD	-0.009	0.929

SDS: standard deviation scores, TRAb: anti-thyroid stimulating hormone receptor antibodies, fT4: free thyroxine, fT3: free tri-iodothyronine, ATD: anti-thyroid drugs, BMI: body mass index

GD is the most common cause of the thyrotoxicosis in children and adolescents accounting for more than 95% of cases (2). However, the occurrence of GD was much lower in our series, accounting for approximately three quarters of all cases. The second most frequent cause of juvenile thyrotoxicosis is hashitoxicosis and its prevalence was reported to range from 0.5% to 22% in different studies (9). Our results were similar to those reported in a recent study from Scotland in which 19.6% of patients with thyrotoxicosis were classified as hashitoxicosis (20). Due to the similarity in the most commonly encountered presenting complaints in patients with GD and hashitoxicosis, the distinction between the two may be difficult, as was observed in this study. Additionally, most patients with hashitoxicosis may not have been diagnosed, probably because of its relatively short thyrotoxic course. The hallmark of GD is the presence of TRAb while patients with hashitoxicosis will typically have anti-TPO and/or anti-Tg (24,25). In the present study, most of the patients with GD were tested for the presence of TRAb and we believe this is one of the strong features of this study. On the other hand, TSHR antibodies may also be present in the sera of patients with hashitoxicosis and some experts consider GD and hashitoxicosis to be basically the same disorder at different ends of a continuum (26).

Graves' ophthalmopathy is an inflammatory disease of the eye and orbital tissues, and its prevalence has been previously reported to range from 17.1-67.6% in children and adolescents with GD (21,27). A relatively high prevalence (86.8%) of eye signs in children with GD was reported in a recent study (20). This finding may be due to inclusion of mild signs such as lid lad in the analysis rather than assessing true proptosis. In the present study, ophthalmopathy was reported in approximately in one third of patients with GD and this finding is in concordance with previous studies. However our data on ocular findings were not based on a

standard protocol for definition of Graves' ophthalmopathy, but we suppose that reported cases presumably are children with moderate or severe ophthalmopathy.

Treatment options for GD in children include ATDs, RAI therapy and surgical thyroidectomy, and each of these treatment approaches is associated with specific risks (2). ATDs are generally the accepted option for initial treatment of GD in children and adolescents in most countries. In this study, almost all patients with GD received ATDs, with most being prescribed MMI, with the exception of a few patients who had been treated with beta-blockers. PTU is not recommended for use in children because of its potential severe hepatotoxicity (28). Thus, there was no patient who received PTU as initial treatment in this patient group. Despite the presence of various definitions of remission and a wide variety of ATD treatment durations from two years and beyond, remission rates after ATD withdrawal are reported to vary from 11% to 49% (7,19,21,22,29). In the present study, remission was achieved in 58.0% of patients at the time when their anti-thyroid treatment was ceased for possible remission. This relative high remission rate should be interpreted with caution due to the retrospective nature of this study and to the fact that there was no standard protocol applied to all patients in this study. The cumulative remission rate was 17.6% when remission rate in all patients who received ATDs for initial treatment was estimated, which is consistent with the literature.

In several studies, a range of prognostic factors have been identified as being associated with lower remission chance in children and adolescents with GD (22,30). These studies have reported that ethnicity, age, pubertal status, BMI (SDS), goiter volume, initial severity (higher fT4 concentration and TRAb levels) and presence of other autoimmune conditions are prognostic factors (14,15,16,31). However, similar to a number of other studies (6,7,17,32), no clinical variable that is constantly associated with a definite outcome was identified in our cohort. The major limitation of these studies was that almost all, including this one, are retrospective. Kaguelidou et al (16) have published the only prospective study which reported that the risk of relapse was higher in very young patients, in patients of non-Caucasian origin and those with high levels of serum TRAb and fT4. In our study, a higher remission rate in patients who received ATDs for less than three years indicates that patients who will achieve remission may be predicted, based on follow up of thyroid function tests and requirement of lower doses of ATDs. Thus, a trial of cessation of ATDs can be tried at an earlier time in such patients.

If remission is not achieved or relapse or severe side-effects occur during ATDs therapy, radioiodine ablation and surgical thyroidectomy are available treatment modes for second line approach in patients with GD. Thyroidectomy is an effective treatment for GD (8). In this study we found that this method had been used slightly more frequently than radioiodine ablation. Various studies reported that hypothyroidism occurs in nearly all children who undergo total thyroidectomy (20,21,33,34). Our observations were concordant with these studies.

In the past, ^{131}I therapy was considered to be contraindicated in children. However, according guidelines from Japan and the American Thyroid Association, ^{131}I therapy can be performed with caution (8,9). Due to the ease of administration of RAI there is a trend towards permanent therapy (10). Remission rates vary due to variability in the dose of ^{131}I used. While many patients are successfully treated with ^{131}I therapy, in approximately one third of patients remission is not achieved or relapse occurs (30). According to our data, 11 of the 17 children who were treated with ^{131}I achieved biochemical remission. Fears about radioiodine ablation, compounded by the lack of access to RAI may have contributed to the relative low rate of ^{131}I therapy in children with GD in this series.

Study Limitations

Our study has potential limitations and strengths. The major limitation of this study is its retrospective nature and the lack of a globally accepted standard protocol for management of thyrotoxicosis in children and adolescents. In addition, adverse events associated with ATD treatment, thyroidectomy or radioactive iodine ablation were not included in the data collected for this study. The lack of detailed information about side effects hampered interpretation of the adverse events and treatment preferences. The strengths of this study are inclusion of data from multiple centers throughout Turkey and its relatively large sample size when compared with previous studies.

Conclusion

In conclusion, clinical manifestations and laboratory findings of patients with GD and hashitoxicosis were found to be essentially similar. A positive TRAb result, elevated radioactive iodine (or ^{99m}Tc) uptake and ophthalmopathy are the key features for diagnosis of GD. Although there is no optimal accepted treatment for GD, we have observed that initial treatment with ATDs and using total thyroidectomy and ^{131}I therapy as a second line treatment for permanent therapy is a generally accepted approach for treatment of patients with GD.

Ethics

Ethics Committee Approval: Ethical approval for this study was obtained from Ethical Committee of the Firat University Medical School (05.10.2017-0015).

Informed Consent: Informed consent was not obtained from patient's parents because this paper does not report on experimental protocol and all data analyzed were collected as part of routine diagnosis and treatment options.

Peer-review: Externally and internally peer-reviewed.

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Evaluation of Unfavorable Cardiovascular and Metabolic Risk Factors in Children and Young Adults with Haemophilia

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What is already known on this topic?

Patients with haemophilia were reported to have a reduced cardiovascular mortality due to a protective effect of having lifelong deficiency of factor 8 or 9. However, there is increasing evidence that this condition does not appear to be preventive.

What this study adds?

Cardiovascular and metabolic risk factors like overweight/obesity, elevated blood pressure/hypertension, prediabetes/diabetes and dyslipidaemia can be detected from early ages in patients with haemophilia.

Abstract

Objective: Increased risk of unfavorable cardiovascular risk factors has been recognised in ageing patients with haemophilia (PwH), but needs further studies in younger patients. The purpose of this study was to assess obesity, hypertension (HT), metabolic variables, insulin resistance and metabolic syndrome in young PwH.

Methods: Forty-eight haemophilia A and B patients and 35 age and sex matched healthy controls were included in the study. Anthropometric measurements, blood pressure (BP), fasting glucose and insulin levels, serum lipids and diet were evaluated. The metabolic syndrome was defined according to the criteria of the International Diabetes Federation for pediatric and adult age groups.

Results: The mean age of PwH was 21 ± 9 years (range, 6-40 years). Of those ≥18 years, 46% were were obese/overweight while there were no obese/overweight cases in the <18 year-old patients. Obesity was more prevalent in PwH with arthropathy (p = 0.017). Seven percent of the PwH between 10 and 18 years-old and 25% of those ≥18 years had metabolic syndrome. There was no difference in metabolic syndrome frequency between PwH and controls >10 years-old (19.5% *vs* 10% respectively, p = 0.34). Fifty percent of the PwH ≥18 years-old had elevated BP or HT. Fasting blood glucose levels of PwH were significantly higher compared to controls (p = 0.02).

Conclusion: Our study showed that obesity, HT and metabolic syndrome are frequent problems, especially in PwH with arthropathy. Early prevention and management of overweight, obesity and their sequelae must be addressed in clinical practice in order to maximize the overall health of the haemophilia population.

Keywords: Haemophilia, obesity, hypertension, metabolic syndrome

Introduction

In recent years, cardiovascular and metabolic risk has been defined in the ageing population of haemophiliacs. Studies in this area in young patients with haemophilia (PwH) are scarce (1,2). With increasing life expectancy of heamophilia patients, mortality and risk determination due to cardiovascular diseases has become an issue. While cardiovascular mortality was reported to be reduced in PwH due to a protective effect of having lifelong deficiency of factor 8 or 9 (3,4), there is an increasing evidence that this condition does not appear to prevent cardiovascular disease (5). Furthermore, Sun et al (2) recently demonstrated a

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significantly inferior microvascular endothelial function in haemophilia patients compared to healthy controls. Besides atherosclerosis, other established risk factors including obesity, hypertension (HT), dyslipidaemia, diabetes mellitus (DM) and family history for cardiovascular diseases are also known to play a crucial role in mortality and morbidity of these patients (1). Recently, Limjoco and Thornburg (6) studied these risk factors in a young haemophiliac population, aged 5-20 years, and identified modifiable risk factors for cardiovascular diseases.

Severe obesity in children and young adults is known to be associated with an increased prevalence of cardiometabolic risk factors, particularly among boys and young men (7). Haemophilia is characterized by progressive arthropathy, functional impairment and chronic joint pain. These are all barriers towards engagement in physical activity and may limit an individual's ability to maintain a healthy weight (8,9). Therefore, obesity is a health issue in PwH as well as an aggravating factor for cardiometabolic and joint health (9).

Prevalence of HT in adults with moderate to severe haemophilia has been shown to be increased (10). Alperstein et al (11) reported an increased prevalence of HT in a hospitalized pediatric hemophilia population compared to a pediatric healthy male population, although this was not statistically significant (1.52% vs. 1.22%, $p = 0.26$). Recently, Limjoco and Thornburg (6) reported high rates of overweight and obesity, (pre) HT and abnormal lipid levels in children and young adults with haemophilia.

The primary aim of this study was to assess obesity, HT, metabolic variables, insulin resistance and metabolic syndrome in children and young adults with haemophilia. We hypothesized that increased risk for cardiometabolic diseases could start from younger ages in PwH.

Methods

Study Design

This cross-sectional study was conducted in the Department of Pediatric Hematology and Oncology of Cerrahpaşa Medical Faculty and Oncology Institute of İstanbul University, between February 2010 and November 2010. Forty-eight PwH and 35 age and sex matched healthy controls were included. The study was approved by İstanbul Clinical Research Ethics Committee No:1 (No: C-009/2010). Informed consent was obtained from parents for age groups 6-12 years, from both subjects and parents for age groups 12-18 years, and from subjects only in those older than 18 years, in accordance with the Declaration of Helsinki.

Patients

During the regular outpatient clinic visit for hemophiliac patients, consecutive patients were asked to participate in the study. Forty-eight male patients, aged 6-40 years, with hemophilia A and B were included in the study irrespective of the severity of their disease. Patients with a coagulation factor level of less than 1% of normal were classified as severe, 1-5% of normal were classified as moderate, and 5-40% of normal were classified as mild (12). The past medical records of the participants were examined. Age of diagnosis, annual spontaneous or traumatic bleeding rate, annual factor consumption, presence of chronic arthropathy, whether physiotherapy or home exercise was undertaken, sports and dietary habits, presence of other systemic diseases and/or other complications of haemophilia, such as carrier status of HBV, HCV, HIV or presence of inhibitor were recorded. Collected data items also included self-reported family history for DM, HT, coronary artery disease (CAD) (male <55 years, female <65 years) and dyslipidaemia. The annual factor consumption per kilogram of body weight (in international units/kg) was determined for each patient for the 12-month period prior to enrollment. Factors used for elective interventional and surgical procedures were not taken into account. Patients with a history of known cardiovascular disease were not included in the study. Blood samples were taken as part of the clinical follow-up of patients.

Control Group

The control group consisted of 35 age-matched, random male subjects, with no history of congenital or acquired bleeding disorder, cardiovascular disease or chronic disease, who presented to the outpatient clinics of the pediatric or internal medicine departments. Age, sports and dietary habits, self-reported family history for DM, HT, CAD (male <55 years, female <65 years) and dyslipidaemia were recorded.

Assessment of exercise and nutritional status: In the study and control groups, for individuals under 18 years, at least three days and 30 minutes regular exercise a week; for individuals aged 18 years and older, at least two days and 30 minutes a week were classified as those who perform regular exercise and others were classified as non-performing.

Dietary intake was evaluated by an experienced dietitian using a 3-day food record. Subjects were given detailed oral and written instructions regarding the completion of a 3-day food record, consisting of two midweek days and one weekend day. In order to determine the amounts of

consumed foods correctly, information was given about measuring cups such as water glass, tea glass, teaspoon, tablespoon, serving spoon and bowl. Energy and nutrient intake was analyzed by a computerized food analysis program adapted for our country (BeBis4 software program, Turkish version, Stuttgart, Germany) and evaluated according to the recommendations of the Turkish Dietary Guidelines (13). A percentage level of sixty-six or less of the references was used as the criterium for inadequate nutritional intake (14). Over 300 mg daily cholesterol intake was considered as high intake.

Anthropometric measurements: All participants underwent a complete physical examination, including standardized measurement of weight, height and waist circumference, in duplicate. Body mass index (BMI) standard deviation (SD) scores values were calculated for children <18 years, using Turkish national reference data (15). Subjects with BMI ≥95% were defined as obese and with BMI ≥85% and <95% as overweight. BMI was calculated for adults and classified as underweight, normal weight, overweight or obese based on World Health Organization classification (16). Waist circumference was evaluated according to NHANES 3 reference limits appropriate for age and sex (17). Blood pressure (BP) was evaluated by three consecutive measurements. In children <13 years of age, HT was defined as systolic or diastolic BP ≥95th percentile according to the recent sex, age and height tables (18). For adolescents ≥13 years and adults, HT was defined as systolic and/or diastolic BP ≥140/90 mmHg and elevated BP was defined as systolic and/or diastolic BP ≥120/80 mmHg according to 2017 guidelines (18,19).

Biochemical assessment: In all subjects, fasting glucose, total cholesterol, low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, triglyceride, uric acid (measured by enzymatic colorimetric method using Abbott Architect c8000 autoanalyzer) and insulin (measured with chemiluminescence, by Abbott Architect i2000) levels were evaluated. Hyperglycemia was defined as a fasting glucose level of ≥100 mg/dL. Insulin resistance was estimated by the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) formula, that is fasting serum insulin (μU/mL) x fasting plasma glucose (mmol/L)/22.5, as described by Matthews et al (20). For adults, a HOMA-IR value above 2.7 was considered as insulin resistance (21). For children and adolescents, insulin resistance was evaluated by HOMA-IR values higher than ≥97th percentile for age and sex (22).

The criteria of the International Diabetes Federation, in children above 10 years (23) and adults (24) were used for assessment of metabolic syndrome.

Statistical Analysis

The data were analyzed using the Statistical Package for Social Sciences program, version 18.0 (IBM Inc., Chicago, IL, USA). For baseline characteristics, a descriptive statistical analysis was performed using percentages for categorical variables, mean ± SD for normally distributed continuous variables and median (range) for skewed continuous variables. Differences between two groups were tested using two-sample t-test or Mann-Whitney U test for continuous variables and chi-square test or Fisher's exact test for categorical variables, as appropriate. Results were evaluated at 95% confidence interval and a p value less than 0.05 was considered statistically significant.

Results

A total of 48 haemophilia patients and 35 age and sex matched healthy controls were included in the analysis. The demographic characteristics between the groups were similar. Mean ages of the haemophilia and control groups were 20.5±9.1 years and 21.4±9.0 years, respectively (p=0.65). There was no difference in weight, height, BMI and waist circumference values of PwH and controls (Table 1). Median age at diagnosis was 11 (1-129) months among haemophilia patients. Sixty-six percent of haemophilia A and 50% of haemophilia B patients were on prophylaxis, others were on demand therapy. Median annual factor consumption for haemophilia A and B patients were 1846 IU/kg (0-3600) and 840 IU/kg (318-2434), respectively. The frequency of PwH with inhibitors was 8.3% and these subjects were mainly severe type haemophilia A cases. Arthropathy was present in 61.9% of the haemophilia A and 66.7% of haemophilia B patients; and mainly in patients of severe type over 18 years old. Only a few of the patients were getting physiotherapy (5% of haemophilia A and none of the haemophilia B patients).

Energy, protein, fat, carbohydrate, fiber and cholesterol intakes by age and sex were calculated in the haemophilia and control groups according to their food consumption records (Table 1). Energy intake was significantly higher in the control group (p=0.02), but other nutrient intakes were similar. Subjects in both study and control groups were exposed to similar amounts of regular exercise. Haemophilia patients of normal weight were taking less regular exercise compared to those with overweight or obesity, 14.3% of the normal weight group compared to 23.1% of the overweight/obese patients although this was not significant (p=0.66).

Biochemical assessment of all participants is shown in Table 2. Fasting blood glucose levels of PwH were higher

compared to controls (p = 0.02). However, serum triglyceride concentrations were significantly lower in the haemophilia group (p = 0.008). Total cholesterol levels were somewhat lower in the haemophilia group, but the difference did not reach statistical significance. Forty-six percent of PwH over 18 years were overweight/obese, however none of the patients younger than 18 years old were overweight/obese. Obesity was more prevalent in PwH with arthropathy (p = 0.017). When metabolic syndrome was assessed in different age groups, none of the patients < 10 years old had metabolic syndrome, 7.7% of patients between 10 and 18 years old and 25% of PwH between 18 and 40 years old had metabolic syndrome. Fifty percent of PwH > 18 years had elevated BP/HT vs 23% of those ≤18 years (p = 0.03). The frequency of elevated BP/HT remained higher in all subjects in the haemophilia cohort, although not statistically significant, when compared to controls (35.5% vs. 28.6%, p = 0.51). When PwH and controls over 10 years were compared for metabolic syndrome, no statistically significant difference was found (19.5% and 10% respectively, p = 0.34). Comparison of metabolic variables among haemophilia patients and controls are shown in Table 3. In addition, the Spearman correlation analysis did not show any correlation between annual factor consumption and any of the metabolic parameters in PwH.

Table 1. Clinical and anthropometric data in patients with haemophilia and in healthy controls

	Haemophilia patients (n = 48) (%)	Controls (n = 35) (%)	p value
Number of subjects by age groups			0.99
6-9.9 years	7 (14.6%)	5 (14.3%)	
10-17.9 years	13 (27.1%)	9 (25.7%)	
18-40 years	28 (58.3%)	21 (60.0%)	
Children (< 18 years)	**(n = 20)**	**(n = 14)**	
Weight (SDS)	0.1 (-2.7 - 2.4)	-0.1 (-2.7 - 1.6)	0.29
Height (SDS)	0.1 (-2.1 - 2.4)	-0.1 (-1.5 - 1.3)	0.56
BMI (SDS)	-0.1 (-2.9 - 1.8)	-0.6 (-2.7 - 1.4)	0.28
Adults (≥18 years)	**(n = 28)**	**(n = 21)**	
Weight (kg)	74 (52 - 95)	81 (55 - 105)	0.06
Height (cm)	175 (166 - 187)	175 (158 - 190)	0.63
BMI (kg/m^2)	24.0 (17.9 - 30.0)	25.1 (18.8 - 30.0)	0.10
Waist circumference (cm)	89 (70 - 111)	92 (71 - 106)	0.27
Exposure to regular exercise	8 (16.7%)	6 (17.1%)	0.95
Energy and nutrient intakes			
Energy (kcal)	1318 (496 - 2530)	1572 (900 - 2573)	0.02
Protein (%)	15.9 ± 2.8	16.7 ± 3.3	0.29
Lipid (%)	36.1 ± 5.2	35.4 ± 3.7	0.51
Carbohydrate (%)	48.1 ± 6.3	47.8 ± 4.5	0.80
Fiber (gr)	12.7 (5.9 - 27.0)	15.2 (6.5 - 23.5)	0.06
Cholesterol (mg)	176.0 (18.7 - 378.7)	178.0 (81.3 - 365.7)	0.18
Haemophilia type			
A (%)	42 (87.5%)	N/A	N/A
B (%)	6 (12.5%)	N/A	N/A
Haemophilia severity			
Mild	4 (8.3%)	N/A	N/A
Moderate	2 (4.2%)	N/A	N/A
Severe	42 (87.5%)	N/A	N/A
Arthropathy	30 (62.5%)	N/A	N/A
Annual factor consumption (IU/kg)	1796 (0 - 3600)	N/A	N/A

Data are presented as mean ± standard deviation, median (range) or n (%).

SDS: standard deviation score, BMI: body mass index, N/A: not applicable

Table 2. Biochemical profile of patients with haemophilia and healthy controls

	Haemophilia patients (n = 48)	Controls (n = 35)	p value
Fasting glucose (mg/dL)	93.9 ± 9.9	88.1 ± 12.5	**0.02**
Fasting insulin (μIU/mL)	7.2 (3.2 - 21.9)	7.2 (0.7 - 29.8)	0.71
HOMA-IR	1.8 (0.7 - 5.5)	1.7 (0.2 - 8.3)	0.32
Total cholesterol (mg/dL)	153.2 ± 36.9	165.1 ± 32.6	0.13
LDL-cholesterol (mg/dL)	84 (36 - 162)	95 (60 - 160)	0.09
HDL-cholesterol (mg/dL)	41.5 (24 - 75)	40 (27 - 65)	0.21
Triglyceride (mg/dL)	66.5 (31 - 261)	93 (42 - 372)	**0.008**
Uric acid (mg/dL)	5.0 ± 1.3	5.4 ± 1.5	0.23

Data are presented as mean ± standard deviation, median (range).

HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low-density lipoprotein, HDL: high-density lipoprotein

Table 3. Frequency of metabolic syndrome components

	Haemophilia patients (n = 48)	Controls (n = 35)	p value
Overweight/obese	13 (27.1%)	11 (31.5%)	0.66
Central obesity*	3 (6.3%)	3 (8.6%)	0.68
Elevated BP/HT	17 (35.5%)	10 (28.6%)	0.51
Dyslipidaemia			
Hypertriglyceridemia	5 (10.4%)	6 (17.1%)	0.28
Low HDL-cholesterol	2 (4.2%)	3 (8.6%)	0.35
Insulin resistance	11 (22.9%)	5 (14.3%)	0.33
Hyperglycemia	14 (29.2%)	3 (8.6%)	**0.02**
Family history of CVD	28 (58.3%)	21 (60.0%)	0.88

*According to waist circumference.

BP: blood pressure, HT: hypertension, CVD: cardiovascular disease, HDL: high-density lipoprotein

Discussion

Our study showed that obesity, HT and metabolic syndrome are frequent problems in PwH, especially in those over 18 years with arthropathy. Early prevention and management of overweight, obesity and their sequelae must be addressed in clinical practice in order to maximize the overall health of the haemophilia population. Therefore, assessment of cardiovascular and metabolic risk factors, beginning from early childhood, is crucial for this specific patient population.

The relationship between haemophilia and cardiovascular risk is not yet well understood (25). In several cohort studies Haemophilia has traditionally been regarded as a protective state for thrombosis due to hypocoagulability (3,4). However, some studies indicated a potential negative effect of haemophilia (26), while some found no substantial effect (27). As obesity is an important risk factor for cardiovascular disease, the impact of obesity in the haemophilia population on cardiovascular disease has been reviewed (9). These authors recommended implementing general guidelines for weight management in the context of the haemophilia care team.

Overall life expectancy and quality of life among the haemophiliac population have increased in recent years, primarily because of the reduction in mortality/morbidity due to infections and advances in factor replacement therapy. However, older patients who had been treated with on demand therapy still have a variety of orthopedic problems. In our study, overweight and obesity were frequent in subjects over 18 years with target joints. This finding may be attributed to reduced engagement in physical activity to prevent bleeding and to protect the joints. Besides, target joints progressively lead to pain, restriction of movement and potentially irreversible structural damage, the hallmarks of haemophilic arthropathy. The associated reduced physical activity results in weight gain. Furthermore, the reduced mobility and loss of muscle function leads to muscle atrophy, which may in turn increase the risk of weight gain (28). Furthermore, in a Dutch haemophilia cohort, it has been reported that overweight/obesity itself increased the number of joint bleeds and reduced function in the lower

limbs (29). Recently, Limjoco and Thornburg (6) reported high rates of overweight and obesity in a relatively younger haemophilia cohort (mean age 12 years, range 5-20 years). However, these authors identified no difference in target joints based on weight category (30% in normal weight vs. 25% in overweight or obese, p = 0.74). They suggested that the impact of overweight and obesity on joint disease may have been offset by the high rate of prophylaxis or that it may manifest over longer periods of time in follow-up.

Another outcome of increase in life expectancy of PwH is experiencing cardiovascular complications. HT is one of the most relevant cardiovascular risk factors that has gained attention, since it is also a major risk factor for intracranial hemorrhage in PwH (30). There are some studies documenting an increased rate of HT in adults with hemophilia (10,31) but little is currently known about its prevalence and severity. Increased prevalence of HT may be the result of the regular visits of these patients to clinics and getting the diagnosis of HT or due to intraparenchymal hemorrhages in the kidneys (32). Recently, a slightly increased prevalence of HT was reported in a pediatric hemophilia population, thus raising awareness of the need for assessment of BP even in young PwH (11). Our study showed that the prevalence of elevated BP and HT was higher, especially in PwH over 18 years, although this finding was not statistically significant. Nevertheless, the clinical difference noted in the hemophiliac group demonstrates a trend and warrants further study. BP measurements should be a part of standard care in PwH early in their life, with the possible consideration of early intervention.

Although none of the patients had DM in our cohort, higher concentrations of fasting blood glucose, which predicts diabetes, were observed. The same subpopulation was affected by both increased blood glucose levels and obesity, as expected. Biere-Rafi et al (1) identified a higher proprotion of PwH with hyperglycemia than controls. In contrast Alperstein et al (11) reported a low prevalence of DM in a pediatric haemophilia population. While the prevalence of dyslipidaemia was similar among haemophiliacs and the control group, mean serum concentrations of triglyceride were significantly lower in our haemophilia group. Additional research is required to determine whether blood glucose and lipid screening should start earlier for children with haemophilia.

The frequency of metabolic syndrome in our pediatric PwH (aged 10-18 years) was higher than Turkish data on healthy schoolchildren, aged 10-19 years, according to the criteria of IDF (7.7% vs 2.3%, respectively) (33). However, for the adult age group of PwH, frequency of metabolic syndrome was comparable with previously reported data (25% in PwH vs. 31.2% in Turkish adult males) (34).

Study Limitations

This study has several limitations. Our haemophilia patients were very heterogeneous with a wide age range (child, adolescent and adult), with both haemophilia A and B and all degrees of severity. Therefore, most of the subgroup analyses could not be performed and the relationship between cardiometabolic risk factors and severity of disease could not be analyzed. Further studies should include a more homogenous study population. Furthermore, our data were collected from past medical records and at only one outpatient clinic visit rather than over time. Repeated BP measurements on different days are needed for the accurate estimation and diagnosis of HT, and the exclusion of "white coat" HT. Although we were not able to collect measurements on different days, we referred all subjects with at least one episode of elevated BP/HT for further assessment. Another limitation of our study was that we did not have information about smoking and alcohol use, which will have an impact on cardiovascular and metabolic parameters. Furthermore, we asked about the subject's routine exercise status, but did not question them about how many hours they spend watching TV, playing computer games and using mobile phones, which are risk factors for the development of obesity. Further studies should be designed to follow-up patients longitudinally.

Conclusion

In conclusion, cardiovascular and metabolic risk factors like overweight/obesity, elevated BP/HT, prediabetes/DM and dyslipidaemia can be detected from very early ages in PwH. Screening from early ages for cardiovascular risk factors and considering early intervention and management might help to improve the general health status of this specific patient group and reduce morbidity.

Ethics

Ethics Committee Approval: The study was approved by İstanbul Clinical Research Ethics Committee No: 1 (No: C-009/2010).

Informed Consent: Informed consent was obtained from parents for ages 6-12 years, from both subjects and parents for age of 12-18 years, and from subjects of ages older than 18 years, in accordance with the Declaration of Helsinki.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Melek Yıldız, Bülent Zülfikar, Hasan Önal, Data Collection or Processing: Melek Yıldız, Nihal Özdemir, Beyza Eliuz Tipici, Bülent Zülfikar, Analysis or Interpretation: Melek Yıldız, Nihal Özdemir, Beyza Eliuz Tipici, Hasan Önal, Literature Search: Melek Yıldız, Nihal Özdemir, Başak Koç, Writing: Melek Yıldız, Başak Koç, Nihal Özdemir.

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Vitamin D Deficiency and Insufficiency According to the Current Criteria for Children: Vitamin D Status of Elementary School Children in Turkey

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What is already known on this topic?

Vitamin D deficiency and insufficiency is a widely observed condition among children, especially in winter and post-winter periods.

What this study adds?

Our study determined the frequency of vitamin D deficiency and insufficiency in a large group of children of elementary school age, based on seasonality. Particularly, this is the first study, determining serum 25(OH)D levels in this age group, subclassified into different seasons.

Abstract

Objective: This study aimed to determine the ratio of seasonal vitamin D deficiency and insufficiency in elementary school children aged between 6-9 years old, living in one of the largest metropols of Europe, İstanbul.
Methods: Serum 25(OH)D levels of 640 children aged 6-9 years old were scanned retrospectively from the hospital information system records between September 2017-August 2018 period. Vitamin D deficiency was defined as a serum 25(OH)D level less than 12 ng/mL (30 nmol/L) and insufficiency as levels between 12 and 20 ng/mL (30-50 nmol/L).
Results: Serum 25(OH)D levels ranged from 3.90 to 64.60 ng/mL, the median value was 25.95 ng/mL for all subjects. Of all the primary school children, 485 (75.78%) had adequate levels of 25(OH)D. Vitamin D deficiency was observed in 36 of children (5.62%), whereas insufficient levels of 25(OH)D were found in 119 children (18.60%). The ratio of vitamin D insufficiency and deficiency together was highest in spring (31.87%) and lowest in summer (13.12%).
Conclusion: Vitamin D deficiency is a widely observed and preventable public health problem among children of different ages. It is necessary to increase the awareness among health professionals, and providing 25(OH)D supplements will yield generations with healthy bone structure and well growth.
Keywords: 25(OH) vitamin D, vitamin D deficiency, vitamin D insufficiency, primary school children, vitamin D levels

Introduction

25-OH vitamin D [25(OH)D] started to gain importance worlwide for its important role in healthy bone structure and calcium and phosphate metabolism. There are many studies showing 25(OH)D deficiency and insufficiency in children worldwide (1). In the presence of 25(OH)D deficiency and insufficiency, absorbtion of both calcium and phosphorus is impaired resulting in reduced bone mineral density (2).

Low levels of 25(OH)D affects an individual's present and future health status, triggering multiple systemic responses reducing bone density and the level of immune response since there are 25(OH)D receptors in a wide range of tissues, and are related with retarded growth, skeletal deformities and secondary hyperthyroidism in the childhood, whereas

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hip fracture in the elderly is observed in individuals with impaired bone structure (3,4,5). Also, there are increasing data explaining the relationship between low levels of 25(OH)D and different types of non-skeletal diseases including some types of cancer, autoimmune, infectious, cardiovascular and psychiatric diseases (6).

Risk factors for 25(OH)D deficiency in children were defined as obesity, intestinal malabsorbtion syndromes, usage of anticonvulsant agents such as Phenytoin, phenobarbital, and carbamazepine, low levels of sun exposure, clothing habits, climatization and seasonality, nutritional choices, dark skin color (5).

In order to determine an individual's vitamin D status, serum 25(OH)D level is measured. There are different threshold points used to determine 25(OH)D status of individuals as suggested by different organizations and in guidelines (7,8). Regular measurement of 25(OH)D levels in the childhood and replace the low levels with vitamin D fortification or supplementation is essential and a public health matter in order to acquire healthy generations with robust bone structure.

The aim of our study was to assess serum 25(OH)D levels in elementary school children aged between 6-9 years old within a year duration and determine 25(OH)D status between different seasons.

Methods

Study Population and Analysis

This is a retrospective study, conducted in one of the largest training hospitals in İstanbul, Turkey. Between September 2017-August 2018, children aged between 6-9 years old who underwent annual check-up for vitamin D status were randomly selected according to the block randomization method in order to create sampling groups of equal sample sizes from hospital information system. All of the participants in our study resided in a large metropolitan area. All were Caucasian and of Turkish origin. Health status of children were controlled from their medical data and children who have a chronic disease, eg. diabetes, inflammatory bowel disease, obesity were excluded. Additionally, children with extremely high and toxic levels of 25(OH)D were excluded, assuming the use of vitamin D supplementation. Of these children fitting to our inclusion criteria, 160 children (80 female, 80 male) were randomly selected from the hospital records for each age group in terms of their sampling and analysis date. One hundred sixty children from each age group were then divided into subgroups of 40 subjects (20 female, 20 male) for each seasons. Seasons in our climate zone are as follows: Fall (September to November), Winter (December to February), Spring (March to May) and Summer (June to August).

The study was approved by the Institutional Ethical Committee of İstanbul Training and Research Hospital (no. 2018/1499).

Serum 25(OH)D levels were measured with chemiluminescence method using Access 25(OH) vitamin D total test (Beckmann Coulter, Inc., USA). Inter-assay and intra-assay CV% as supplied by the manufacturer were between 5.1-8.1% and 2.2-4.7%, respectively. The analysis laboratory was a participant of the RIQAS Immunoassay Speciality-1 External Quality Assurance program (Randox Laboratorıes Ltd., United Kingdom) for 25(OH)D and no deviation in the internal and external quality control results were observed within the study duration.

Determination of Vitamin D Status

Different classification algorithms were recruited for the determination of vitamin D status in the literature, so far. In our study, we used the widely used cut-off points as suggested by Munns et al (8). According to their criteria, vitamin D deficiency was defined as a serum 25(OH)D level of <12 ng/mL (<30 nmol/L) and insufficiency as a 25(OH)D level between 12 and 20 ng/mL (30-50 nmol/L). 25(OH)D levels higher than 20 ng/mL (50 nmol/L) was accepted as adequate.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation. In the tables, the lowest and highest values were defined as well as the medians. The rate of deficiency, insufficiency and adequacy were shown as the number and percentage of the cases within eacy subgroup. Statistical analyses were done using the SPSS for windows (SPSS Inc, Chicago, IL). Comparisons of means between two groups were done using Student's t-tests. The ratios of vitamin D deficiency between groups were compared using the χ^2 test. The results were evaluated using a significance value of $p<0.05$.

Results

Table 1 presents the mean serum 25(OH)D concentrations among different age groups of the study population. According to these measurements, we could not find a statistically significant difference between different genders of children in same age groups. When all age groups were compared in terms of gender, and independent of the seasonality, we could not find a difference between boys and girls. When all 640 children were evaluated together, serum 25(OH)D levels ranged from 3.90 to 64.60 ng/mL;

the median value was 25.95 ng/mL. Division of children into subgroups in terms of age, gender, and seasonality did not yield a statistically significant difference between boys and girls of the same group (Table 2).

Mean 25(OH)D levels of all subjects were found to be 32.11 ± 11.24 in fall, 24.24 ± 7.95 in winter, 25.18 ± 10.09 in spring and 29.69 ± 11.53 in summer seasons. When mean levels of 25(OH)D were compared between seasons, levels measured in winter and spring were significantly lower than the levels in summer and fall ($p < 0.001$) (Figure 1).

Analysis of the vitamin D status in terms of age and gender was shown in Table 3. Of all the 640 primary school children, 485 (75.78%) had adequate levels of 25(OH) D. Vitamin D deficiency was observed in 36 of children (5.62%), whereas insufficient levels of 25(OH)D were detected in 119 children (18.60%). According to our data, the highest rates of deficiency and insufficiency were found in the 8-year old children's group (8.12%; 21.25%, respectively). Furthermore, when the children were divided into subgroups in terms of age and gender, we detected a statistically significant difference between 6-year old girls and boys for the rate of deficiency, and 7-year old girls and boys for the rate of insufficiency (Table 3).

The rate of vitamin D insufficiency and deficiency together was highest in spring, and lowest in summer season (13.12%) (Table 4). Comparison of insufficiency and deficiency rates between the seasons fall-winter, winter-summer, spring-summer, fall-spring yielded statistical significance (Figure 2).

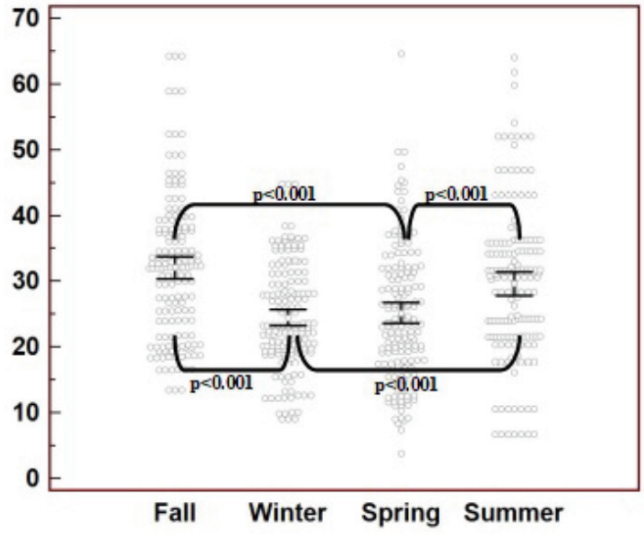

Figure 1. Distribution of 25(OH)D in different seasons. The y axis represents the 25(OH)D levels and their seasonal distribution. Significant differences were depicted with conjuncted lines between the seasons using p values

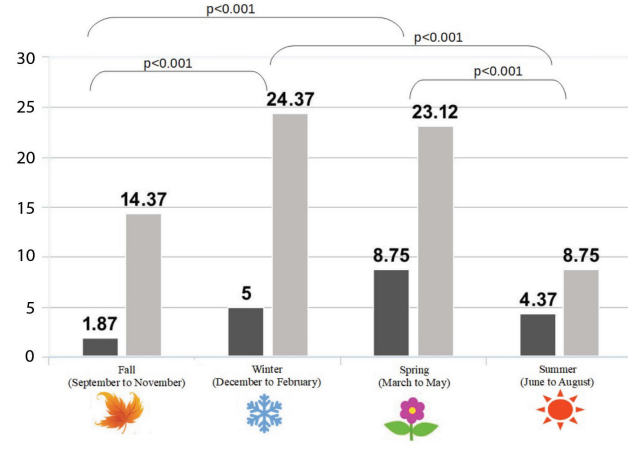

Figure 2. Rate of vitamin D deficiency and insufficiency by season. 25(OH)D < 20 ng/mL accepted as deficiency (black color); 25(OH)D between 21-29 ng/mL accepted as insufficiency (grey color). Significant differences were depicted with conjuncted lines between the seasons using p values

Table 1. 25(OH)D levels of children for different age groups

Age (years); number per group	Serum 25(OH)D levels (ng/mL) Mean ± SD (lowest value-highest value; median)			
	Girls	Boys	p	Total
6 **(Girls n = 20; Boys n = 20; Total n = 40)**	28.81 ± 10.40 (12.60-61.90; 26.85)	30.57 ± 13.67 (9.2-64.30; 30.90)	0.56	29.81 ± 12.30 (9.2-64.30; 28.90)
7 **(Girls n = 20; Boys n = 20; Total n = 40)**	25.43 ± 11.22 (10.40-64.60; 21.70)	28.27 ± 8.57 (9.1-46.5; 29.2)	0.29	26.61 ± 10.22 (9.10-64.60; 24.90)
8 **(Girls n = 20; Boys n = 20; Total n = 40)**	26.37 ± 10.95 (6.70-58.90; 24.10)	25.13 ± 10.83 (3.90-47.50; 23.60)	0.59	25.79 ± 10.85 (3.90-58.90; 24.00)
9 **(Girls n = 20; Boys n = 20; Total n = 40)**	27.20 ± 9.87 (8.90-45.10; 30.20)	28.57 ± 10.75 (1230-52.10; 25.85)	0.63	27.84 ± 10.21 (8.90-52.10; 28.20)
Overall **(Girls n = 80; Boys n = 80; Total n = 160)**	26.83 ± 10.65 (6.70-64.60; 25.15)	28.08 ± 11.63 (3.90-64.30; 28.20)	0.36	27.51 ± 11.23 (3.90-64.60; 25.95)

SD: standard deviation, 25(OH)D: 25-OH vitamin D

Table 2. 25(OH)D levels of children in different age groups subdivided into seasonal categories

Age (years); number per group	Fall-Serum 25(OH)D levels (ng/mL) Mean ± SD (lowest value-highest value; median)			
	Girls	Boys	p	Total
6 (Girls n = 20; Boys n = 20; Total n = 40)	31.36 ± 7.76 (20.00-38.00; 34.60)	38.07 ± 20.76 (13.5-64.3; 37.25)	0.52	34.34 ± 14.29 (13.5-64.3;36.80)
7 (Girls n = 20; Boys n = 20; Total n = 40)	25.16 ± 11.00 (16.5-39.4; 18.5)	40.30 ± 5.48 (36.1-46.5; 38.30)	0.072	34.34 ± 14.29 (16.5-46.5; 35.4)
8 (Girls n = 20; Boys n = 20; Total n = 40)	32.41 ± 13.84 (18.4-58.9, 27.7)	31.88 ± 10.71 (19.4-44.7; 29.6)	0.93	32.21 ± 12.38 (18.4-58.9; 28.65)
9 (Girls n = 20; Boys n = 20; Total n = 40)	31.61 ± 5.93 (21.5-39.8; 32.55)	31.15 ± 9.58 (19.9-49.3; 32.10)	0.91	31.34 ± 8.06 (19.9-49.3; 32.40)
Overall (Girls n = 80; Boys n = 80; Total n = 160)	30.7 ± 10.76 (16.5-58.9; 30.90)	33.77 ± 11.80 (13.5-64.3; 34.00)	0.34	32.11 ± 11.24 (13.5-64.3; 32.30)
Age (years); number per group	Winter-Serum 25(OH)D levels (ng/mL) Mean ± SD (lowest value-highest value; median)			
	Girls	Boys	p	Total
6 (Girls n = 20; Boys n = 20; Total n = 40)	20.66 ± 4.92 (15.5-25.3; 21.20)	30.00 ± 7.67 (19.2-44.9; 31.30)	0.069	28.14 ± 8.04 (15.5-44.9; 30.00)
7 (Girls n = 20; Boys n = 20; Total n = 40)	22.17 ± 5.66 (12.2-32.6; 20.80)	24.72 ± 7.89 (9.1-34.8; 27.45)	0.45	23.37 ± 6.70 (9.1-34.8; 23.30)
8 (Girls n = 20; Boys n = 20; Total n = 40)	23.30 ± 6.51 (9.9-36.8; 22.70)	23.68 ± 8.72 (12.7-36.2; 21.95)	0.91	23.46 ± 7.25 (9.9-36.8; 22.70)
9 (Girls n = 20; Boys n = 20; Total n = 40)	24.67 ± 14.29 (10.1-38.4; 25.10)	18.32 ± 5.65 (12.3-26.1; 19.50)	0.38	21.14 ± 10.19 (10.1-38.4; 19.50)
Overall (Girls n = 80; Boys n = 80; Total n = 160)	22.85 ± 7.25 (9.9-38.4; 22.35)	25.42 ± 8.43 (9.1-44.9; 26.10)	0.21	24.24 ± 7.95 (9.1-44.9; 22.90)
Age (years); number per group	Spring-Serum 25(OH)D levels (ng/mL) Mean ± SD (lowest value-highest value; median)			
	Girls	Boys	p	Total
6 (Girls n = 20; Boys n = 20; Total n = 40)	27.72 ± 9.90 (12.6-49.7; 26.7	23.60 ± 9.85 (9.2-43-7; 21.85)	0.26	25.52 ± 9.92 (9.2-49.7; 25.35)
7 (Girls n = 20; Boys n = 20; Total n = 40)	25.53 ± 12.26 (10.4-64.6; 22.50)	26.47 ± 8.98 (13.5-37.7; 29.20)	0.83	25.84 ± 11.1 (10.4-64.6; 23.30)
8 (Girls n = 20; Boys n = 20; Total n = 40)	23.61 ± 9.27 (9.1-45.4; 22.50)	23.03 ± 13.21 (3.9-47.5; 22.7)	0.88	23.32 ± 11.23 (3.90-47.5; 22.70)
9 (Girls n = 20; Boys n = 20; Total n = 40)	25.35 ± 10.17 (8.9-45.1; 25.45)	28.4 ± 9.43 (17.5-44.6; 26.30)	0.48	26.37 ± 9.83 (8.9-45.1; 25.45)
Overall (Girls n = 80; Boys n = 80; Total n = 160)	25.48 ± 10.38 (8.9-64.6; 24.30)	24.72 ± 10.74 (3.9-47.5; 23.20)	0.70	25.15 ± 10.50 (3.90-64.6; 23.80)
Age (years); number per group	Summer-Serum 25(OH)D levels (ng/mL) Mean ± SD (lowest value-highest value; median)			
	Girls	Boys	p	Total
6 (Girls n = 20; Boys n = 20; Total n = 40)	28.45 ± 13.27 (18.4-61.9; 28.45)	43.20 ± 16.26 (21.6-64.1; 39.4)	0.17	37.33 ± 15.28 (18.4-64.1; 31.10)
7 (Girls n = 20; Boys n = 20; Total n = 40)	35.06 ± 17.51 (16.20-50.8; 38.20)	29.96 ± 3.33 (24.8-33.1; 30.30)	0.52	31.87 ± 10.04 (16.2-50.8; 31.45)
8 (Girls n = 20; Boys n = 20; Total n = 40)	28.2 ± 13.06 (6.70-46.9; 26.20)	28.2 ± 13.06 (6.70-46.90; 24.30)	0.50	36.32 ± 10.01 (6.70-46.90; 24.30)
9 (Girls n = 20; Boys n = 20; Total n = 40)	33.85 ± 2.89 (31.80-35.91; 33.85)	43.35 ± 12.37 (34.60-52.10; 43.35)	0.40	3860 ± 9.16 (31.80-52.10; 35.25)
Overall (Girls n = 80; Boys n = 80; Total n = 160)	31.24 ± 12.62 (6.70-61.90; 29.70)	32.53 ± 12.98 (10.60-64.10; 30.90)	0.73	31.94 ± 12.69 (6.70-64.10; 30.75)

SD: standard deviation, 25(OH)D: 25-OH vitamin D

Table 3. The rate of vitamin D deficiency and insufficiency according to age and gender				
	Girls n (%)	Boys n (%)	p value	Overall n (%)
	Vitamin D status of 6 year-old children			
Deficiency [Serum 25(OH)D < 12 ng/mL]	0 (0%)	6 (7.5%)	**< 0.05**	6 (3.75%)
Insufficiency [Serum 25(OH)D = 12-20 ng/mL]	11 (13.75%)	10 (12.5%)	1.00	21 (13.12%)
Adequate levels [Serum 25(OH)D > 20 ng/mL]	69 (86.25%)	64 (80.0%)	0.39	133 (83.13%)
	Vitamin D status of 7 year-old children			
Deficiency [Serum 25(OH)D < 12 ng/mL]	2 (2.5%)	3 (3.75%)	1.00	5 (3.12%)
Insufficiency [Serum 25(OH)D = 12-20 ng/mL]	25 (31.25%)	13 (16.25%)	**< 0.05**	38 (23.75%)
Adequate levels [Serum 25(OH)D > 20 ng/mL]	53 (66.25%)	64 (80.0%)	0.07	117 (73.13%)
	Vitamin D status of 8 year-old children			
Deficiency [Serum 25(OH)D < 12 ng/mL]	7 (8.75%)	6 (7.5%)	1.00	13 (8.12%)
Insufficiency [Serum 25(OH)D = 12-20 ng/mL]	15 (18.75%)	19 (23.75%)	0.56	34 (21.25%)
Adequate levels [Serum 25(OH)D > 20 ng/mL]	58 (72.5%)	55 (68.75%)	0.72	113 (70.63%)
	Vitamin D status of 9 year-old children			
Deficiency [Serum 25(OH)D < 12 ng/mL]	9 (11.25%)	3 (3.75%)	0.13	12 (7.5%)
Insufficiency [Serum 25(OH)D = 12-20 ng/mL]	9 (11.25%)	17 (21.25%)	0.13	26 (16.25%)
Adequate levels [Serum 25(OH)D > 20 ng/mL]	62 (77.5%)	60 (75.0%)	0.85	122 (76.25%)

25(OH)D: 25-OH vitamin D

Table 4. The rate of overall vitamin D deficiency and insufficiency in different seasons			
Seasonality	**Vitamin D status of children n (%)**		
	Deficiency (< 12 ng/mL)	**Insufficiency (12-20 ng/mL)**	**Adequate levels (> 20 ng/mL)**
Fall	3 (1.87%)	23 (14.37%)	134 (83.75%)
Winter	8 (5.0%)	39 (24.37%)	113 (70.63%)
Spring	14 (8.75%)	37 (23.12%)	109 (68.13%)
Summer	7 (4.37%)	14 (8.75%)	139 (86.88%)

Discussion

Our study includes Turkish elementary school children of both genders between 6-9 years old, residing in the largest metropol of Turkey, and one of the largest metropols of Europe, İstanbul. The subjects were chosen among the healthy children, who underwent laboratory tests for their routine annual check-up.

There are different suggested cut-off points for evaluation of vitamin D status. The Endocrine Society accepts a threshold of 12-20 ng/mL for insufficiency, and > 20 ng/mL to represent sufficiency (8). However, the Institutes of Medicine claims that 25(OH)D levels above 20 ng/mL does not supply an additional benefit for bone health (7). Based on these approaches, the prevalence of insufficient and deficient individuals highly vary between the studies worldwide. In

our study, we used the cut-off points determined by the Endocrine Society, since that classification would be most appropriate for our group.

Our study demonstrated that, taken together, a total of 155 children out of 640 (24.21%) had deficient and insufficient levels of 25(OH)D in their blood serum. We also found that, the 25(OH)D levels were significantly differed depending on the seasonality. The rate of deficient and insufficient levels of 25(OH)D were higher in the winter and spring, when compared to other seasons. Additionally, while we compared the number of children with inadequate levels of 25(OH)D, we found that 78 (12.18%) of were girls, whereas 77 (12.03%) of were boys.

This is the first and wide analysis of vitamin D status among primary school age group of children in Turkey. There is a range of studies showing deficient or inadequate levels of 25(OH)D in children, worldwide. The findings of our study have similar and different findings with other studies of Turkish and European origin.

In their study with Turkish children of 11-18 ages, Karagüzel et al (9) used the cut-off value 20 ng/mL for deficiency, and found the prevalence of vitamin D deficiency 93% during spring and 71% during autumn seasons, with an overall prevalence of 82%. While we used cut-off point 12 ng/mL for deficiency in our study group, we found vitamin D deficiency was 8.75% during spring, and 1.87% during autumn. Additionally, insufficient levels of vitamin D were detected in the spring and autumn seasons, with a rate of 23.12% and 14.37%, respectively. The difference between their study and ours might be due to the following reasons: Firstly, their study group consisted of a different age group than our group, who are teenagers, among which are girls wearing traditional clothing covering their body as a result of regional beliefs. Secondly, their study was conducted in the northeastern part of Turkey with a colder and less sunny seasonal times when compared to İstanbul (10). Lastly, their cut-off points are higher than the values we recruited for the classification of our subjects.

Erol et al (11) also measured the 25(OH)D levels of 280 children aged 3-17 years old, living in İstanbul, Turkey, the same region our study group is located. They used the classification suggested by American Pediatric Endocrine Association defining a serum 25(OH)D level less than 15 ng/mL as deficiency, and levels between 15 and 20 ng/mL as insufficiency. Of the individuals, they found 80.36% rate of deficiency and 11.79% rate of insufficiency in the end of winter samples.

In a study from Kuwait, recruiting the similar age group subjects, the defined that being ≤8.5 years old is a significant risk factor for vitamin D deficiency (12). This finding is consistent with our data showing higher rate of deficiency and insufficiency in 8 years old children. The similarity between two studies might be a result of accelerated growth in children of this age.

In their study analyzing vitamin D status' of a large group of Greek children between 9-13 years old, and using the same cut-off points with our study, Manios et al found that the overall prevalence of vitamin D deficiency and insufficiency were 5.2% and 52.5%, respectively (13). The lower rate of vitamin D deficient children in our study group might be a result of their sampled age group and this group's increased demand to 25(OH)D as a result of accelerated growth. Additionally, they did not include the samples on the summer season, since they collected samples from schools, and the schools were on summer break from June to September. They also observed a higher prevalence of low 25(OH)D levels in girls, when compared to boys. Female gender is a well-known reason of both vitamin D deficiency and insufficiency, and data discussing this issue suggested different reasons. Traditional clothing as a result of religional beliefs is one of the arguments that is put forward (14). However, 25(OH)D levels were still found to be lower in girls residing in countries that traditional clothing is not widely used (13,15,16). Studies collecting dietary 25(OH)D intake of children revealed that mean intake of 25(OH)D with food consumption is lower in girls when compared to boys of same age (17). Additionally, tendency to outdoor activities and time spent under direct sunlight is lower in girls (9,11). Recently, female sex hormones (mainly estradiol and estrogens) were shown to be affecting the 25(OH)D levels in females altering the synhesis and metabolism (18).

With the increased level of knowledge and awareness on vitamin D and its relation to growth and disease susceptibility, 25(OH)D fortified foods were started to be sold in public markets in some countries (19). In Turkey, 25(OH)D fortified foods are not common in markets and their prices are higher when compared to the similar group of foods. Thus, for the individuals with low income, vitamin D synthesis through proper and adequate sun exposure remains the sole choice. It has been suggested that sunlight exposure of dorsal body areas for 15 minutes at least three times a week is sufficient to maintain adequate levels of 25(OH)D for adults. In case of diminished or decreased sun exposure due to different reasons including low level of outdoor activities, clothing habits, climate changes, 25(OH)D supplementation is required if the consumed amount of 25(OH)D with foods is not sufficient (1). There are data presenting higher mean blood 25(OH)D levels and lower

prevalence of insufficiency and deficiency in children from colder regions of world, suggesting that the higher rate of consumption of fish oil and fish types living in cold sea habitats helped those children to maintain the blood 25(OH) D levels within adequate limits, despite their less exposure to sunlight when compared to the children of warm regions (20,21).

A five-year nationwide 'vitamin D prophylaxis augmentation programme' was initiated in 2005 with a collaboration between Turkish Pediatric Endocrine Society and Ministry of Health of Turkey, recruiting free distribution of vitamin D drops to all 0-12 months old children. Consequently, these efforts resulted in a decline in the prevalence of rickets from 6% in 1998 to 0.1% in 2008 in children under 3 years of age (22). However, our study and other forementioned studies reveal the need for vitamin D supplementation for children of different age groups. Although vitamin D supplements are sold for very low prices, and the drop, powder, ampoule forms are covered by government insurance and can be obtained free, there is still need for public awareness in order to provide adequate levels of 25(OH)D for children. Since the administration of supplements to children is under the control of their parents, parent integration is highly essential even though medical and legal authorities provide the sufficient support.

Study Limitations

Our study has several limitations. Since we used a retrospective data obtained from hospital records of our study group, there is lack of data regarding BMI, social status, time spent under daylight, parathormone levels, dressing habits and daily 25(OH)D intake of children. Additionally, this is a one center study held in İstanbul, thus it does not reflect the status of all Turkish children.

One of the strengths of our study is that it is distinctive for comprising large number of healthy elementary school children aged between 6-9 years and grouping them according to gender and age.

Conclusion

Our findings reveal that vitamin D deficiency and insufficiency is a common condition on winter and spring times, among children of elementary school age. 25(OH) D supplementation and close follow-up of vitamin D status especially in the winter and post-winter period are required to supply a strong bone structure and healthy growth. Children with adequate levels of 25(OH)D and healthy skeleton will benefit in their adult years, thus this is a public health issue, and should be taken into consideration by authorities using the recommendations from appropriate guidelines.

Ethics

Ethics Committee Approval: The study was approved by the Institutional Ethical Committee of İstanbul Training and Research Hospital (no. 2018/1499).

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

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Devrim Sarıbal, Design: Devrim Sarıbal, Data Collection or Processing: F. Sinem Hocaoğlu-Emre, Osman Oğuz, Analysis or Interpretation: Devrim Sarıbal, Osman Oğuz, Literature Search: Devrim Sarıbal, Osman Oğuz, Writing: F. Sinem Hocaoğlu-Emre.

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ORIGINAL ARTICLE

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Effect of Education on Impaired Hypoglycemia Awareness and Glycemic Variability in Children and Adolescents with Type 1 Diabetes Mellitus

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What is already known on this topic?

Impaired hypoglycemia awareness and glycemic variability are important problems causing acute and chronic complications in children and adolescents with type 1 diabetes.

What this study adds?

Professional continuous glucose measurement system is a valuable tool to diagnose impaired hypoglycemia awareness (IHA) in type 1 diabetic children and adolescents. IHA, glycemic variability and time in range can be improved by education-based intervention.

Abstract

Objective: The aim of this study was to determine the prevalence of impaired hypoglycemia awareness (IHA) in children and adolescents with type 1 diabetes mellitus using a professional continuous glucose monitoring (CGM) system and to show the effect of structured education on glycemic variability (GV) in children and adolescents with IHA.

Methods: Forty type 1 diabetic children and adolescents with a diabetes duration of at least five years were eligible for inclusion in this prospective, quantitative study. All subjects were asked about their history of being aware of the symptoms of hypoglycemia using a questionnaire. Professional CGM was conducted in all of the patients for six days. The frequency of IHA detected by comparison of CGM and logbook reports were analyzed. Patients with identified IHA underwent a structured training program. After three months, CGM was re-applied to patients with IHA.

Results: The study was completed by 37 diabetic children and adolescents. After the initial CGM, nine patients (24.3%) were found to have had episodes of IHA. Area under the curve (AUC) for hypoglycemia and number of low excursions were; 1.81 ± 0.95 and 8.33 ± 3.60 for the IHA group at the beginning of the study. AUC for hypoglycemia was 0.43 ± 0.47 after three months of structured education the IHA patients ($p = 0.01$). Coefficient of variation which shows primary GV decreased significantly although unstable at the end of education in IHA patients ($p = 0.03$).

Conclusion: CGM is a valuable tool to diagnose IHA. IHA, GV and time in range can be improved by education-based intervention.

Keywords: Continuous glucose monitoring, education, impaired hypoglycemia awareness, glycemic variability, type 1 diabetes, children

Introduction

Hypoglycemia is the most common acute complication of type 1 diabetes with adverse effects on both the quality of life of patients and the management of their diabetes (1,2). Hypoglycemia is usually defined as a plasma glucose level < 70 mg/dL (3.9 mmol/L) (3). The following classification of hypoglycemia, based on clinical evaluation, is worth considering (4). Level 1: a hypoglycemia alert glucose value of < 70-54 mg/dL (3.9-3.0 mmol/L) with or without symptoms. Level 2: a glucose level of < 54 mg/dL (< 3.0 mmol/L) with our without symptoms. This glucose level should be considered clinically significant hypoglycemia requiring immediate attention. Level 3: severe hypoglycemia. This denotes cognitive impairment requiring external assistance for recovery but is not defined by a specific glucose value.

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The main symptoms of hypoglycemia occur as a result of neuroglycopenic and autonomic activation (5). Neuroglycopenic symptoms occur as a result of hypoglycemic activation of the autonomic nervous system and these symptoms are often severe enough so that hypoglycemia will be noticed by the patient, thus providing protection from complications related to hypoglycemia (6). Nocturnal hypoglycemia is often asymptomatic and mild hypoglycemia during the day may also not be noticed by the patient. Therefore it is difficult to determine the true frequency of hypoglycemia. As efforts to achieve optimal glucose control increase in order to prevent the chronic complications of diabetes, the risk of hypoglycemia increases. Recurrent antecedent hypoglycemia induces sympathoadrenal responses and unawareness of hypoglycemia (6,7,8,9,10). This is known as impaired hypoglycemia awareness (IHA) and can be defined as the inability to perceive the onset of hypoglycemia.

Typically, autonomic symptoms are lost before neuroglycopenic symptoms, which then predominate (3). Type 1 diabetic patients with IHA and impaired counter-regulation are more likely to suffer from severe hypoglycemia, have longer diabetes duration and, interestingly, lower hemoglobin A1c (HbA1c). In addition IHA is a major limitation to achieving tight metabolic control of type 1 diabetes and reduced quality of life. The perception of adrenergic symptoms are reduced or disappear completely in these patients (6,7). It has been reported that careful glucose monitoring, individualized blood glucose targets and structured education programs are important in preventing and managing IHA (6,7,8,9,10). Real time continuous glucose monitoring (CGM) systems reduce IHA in children, adolescents and adults with type 1 diabetes (6,10).

The aim of this study was to determine the prevalence of IHA in children and adolescents with type 1 diabetes mellitus attending a single center by using a professional CGM system. A further aim was to examine the effect of structured education on glycemic variability (GV) in children and adolescents with IHA.

Methods

Type 1 diabetic children and adolescents with a diabetes duration of at least five years were eligible for inclusion in this prospective, quantitative study. Patients were selected regardless of their metabolic control. The study was approved by the Ege University Medical Ethics Committee (approval number: 14-7/15). Written, informed consent was obtained from all participants and their parents.

All subjects were asked about their history of being aware of the symptoms of hypoglycemia prior to starting CGM with the following question: "Do you feel the symptoms of hypoglycemia". Possible answers were: "yes", "no" or "sometimes". All subjects and their parents were invited to the outpatient clinic for a two hour training and evaluation session. CGM sensors used for all subjects were Medtronic iPro®2 professional CGM system (MiniMed Medtronic, Northridge, USA). Sensor placement was performed by one of the experienced Diabetes Educators. Calibration of the sensor was accomplished by following the protocol established and outlined in the MiniMed CGM manual.

During CGM, patients and parents were asked to measure a minimum of four finger-stick blood glucose levels per day and to record glucose values, meals, insulin doses, exercise periods and symptomatic hypoglycemia in a logbook. Patients used the same brand of glucometer during the monitoring period (Accu Chek performa Nano, Roche Diagnostics, Germany).

At the completion of the six-day CGM period, the system was returned and the data downloaded to determine glucose patterns together with data from the logbooks. Glucose data from each day were analyzed at two different time periods: day and night. Responses to hypoglycemia and exercise, the presence of unrecognized hypoglycemia and the number of high and low patterns seen with the CGM were evaluated from the information collected. Hypoglycemia was defined as a value below 70 mg/dL of glucose. Patients noted the events of symptomatic hypoglycemia occurring over the six days. These notes were compared with the data obtained from CGM.

Data on mean annual HbA1c values were obtained from medical records. HbA1c was measured by turbidimetric inhibition immunoassay (Roche Cobas c513 analyzer using the Tina quant® HbA1c Gen. 3 assay, Germany) before the monitoring period and three months after modifications were made.

The frequency of IHA detected by compariosn of CGM and logbook reports were analyzed. Patients with IHA diagnosed by CGM underwent a structured training program (administration of insulin, hypoglycemia training, safe exercise management and ideal blood sugar levels) and the patients were seen weekly for three months. More frequent capillary blood glucose measurements were performed (4-6 times daily). After three months, CGM was re-applied to patients with IHA.

Statistical Analysis

Data were evaluated using SPSS for Windows, version 16.0 statistical package program (IBM Inc., Chicago, IL., USA). Participants' gender, nutrition, hypoglycemia insensitivity

and hypoglycemia insensitivity according to their sex status, duration of grouped diabetes and hypoglycemia insensitivity to diabetes, and hypoglycemia symptoms were analyzed by chi-square test. HbA1c levels before and after the study, the number of blood glucose measurements at the beginning of the study and the t-test for independent groups were used for the analysis of the CGM at the beginning of the study. Mann-Whitney U test was used to analyze the baseline data of the participants. Wilcoxon sorting test was used for the analysis of the CGM data before and after the study. A $p < 0.05$ was considered significant.

Results

Forty patients were recruited for the study. Three patients withdrew because of poor sensor compliance. Thus the study was completed by 37 diabetic children and adolescents. Mean ± standard deviation age of the patients and mean diabetes duration were 13.80 ± 2.42 and 7.67 ± 1.66 years respectively. 41% were male, 59% were female. Mean HbA1c was 8.0 ± 1.2% for the total group. Twenty five patients were on multiple daily insulin (MDI) therapy while the rest were on continuous subcutaneous insulin infusion (CSII) without sensor. No significant difference was found between CSII and MDI patients when comparing mean HbA1c at the start of therapy.

After the initial CGM, nine (six female) patients (24.3%) had episodes of IHA. Seven (77.7%) of the IHA patients were on MDI and two were on CSII. Six (66.6%) of the IHA patients had relatively shorter duration of diabetes (between five and eight years) while the remainder had a longer duration ranging from nine to eleven years. Seven (77.7%) of the IHA patients had completed puberty; one was Tanner stage 3 and the other Tanner stage 1. Mean HbA1c and glucose levels of the patients with and without IHA within the preceding year are given in Table 1.

Eight (21.6%) of the patients diagnosed as IHA with CGM filled out the questionnaire as 'I always feel the symptoms' and one (2.7%) of the patients who answered the questionnaire as 'I sometimes feel the symptoms' was diagnosed as IHA with CGM. There was no significant correlation between the true presence of IHA and the declared awareness of hypoglycemia, as given in the questionnaire responses.

IHA cases were hypoglycemic (blood glucose < 70 mg/dL) for 11.44 ± 5.12 hours while patients without IHA were hypoglycemic for a significantly shorter time (1.93 ± 2.23 hours) at the beginning of the study ($p < 0.01$). Area under the curve (AUC) for hypoglycemia and number of low excursions at the beginning of the study were 1.81 ± 0.95 and 8.33 ± 3.60, respectively for the IHA group and significantly less ($p < 0.01$) for the others with values of 0.23 ± 0.31 and 2.68 ± 2.05, respectively.

In the patients with IHA the proprotion of time spent with a blood glucose of < 70 mg/dL for the postprandial periods were; 19.1% at breakfast, 27.6% at lunch, 24.4% at dinner, 25.4% between 20.00-24.00 hours and 34.6% between 24:00-07:00 hours.

After three months of structured education the IHA patients were hypoglycemic for 4.44 ± 3.78 hours, AUC for hypoglycemia was 0.43 ± 0.47 and the number of low excursions were 5.22 ± 3.99. Though AUC and hypoglycemia duration statistically decreased compared to the initial findings ($p = 0.01$ and $p < 0.01$ respectively), the number of hypoglycemic excursions did not change with structured education. HbA1c levels in IHA patients increased from 7.93 ± 0.90% to 8.20 ± 0.85% with three month educational intervention although this was not statistically significant ($p = 0.35$).

When key metrics for CGM were assessed; AUC per 24 hours (mg/dL x day) and time spent for level 1 and level 2 hypoglycemia and percentage of time spent in level 1 hypoglycemia decreased significantly with structured education. AUC per 24 hours (mg/dL x day) and percentage of time spent in level 1 and 2 hyperglycemia did not change (see Table 2 and 3). Percentage of change in AUC (mg/dL x day) for level 1-2 hypo and hyperglycemia and time in range is shown in Figure 1. Level 2 hypoglycemia decreased by 80% while level 1 hypoglycemia increased by 12% and time in range increased by 17.7% ($p < 0.05$, for all of them). Coefficient of

Table 1. Hemoglobin A1c, diabetes duration, age and mean blood glucose levels of patients with and without impaired hypoglycemia awareness

	Diabetes duration (years)	Age (years)	Initial HbA1c (%)		Average sensor glucose (mg/dL)	
			CSII	MDT	CSII	MDT
With IHA (n = 9)	7.63 ± 1.45	14.82 ± 2.13	7.25 ± 0.35	8.13 ± 0.94	134.2 ± 21.3	169.4 ± 19.2
Without IHA (n = 28)	7.69 ± 1.74	13.59 ± 2.47	7.60 ± 0.95	8.76 ± 1.51	178.6 ± 17.4	209.7 ± 29.3
p	0.91	0.19	0.25		0.59	

Data were presented as mean ± standard deviation.

HbA1c: hemoglobin A1c, CSII: continuous subcutaneous insulin infusion, IHA: impaired hypoglycemia awareness, MDT: multiple daily injections

variation (CV), which is a measure of primary GV, decreased significantly, although it was unstable at the end of three months, with education in IHA patients (p = 0.03) (Figure 2).

Discussion

Impaired hypoglycaemia awareness is defined as poor alertness and therefore poor responsiveness to the signs and symptoms of hypoglycaemia (3). IHA is a major risk factor for serious hypoglycemia. A significant decrease in autonomic signs has been reported in even very brief periods of hypoglycemia in subjects with hypoglycemia unawareness (8).

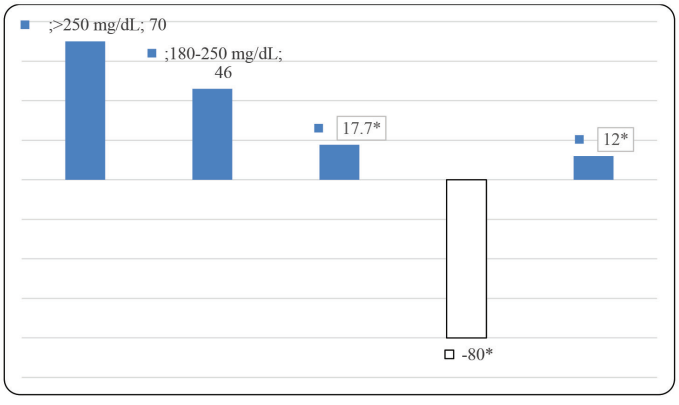

Figure 1. Percentage change in area under the curve (mg/dL x day) after education for impaired hypoglycemia awareness

**p < 0.05*

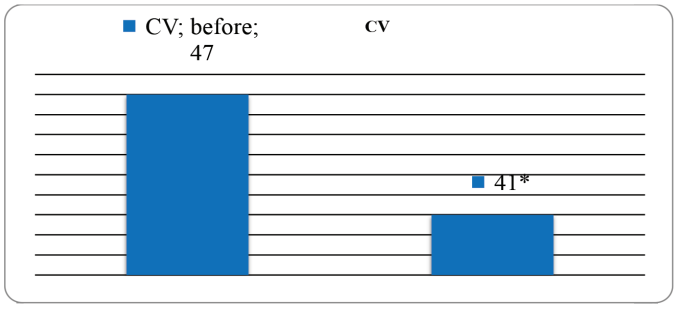

Figure 2. Change in coefficient of variation after education for impaired hypoglycemia awareness

**p = 0.03, CV: coefficient of variation*

Table 2. Area under the curve per 24 hours (mg/dL x day)

Glucose levels	Before education	After education	p
< 54 mg/dL (level 2)	302.5 ± 2.6	68.8 ± 116.6	0.01
54-70 mg/dL (level 1)	94.2 ± 7.3	105.7 ± 6.9	0.004
70-180 mg/dL	321.4 ± 39.7	450.4 ± 55.5	0.02
180-250 mg/dL (level 1)	111.2 ± 47.0	162.5 ± 73.3	0.08
> 250 mg/dL (level 2)	50.5 ± 32.9	86.0 ± 11.4	0.17

Data are presented as mean ± standard deviation

IHA is reported frequently in adults with type 1 diabetes (11). In The Diabetes Control and Complications Trial study, 36% of serious hypoglycemia incidents were attributed to hypoglycemia unawareness (12). Cryer et al (13) and Pramming et al (14) reported loss of autonomic signs in 50% of type 1 diabetic adult patients with 15-20 years of disease duration in the questionnaire-based studies they conducted. Gold et al (15) detected IHA in 29 cases (48%) with a mean age of 48.4 ± 11.0 years and a mean duration of 21 ± 8 years. Hepburn et al (11) reported lower rates of IHA in 111 subjects out of 305 (36.4%) type 1 diabetic patients in a questionnaire-based study.

However it is not clear whether frequency of IHA is the same among pre-pubertal children and adolescents. Gravelling et al (8) carried out a questionnaire study of 98 pediatric diabetic patients assessed by scale. They found hypoglycemia unawareness in 22 cases (22.4%) in subjects with a median age of 8.2 (5.7-10.5) years and mean diabetes duration of 3.2 ± 2.0 years. In a large study of 650 children with type 1 diabetes mellitus which included a questionnaire, IHA was reported in 30% of subjects which is similar to results reported for adults with type 1 diabetes (16). In our study, IHA was detected in 24.3% of 37 children and adolescents with type 1 diabetes mellitus.

Davis et al (17) showed that sex is a risk factor because females are more likely to have a suppressed hormone response to hypoglycemia. It has been suggested that estrogen is an intermediary for this. In our study, six of the nine IHA patients were female and four of the six female patients were at Tanner stage 5. Although the number of IHA patients is too few to draw a conclusion about estrogen, the number of female IHA patients was twice that of males with IHA.

Existence of a relationship between high rates of serious hypoglycemia, a decreased ability to detect hypoglycemia together with prolonged duration of type 1 diabetes and

Table 3. Percentage of time spent with glucose levels in specific glucose ranges

Glucose levels	Before education	After education	p
< 54 mg/dL (level 2)	5.1 ± 3.3	0.6 ± 0.9	0.008
54-70 mg/dL (level 1)	6.7 ± 3.6	4.2 ± 2.8	0.13
70-180 mg/dL	30.4 ± 11.3	31.2 ± 15.0	0.51
180-250 mg/dL (level 1)	22.1 ± 7.0	26.2 ± 8.0	0.08
> 250 mg/dL (level 2)	14.9 ± 7.1	21.9 ± 13.0	0.2

Data were presented as mean ± standard deviation

development of IHA has been reported frequently in the adult literature (13,14,15,18,19,20,21,22,23). In our study, IHA was detected in patients with shorter disease duration (27.3%) compared to patients with longer disease duration (20%). This finding may be due to the relatively closer duration of diabetes in the two groups and shorter duration of diabetes as compared to the adult studies.

The adoption of more flexible HbA1c targets, especially for diabetic patients who have a history of serious nocturnal hypoglycemia and those who are unable to express hypoglycemic symptoms at younger ages is needed in order to decrease the frequency of hypoglycemia (6,24). The target value for HbA1c in the ISPAD guidelines is < 7%, regardless of patient age (25). However, HbA1c levels are not an indicator for frequency of hypoglycemia. In our study, mean HbA1c and mean glucose levels were lower in the IHA group. Considering lower HbA1c values, mean blood glucose levels and continuous subcutaneous glucose monitoring data, presence of IHA has an association with reduced mean blood glucose levels and decreased HbA1c levels. Although not statistically significant, hypoglycemia unawareness tends to occur more frequently in the group with lower HbA1c levels.

In the Type 1 Diabetes Exchange study, the frequency of serious hypoglycemia was lower in pump users (26). It was thought that insulin pump therapy decreased HbA1c without increasing hypoglycemia frequency and the risk of hypoglycemia unawareness (26). In our study only two of the nine IHA patients were on pump therapy without sensors.

Gold et al (15) reported that participants usually experienced hypoglycemic symptoms in the morning. These patients stated awareness of neuroglycopenic symptoms during hypoglycemia. In our study, when subjects were asked the question "Do you experience hypoglycemia signs?", among subjects with hypoglycemia unawareness diagnosed with continuous subcutaneous glucose monitoring, 21.6% replied 'yes, I do experience' and 2.7% replied 'I sometimes experience'. Not one of the subjects said that they were unaware of hypoglycemia. According to continuous subcutaneous glucose monitoring data over 24-hours, it was evident that subjects who had IHA, mostly experienced hypoglycemia between 24:00-07:00 hours (34.6%) with a further 27.6% detected in the postprandial period at noon and this dropped further to 25.4% between 20:00-24:00 hours. Among subjects who did not experience IHA with continuous subcutaneous glucose monitoring, 54.1% said "I do experience" where 16.2% said "I do not experience". The difference between continuous subcutaneous glucose monitoring data and answers to the question "Do you experience hypoglycemia signs?" suggested that symptoms indicating hypoglycemia were not noticed, individuals' perceptions of indications were insufficient for detection of hypoglycemia and individuals replied to the questionnaire based on their emotions at the time of survey rather than their true experience. Continuous subcutaneous glucose monitoring data is more robust because of the elimination of subjective impressions and being reliably quantitative.

Avoiding hypoglycemia for three weeks is sufficient for the abolition of IHA and partial restoration of the adrenal response to hypoglycemia (6,22,27,28). In our study, hypoglycaemia awareness improved at the end of a three month structured training programme which included hypoglycemia and insulin management, safe exercise management and increased target blood glucose levels.

In the Hypo COMPaSS Trial GV was improved within 24 weeks in adults with long-standing type 1 diabetes, complicated by IHA and recurrent severe hypoglycemia, with the help of education based intervention shown by blinded CGM (29). In the study IN CONTROL real time CGM increased time spent in normoglycaemia and reduced severe hypoglycaemia in adult patients with type 1 diabetes and impaired awareness of hypoglycaemia, compared with self monitoring blood glucose (30). In our study we have shown that in type 1 diabetes mellitus with structured education, frequency of level 2 hypoglycemia decreased with more time spent in normoglycemia and produced less glucose variability, as shown by decreased CV, without a change in metabolic control assessed by HbA1c.

Study Limitation

Shortness of the follow-up period and the low number of cases can be listed as the limitations of this study.

Conclusion

We have shown that professional CGM is a valuable tool to diagnose impaired awareness of hypoglycemia and that GV can be improved in pediatric type 1 diabetes patients complicated by IHA with the help of education-based intervention.

Ethics

Ethics Committee Approval: The Clinical Research Ethics Committee of Ege University Medical Ethics Committee (Approved number:14-7/15).

Informed Consent: Written, informed consent was obtained from all participants and their parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Günay Demir, Samim Özen, Hafize Çetin, Damla Gökşen, Concept: Günay Demir, Samim Özen, Damla Gökşen, Design: Samim Özen, Damla Gökşen, Şükran Darcan, Data Collection or Processing: Günay Demir, Samim Özen, Hafize Çetin, Damla Gökşen, Analysis or Interpretation: Günay Demir, Samim Özen, Hafize Çetin, Damla Gökşen, Şükran Darcan, Literature Search: Günay Demir, Samim Özen, Damla Gökşen, Şükran Darcan, Writing: Günay Demir, Samim Özen, Damla Gökşen, Şükran Darcan.

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CASE REPORT

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A Novel Homozygous *CYP19A1* Gene Mutation: Aromatase Deficiency Mimicking Congenital Adrenal Hyperplasia in an Infant without Obvious Maternal Virilisation

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What is already known on this topic?

In aromatase deficiency, the accumulated androgens may cause signs of maternal virilisation during pregnancy. Large multiple cysts have been described in aromatase deficient girls during infancy and childhood. In previous reports of aromatase deficiency, cases were term neonates of average weight for gestational age.

What this study adds?

In this report, we describe a case of aromatase deficiency showing disorder of external genital development in a preterm infant born at age 23 weeks. A report a novel large deletion in the *CYP19A1* gene was shown. Maternal virilisation was not a marked finding in our case, except for a mild deep voice. The absence of virilisation in our patient's mother could likely be due to the premature delivery of the patient.

Abstract

Aromatase deficiency is a rare, autosomal recessive disorder in which affected patients fail to synthesize normal estrogen. Herein, we report a 46, XX patient born with virilised external genitalia. A novel homozygous mutation in the *CYP19A1* gene, causing aromatase deficiency, was detected. A 30-day infant registered as a male was referred to pediatric endocrinology because of a uterus detected on ultrasonography. The infant was born at 23 gestational weeks by C-section because of preeclampsia and premature membrane rupture. The parents were consanginenous. There was no evidence of virilisation, such as acne, hirsutism, deep voice or clitoral enlargement in the maternal history. Physical examination of the infant revealed complete scrotal fusion and a single urogenital meatus, consistent with Prader stage-3. A standard dose adrenocorticotropic hormone (ACTH) test revealed an inadequate cortisol response and high 17-hydroxy progesterone levels, suggesting simple virilising congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. However, no mutation in the *CYP21A2* gene was detected. At age 2.5 years the ACTH test was repeated, after suspension of hydrocortisone treatment for 48 hours, when resulting cortisol and androgen levels were normal. The patient was re-evaluated in terms of 46, XX disorders of sex development (DSD), especially with a suspicion of aromatase deficiency. A novel, homozygous, exon 6 deletion was identified in the *CYP19A1* gene. Aromatase deficiency may be confused with CAH in the newborn period. In this case 46, XX DSD aromatase deficiency was present in the absence of a history of maternal virilisation or large and multicystic ovaries.
Keywords: 46, XX disorders of sex development, *CYP19A1* gene, aromatase deficiency

Introduction

Aromatase (cP450arom) catalyses the conversion of androgens to estrogens. The biological importance of aromatase is related not only to its role in estrogen biosynthesis, but also to its potential influence on the balance of the androgen-estrogen ratio in different tissues. In humans, cP450arom is encoded by a single gene *(CYP19A1)*, that is located on chromosome 15q21.1. The protein-coding sequence is contained within nine exons (E2-E10), spanning approximately 35 kb (1,2,3). The CP450arom enzyme is mainly located in the endoplasmic reticulum of estrogen-

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producing cells in the ovary, placenta, testis, brain, adipose tissue, liver, muscle and hair follicles (4,5).

Aromatase deficiency is a rare, autosomal recessive disorder in which affected patients do not have normal estrogen synthesis (1). During pregnancy, dehydroepiandrosterone sulphate (DHEAS) and 16OH-DHEAS, arising from the fetal adrenal gland and liver, respectively, become important sources for the synthesis of placental estrogens (4,5,6). Fetuses lacking aromatase activity are not able to convert DHEAS to estrogens in the placenta. DHEAS is therefore converted to testosterone, resulting in the virilization of both fetus and mother. Since the first description of aromatase deficiency by Shozu et al (7) in 1991, around 40 cases have been reported (1,3,4,5,7,8,9,10,11,12,13,14,15,16,17,18,19,20).

In aromatase deficiency, the accumulated androgens may cause signs of maternal virilisation (acne, deep voice, clitoral enlargement) during pregnancy. After delivery these symptoms usually disappear gradually. In the postpartum period, some clinical and laboratory findings of androgen excess regress and androgen levels return to normal levels. In most female infants exposed *in utero* to excessive androgen levels, ambiguous genitalia have been reported. Delayed skeletal maturation has been described and most affected girls have multiple ovarian cysts and failure of breast development at puberty (3,5).

Herein, we report a 46, XX patient born with virilised external genitalia. A novel homozygous mutation in the *CYP19A1* gene, causing aromatase deficiency, was detected.

Case Report

A 30-days old infant with a male-dominant genital appearance was referred to pediatric endocrinology because of a uterus, detected on ultrasonography. The infant was born at 23 weeks of gestation by C-section because of preeclampsia and premature membrane rupture. The parents were consanginenous. Birth weight was 680 gr. The infant was intubated, given surfactant treatment and required mechanical ventilation support. Bilateral cryptorchidism and hypospadias were thought to be associated with the severe prematurity. Since gender assessment at birth was made as male, the baby received a male name and identity card. He was the first baby of a 25-year old healthy mother and a 27-year old healthy father who were first cousins. The mother had had two abortions in the past, so she was treated with progesterone for one month between the 16th and 20th gestational weeks and also with salicylic acid throughout the pregnancy. There was no evidence of virilisation, such as acne, hirsutism, deep voice or clitoral enlargement in the maternal history. Physical examination of the infant revealed complete labioscrotal fusion and a single urogenital meatus, consistent with Prader stage-3. Gonads were not palpable, a chorda was present and the phallus was measured as 2x1 cm on the dorsal and 1.6x1 cm on the ventral side. At the time of the investigation the patient was still being followed in the neonatal intensive care unit and having mechanical respiratory support. On postnatal day 30, the patient's hormone levels were as follows: 17-hydroxy progesterone (17OHP): 41 ng/mL [normal limits (NL) <35.5 ng/mL], DHEA sulphate (DHEASO4): 1500 µg/dL (NL 123-882 µg/dL), testosterone: 2.94 ng/mL (NL 0.05-0.16 ng/mL), FSH: 1.3 IU/L (NL 0.3-2.6 IU/L), LH: 0.48 IU/L (NL 0.1-8.5 IU/L), estradiol <10 pg/mL (NL <15 pg/mL), progesterone: 4.7 ng/mL (NL 0.18-6.4 ng/mL). Karyotype was 46, XX. A standard dose adrenocorticotropic hormone (ACTH) test (30 µg/kg/dose) revealed an inadequate stimulated cortisol and high 17OHP levels, suggesting simple virilising congenital adrenal hyperplasia (CAH) likely due to 21-hydroxylase deficiency (Table 1). Additionally there were several other problems, such as septicemia, surfactant deficiency and respiratory distress. The patient was on mechanical ventilation due to severe prematurity at this time. Although the classical findings of adrenal insufficiency were not present, the decision was taken to administer hydrocortisone® replacement since cortisol deficiency could not be excluded. Hydrocortisone® was commenced at a dose of 10 mg/m^2/day, three times a day. The name and identity card of the baby were changed to female with the agreement of the parents and the decision of multidisciplinary gender assessment committee.

Over the next two years, androgen levels were quite low, despite hydrocortisone doses as low as 6-7 mg/m^2/day, and no mutation in *CYP21A2* gene was detected. This unusual clinical condition and lack of a mutation in *CYP21A2* gene led to doubt concerning the security of the diagnosis of 21-hydroxylase deficiency. At the age of two years and six months the standard dose ACTH test was repeated, after suspension of hydrocortisone treatment for 48 hours. The results of this test showed the cortisol and androgen levels were normal (Table 1). When maternal history was re-evaluated, the mother remembered that she had a mild deep voice during pregnancy. The patient was re-evaluated in terms of 46, XX disorders of sex development (DSD), especially with the suspicion of aromatase deficiency (Table 2). Finally, aromatase deficiency was confirmed by genetic analysis (Figure 1).

At the last clinical visit, the patient was 4.3 years old, height was 95.5 cm (-2.3 SD), weight 14.5 kg (-1.27 SD) and breast

development was Tanner stage-1. Further examinations were performed for disorders which could be associated with aromatase deficiency (Table 2).

Informed consent was obtained from the parents of the patient for publication of this case.

Genetic Analysis

An Ethylenediaminetetraacetic acid blood sample was taken for *CYP19A1* gene sequence analysis. At the PCR step, as the very large region including exon 6 could not be amplified, a long PCR and sequence analysis was planned to detect exact breakpoints. Sequence analysis with a Next Generation Sequencing Method (Illumina-MISEQ, San Diego, CA, USA) was done and a 3212 bp deletion within chromosome 15:51.511.985-51.508.774 was detected (NM_000103.3:c.629-1453_744-486del). This large deletion was evaluated as a likely "pathogenic" variant due to ACMG criteria.

The *CYP19A1* gene contains 10 exons and exon 6 was largely deleted with some parts of introns of both sites and two canonical splice sites. This was a null variant. The allele was not found in gnomAD exomes. This is a conserved region in different species. This was a novel variant.

Discussion

In this report, we describe a case of aromatase deficiency in a 23-week preterm infant with a disorder of external

Table 1. Results of classical adrenocorticotropic hormone stimulation test at ages 60 days and 2 years

	Postnatal 60 days		Age 2 years		
Time of blood sampling (minutes)	**0'**	**30'**	**0'**	**30'**	**60'**
Cortisol (µg/dL) (NL: 0': 6.5/30': 20)	1.7	12.8	6.5	20.3	21
17OHP (ng/mL) (NL: 0':1.2-8.4/60': 40)	65	80	0.28	1.35	1.4
Progesterone (ng/mL) (NL: 0': 0.34/60': 1.0)	7.9	9.5	0.1	0.8	1.0
Testosterone (ng/mL) (NL: 0.05-1.6)	0.9	2.1	< 0.13	< 0.13	< 0.13
Δ^4 androstenodione (ng/mL) (NL: 0.1-0.3)	1.5	2.3	< 0.3	-	< 0.3
ACTH (IU/L) (NL: 0-63)	12	-	12	-	-
DHEAS (µg/dL) (NL: 123-882)	1500	-	5.5	-	-
Renin (ng/mL/hour) (NL: 0.48-4.8)	5.4	-	0.8	-	-
Aldosterone (pg/mL) (NL: 35-300)	375	-	144	-	-

17OHP: 17-hydroxy progesterone, ACTH: adrenocorticotropic hormone, DHEAS: dehydroepiandrosteron sulphate, NL: normal level

Table 2. Laboratory findings of the patient at diagnosis and follow-up

	2 months	26 months	52 months
LH (IU/L)	0.48	2.43	0.61
FSH (IU/L)	1.3	46.67	32.8
Estradiol (pg/mL)	< 10	10	< 10
IGF-1 (ng/mL)	-	117	144
Bone age (years)	-	2	3
Pelvic ultrasonography	Uterus: 20 mm	Uterus: 16 mm, right ovary: 0.6 mL, left ovary: 0.5 mL	Uterus: 30 mm, right ovary: 0.4 mL, left ovary: could not be detected
BMD	-	-	-1.4 SD

LH: luteinizing hormone, FSH: follicle stimulating hormone, IGF-1: insulin-like growth factor-1, BMD: bone mineral density, SD: standard deviation

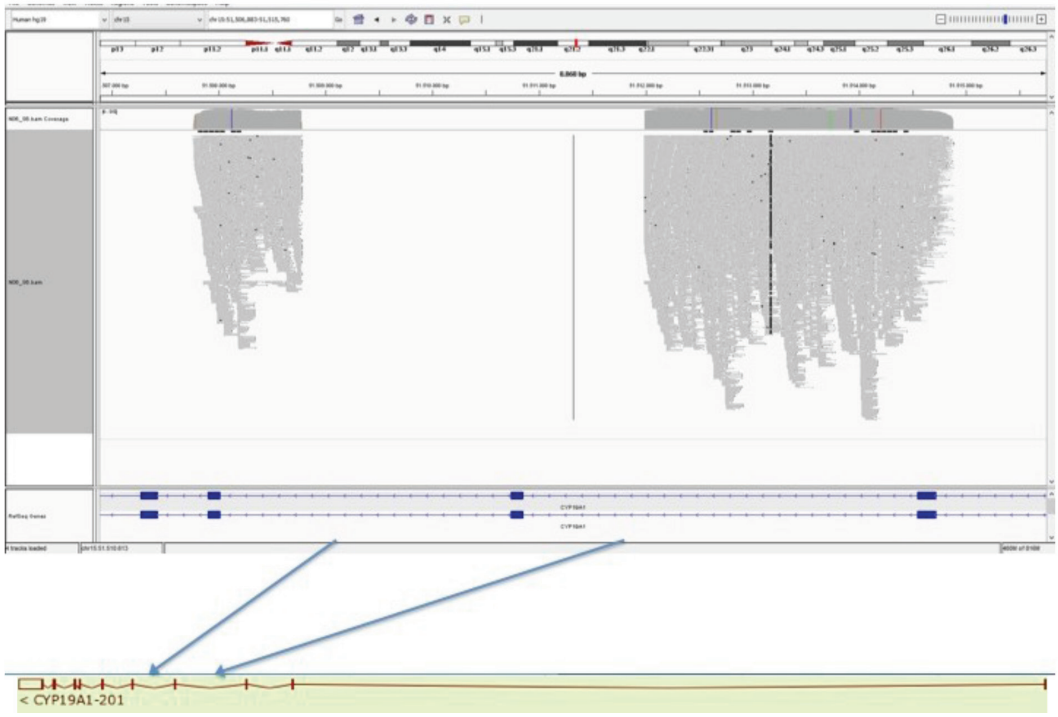

Figure 1. Identification of deletion in next generation sequencing, as visualized by integrative genomics viewer software. A 3212 bp deletion represented by blue arrows, was detected within chr15: 51.511.985–51.508.774 (NM_000103.3:c.629-1453_744-486del)

genital development. We report a novel large deletion in the *CYP19A1* gene (Figure 1). To date, more than 33 different mutations in the *CYP19A1* gene have been reported in patients with aromatase deficiency. These mutations include mis-sense, splice site, non-sense, insertions and small deletions and one large intragenic deletion (1,3,4,5, 8,9,10,11,12,13,14,15,16,17,18,19,20,21,22). The majority of the mutations reported are located in exons 9 and 10, which encode the substrate-binding site and haem-binding domains, respectively (12). Our patient had a large deletion in exon 6. Although we did not make a functional study, this variant is a null variant and classified as a likely pathogenic variant due to ACMG criteria. The fact that we were not able to conduct a functional study related to the mutation we identified was the limiting factor of our study.

Aromatase deficiency causes virilisation (acne, deep voice, clitorial enlargement) in the mother because placental androgens cannot be converted to estrogens, resulting in excessive androgen levels and virilisation of the mother during pregnancy (16). While it is an important clue, maternal virilisation is not a rule. In the study of Marino et al (3), three of six cases had a history of gestational virilisation. Maternal virilisation was not a marked finding in our case, except for a mild deep voice. Why some mothers do not have signs of virilisation can be explained by either a lower fetal adrenal androgen secretion or adequate placental estrogen production (16). Grumbach and Auchus (23) reported that a placental aromatase activity as low as 1% of normal is enough to prevent maternal virilisation. Furthermore, substantial placental estrogens are produced, especially in the 3rd trimester of gestation (16). The absence of virilisation in our patient's mother could likely be due to premature delivery or to the presence of partial aromatase activity.

In most female cases of aromatase deficiency, disorders of external genitalia with various degrees of masculinization have been reported. Gonads were non-palpable and internal genitalia differentiation was normal female in these cases (1,3,4,5). For this reason, these patients can be diagnosed as CAH, which is the most common cause of virilisation in a female fetus (1). Similarly, our patient was considered to have CAH because of physical findings and also because of quite high androgen levels. In addition the severe prematurity and the lack of data about normal androgen levels in such neonates led to initial diagnostic confusion in our patient. Low androgen levels, despite low hydrocortisone doses on the follow-up, are very unusual in classical CAH patients. This important observation, together with the lack of mutation in *CYP21A2*, encouraged us to reconsider a diagnosis of 21-hydroxylase deficiency.

A clinical phenotype, including changes in the hypothalamic-pituitary-gonadal axis, ovarian cyst development, skeletal

maturation and growth, as well as changes in insulin sensitivity and lipid profile, has been reported in aromatase deficiency (5). Marino et al (3) investigated the hypothalamic-pituitary-gonadal axis and described high levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the neonatal period. A two-month old girl, reported by Mullis et al (17), had elevated FSH levels (baseline and GnRH-stimulated) but normal LH levels (baseline and GnRH-stimulated). Contrary to these findings, our patient had normal gonadotropin levels on postnatal day 60 (Table 2). LH and FSH levels were elevated at the ages of 26 and 52 months (Table 2). In previous reports of aromatase deficiency, cases were term neonates with average weights for gestational age (5,7,17,18). Our patient was born at the 23rd week of gestation, thus gonadotropin levels in early infancy would probably not be helpful in the diagnosis. In premature infants without aromatase deficiency, gonadotrophin levels are very high after birth, but a sharp decrease in FSH levels is seen around term age. Also, in term neonate without aromatase deficiency, gonadotrophin levels are low at birth and increase progressively afterwards (24,25). Since we measured gonadotropin levels at a near term-equivalent age, it might have been coincidental with the time of rapid decrease.

Large multiple cysts have been described in aromatase deficient girls during infancy and childhood due to chronic stimulation by gonadotropins that cannot be suppressed because of estrogen deficiency (3,5,17,18). Marino et al (3) reported a case series of five patients. Four of them, aged 18, 7, 12 and 10 years, had increased ovarian size with large cysts. They were at a pubertal stage except for the 7-year old. Only one, a 3-year old girl, had normal ovaries. There are some patients who do not have large cysts and may even have hypoplastic ovaries. To date, seven patients with hypoplastic ovaries have been reported (4,12,16,19,20). Thus, there is no consistent ovarian phenotype in patients with aromatase deficiency since some were reported to have large and polycystic ovaries, while others had normal ovarian morphology (16). Despite quite elevated levels of FSH, we did not observe any ovarian cysts in periodic ultrasonographic screening of our patient and she continues to show normal ovarian morphology.

Little is known about the bone phenotype of girls with aromatase deficiency (26). It is accepted that estrogens are important in preserving adequate bone mineral density (BMD) (5). However, data on the role of estrogens on bone mineralization during childhood are scarce. Janner et al (26) found decreased BMD in a 3.5-year old patient, while Belgorosky et al (18) reported normal BMD in a 6-year old patient. It may be true that some expression of cP450arom protein might be enough to maintain a normal mineral bone density. On the other hand, men with aromatase deficiency show a distinct bone phenotype characterized by osteopenia (27). We performed a BMD measurement in our patient at the age of four years, revealing osteopenia (-1.4 SDS) when re-calculated for the patient's height age.

The usefulness of estrogen treatment during infancy and childhood in affected female patients is not clear. Mullis et al (17) reported that low doses of estradiol in a 3-year-old affected girl resulted in normalization of serum gonadotropins, regression of enlarged ovaries and improvement in BMD. Janner et al (26) showed the positive impact of oral 17-β estradiol treatment on longitudinal growth, bone age maturation, regulation of pituitary gonadotropin feedback, improving multicystic ovaries and bone density in the long-term follow-up of a girl with a compound heterozygote mutation in *CYP19A1* gene. So far, our patient has not been started on estrogen treatment, since her ovaries are still normal and existing data on estrogen treatment for these patients are inadequate.

In conclusion, a novel mutation in *CYP19A1* gene explains the virilisation of our patient. Aromatase deficiency could easily be confused with CAH, especially in a preterm infant. In a case of 46, XX DSD, aromatase deficiency can present without a history of maternal virilisation or in the absence of large and multycystic ovaries. The absence of virilisation in our patient's mother could likely be due to premature delivery of the patient or presence of partial aromatase activity. It is not clear if premature delivery and aromatase deficiency are related or are coincidental in our patient. The effect of inadequate androgen-estrogen conversion in placenta on the continuation of the gestational process in aromatase deficiency is not clear. In our opinion further investigation of this relationship and possible mechanisms are warranted.

This case report emphasizes the importance of considering aromatase deficiency as a very rare cause of 46, XX DSD and the need to perform genetic analyses in patients, especially in the absence of a definite diagnosis. Further published cases will enhance our knowledge of the phenotypic spectrum of aromatase deficiency.

Ethics

Informed Consent: Informed consent was obtained from the parents of the patient for publication of this case.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Fatma Dursun, Design: Fatma Dursun, Data Collection or Processing: Fatma Dursun, Serdar Ceylaner,

Analysis or Interpretation: Fatma Dursun, Serdar Ceylaner, Literature Search: Fatma Dursun, Writing: Fatma Dursun, Serdar Ceylaner.

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CASE REPORT

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Efficiency of Single Dose of Tolvaptan Treatment During the Triphasic Episode After Surgery for Craniopharyngioma

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What is already known on this topic?

Hyponatremia in patients with inappropriate antidiuretic hormone (ADH) syndrome (SIADH) is caused by the combination of excess ADH-induced water retention and secondary solute loss. Vaptans, arginine-vasopressin receptor antagonists, are an alternative for use in SIADH in adults. In children, vaptan treatment has not been approved for SIADH.

What this study adds?

We report successful tolvaptan treatment in a child with SIADH. Furthermore, we report that only one dose of tolvaptan in triphasic episode was effective.

Abstract

Inappropriate antidiuretic hormone syndrome (SIADH) may develop after intracranial surgery. SIADH in the pediatric age group is usually encountered in patients with an intracranial mass both before and after surgery. Fluid restriction is the standard therapy in SIADH. However, a resistant, hyponatremic pattern may be encountered in some cases. Vaptans have been recently introduced for treatment of hyponatremia due to SIADH. There is inadequate data concerning tolvaptan treatment in pediatric patients. We present a 13 year-old female with SIADH of triphasic episode who was transferred to our clinic after surgery for craniopharyngioma. Resistant hyponatremia did not resolve despite fluid restriction and hypertonic saline support. The patient responded rapidly to a single dose of tolvaptan, with no adverse effect, which resulted in successful control of her SIADH.

Keywords: Inappropriate antidiuretic hormone syndrome, tolvaptan, children

Introduction

The syndrome of inappropriate antidiuretic hormone (ADH) secretion (SIADH) is a disorder of impaired water excretion caused by the inability to suppress secretion of ADH (1). SIADH is clinically serious and one of the causes of hyponatremia in hospitalized patients (2). The etiology of SIADH involves excess ADH production due to cranial surgery, malignancies, meningitis-encephalitis, hemorrhage, other cerebral pathologies, pulmonary malignancies and drugs (2,3).

In clinical practice, the gold standard approach to SIADH is fluid restriction and it is widely used (4,5,6). In more severe forms, with neurological symptoms, administration of hyperosmolar saline combined with furosemide may be required. Additionally, the underlying etiology should be treated if possible (3,5,6,7,8,9,10). However, even these treatments may be inadequate for some patients with SIADH.

In the last decade, a new alternative treatment for SIADH, vaptans which are arginine-vasopressin receptor antagonists, has become available (8). Vaptans, act by preventing the insertion of aquaporin 2 water channels into the apical membrane, promoting reabsorption of water and resulting in excretion of diluted urine (3,5,11).

Tolvaptan is one of the vaptans and is a selective V2 receptor antagonist, whereas conivaptan is a non-selective V1/V2 receptor antagonist (3,5,6,10). Conivaptan is approved by the United States Food and Drug Administration (FDA) for

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hypervolemic (nephrotic syndrome, cardiac failure and cirrhosis) or euvolemic hyponatremia (SIADH) treatment in adults, but not in children (10). Peters et al (10) described the first successful treatment with conivaptan in a pediatric refractory SIADH patient. It was also reported that conivaptan played a key role in the management of their case with SIADH and that no adverse effects had developed (10).

Tolvaptan has been approved by the FDA for adults since 2009 and has been successfully used in the treatment of hyponatremia due to SIADH and autosomal dominant polycystic kidney disease (1,9,11,12). However data on the safety, efficacy and optimal dose of tolvaptan in pediatric patients are limited. There are a few case reports concerning tolvaptan therapy for pediatric SIADH (3,5,6,13). Similarly, there is little evidence of the use of tolvaptan in the treatment of pediatric hypervolemic hyponatremia, such as that observed in cardiac failure and nephrotic syndrome (14,15,16,17). Successful tolvaptan treatment has been reported in three children with SIADH (ROHHAD syndrome, large sellar-suprasellar tumor and surgery of astrocytoma) (5), in a patient with intracranial lymphoma (3) and in a child with nephrotic syndrome (17). In addition 28 pediatric cases with cardiac failure, treated with tolvaptan have been reported (15). All these case series reported that tolvaptan therapy was effective, safe and well tolerated in hyponatremic children.

Here, we report the first pediatric case of severe and symptomatic hyponatremia due to SIADH, successfully treated with single dose tolvaptan in Turkey.

Case Report

A 13-year-old girl with a 3-week history of headache and reduction in vision was referred to our practice because of possible endocrine problems due to craniopharyngioma. She was the third child of non-related parents. Her birth history was unremarkable. Her height was 150.8 cm [-1.19 standard deviation (SD)] and her weight was 60.2 kg (1.23 SD). Physical examination was normal except for right eye exotropia and accompanying reduction in vision.

No endocrine abnormalities were detected before the craniopharyngioma operation (see Table 1). On the first postoperative day, dexamethasone treatment for brain-associated surgery was started by the neurosurgeon. Therefore no additional steroid treatment was given in case of central adrenal insufficiency. Furthermore, the patient was polyuric (5.6 mL/kg/h), plasma sodium was 146 mmol/L (reference range 135-145), plasma osmolality was 303 mOsm/kgH_2O and urinary density was 1002. Desmopressin acetate (0.1 µg/kg/day, melt form) treatment was started for diabetes insipidus (DI). Desmopressin treatment improved her polyuria and plasma sodium concentration. On the fourth postoperative day, levothyroxine (100 µg/day) replacement therapy was started for central hypothyroidism. The patient had also developed hyponatremia, starting on postoperative day four, which gradually worsened. On the fifth postoperative day, urinary output of the patient decreased to 0.7 mL/kg/h. Evaluation of laboratory findings (plasma sodium 128 mmol/L, plasma osmolality 267 mOsm/kgH_2O, urinary density 1039) led to the diagnosis of SIADH. Plasma copeptin/ADH levels could not be measured. The findings suggested that SIADH was the second stage of the triphasic condition encountered after cranial surgery. Initial management included fluid restriction (administered fluid: total 800 mL/m^2/day) and cessation of desmopressin treatment. Despite fluid restriction for four days, the patient's blood sodium levels continued to decreas, to 118 mmol/L, and urine density was 1039. Hypertonic saline therapy (3% saline to raise the serum sodium by 10 mEq/L) was also added due to persistence of hyponatremia. However, SIADH could not be controlled and severe hyponatremia continued. In addition, the patient's condition began to worsen and mild loss of consciousness occurred. Therefore, it was decided to start low-dose oral tolvaptan treatment (0.13 mg/kg/day) on the eighth postoperative day. A written consent form was obtained from the parents for the use of tolvaptan.

Table 1. Laboratory findings of the patient

Analyte (normal range)	Preoperative	Postoperative
Free T4 (0.67-1.12 ng/dL)	0.99	0.47
TSH (0.5-5.5 mIU/L)	1.00	0.002
IGF-1 (192-568 ng/mL)	146.1	57.1
Prolactin (3.3-26.7 ng/mL)	5.92	4.43
ACTH (10-50 pg/mL)	19.5	7.42
Cortisol (6.7-22.6 mg/dL)	15.98	4.86
FSH (1.5-12.8 mIU/mL)	3.49	0.08
LH (0.10-12.0 mIU/mL)	1.83	0.02
Estradiol (7-60 pg/mL)	48	<5
Glucose (60-100 mg/dL)	88	83
BUN (5-22 mg/dL)	11	9
Creatinine (0.3-1 mg/dL)	0.51	0.53
Serum uric acid (1.9-5.4 mg/dL)	NA	1.1
Urine sodium (mmol/L)	NA	106
AST (15-40 U/L)	25	22
ALT (8-39 U/L)	24	28

NA: not available, TSH: thyroid-stimulating hormone, IGF-1: insulin-like growth factor-1, ACTH: adrenocorticotropic hormone, FSH: follicle-stimulating hormone, LH: luteinizing hormone, BUN: blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase

One hour after oral intake of Tolvaptan, the urine output and plasma sodium levels of the patient began to correct. Urinary output increased to 8.1 mL/kg/h, urinary density reduced to 1001. One dose of tolvaptan administered to the patient was sufficient to control SIADH and no further treatment was given. Moreover, desmopressin treatment was restarted because of the development of DI 42 hours after the administration of tolvaptan (plasma sodium 138 mmol/L, plasma osmolality 296 mOsm/kgH_2O, urinary output 6.6 mL/kg/h and urinary density 1002). The patient has had persistent DI on follow up which has required desmopressin therapy (Figure 1).

Discussion

Here we report successful tolvaptan administration in a patient who developed severe and uncontrolled hyponatremia due to SIADH. To our knowledge, this is the first report from Turkey of successful pediatric tolvaptan treatment.

Hypothalamus and/or tract damage due to neurosurgery or trauma may frequently result in a typical triphasic response (18,19,20). First, transient DI develops, beginning within 24 hours and lasting from four to five days. The DI is due to reflex inhibition of ADH release because of hypothalamic dysfunction. Following that, on days 6-10, a transient SIADH develops, caused by release of stored ADH from the disrupted posterior pituitary. Finally, DI reoccurs, after the posterior pituitary ADH stores are consumed. This third phase DI may be permanent or transient (19,20). Our patient exhibited this triphasic response.

There are two different approaches to managing central DI in patients who have undergone cranial surgery. The first approach is to employ fluids and avoid the use of vasopressin. This approach may be particularly useful for managing acute postoperative DI in young children. Vasopressin therapy may mask the emergence of the second SIAD phase of the triple phase neurohypophyseal response to neurosurgical injury (21). The other approach is treatment with vasopressin. Our patient was 13-years-old with a weight of 60.2 kg which is the same weight as some adults. For this reason, we preferred to use vasopressin for the DI, and did not observe the masking of the emergence of the SIAD when DI was thought to persist transiently for four or five days (19,20).

Also, a surgery of longer duration has been associated with developing the triphasic response (22). Our patient's surgery took about 10.5 hours. However, in the second transient SIADH phase, the patient developed severe and uncontrollable, symptomatic hyponatremia. For this reason, even though it was probable that this second phase was temporary, intervention was unavoidable because of the severe hyponatremia together with resistance to fluid restriction and hypertonic saline therapy. Vaptans were the most appropriate choice as an alternative treatment. We preferred to use tolvaptan in this case, which is a selective V2 receptor antagonist, as it may be more effective and cause fewer side effects. Her resistant hyponatremia improved dramatically and rapidly following a single low-dose tolvaptan administration.

Marx-Berger et al (6) reported the use of tolvaptan treatment in two infants with non-improving hyponatremia due to SIADH. The treatment was initiated at a dose of 0.8 mg/kg/day, and the dose was reduced to 0.22 mg/kg/day upon the onset of hypernatremia on the second day of treatment in one infant. Tolvaptan was used for seven months in one

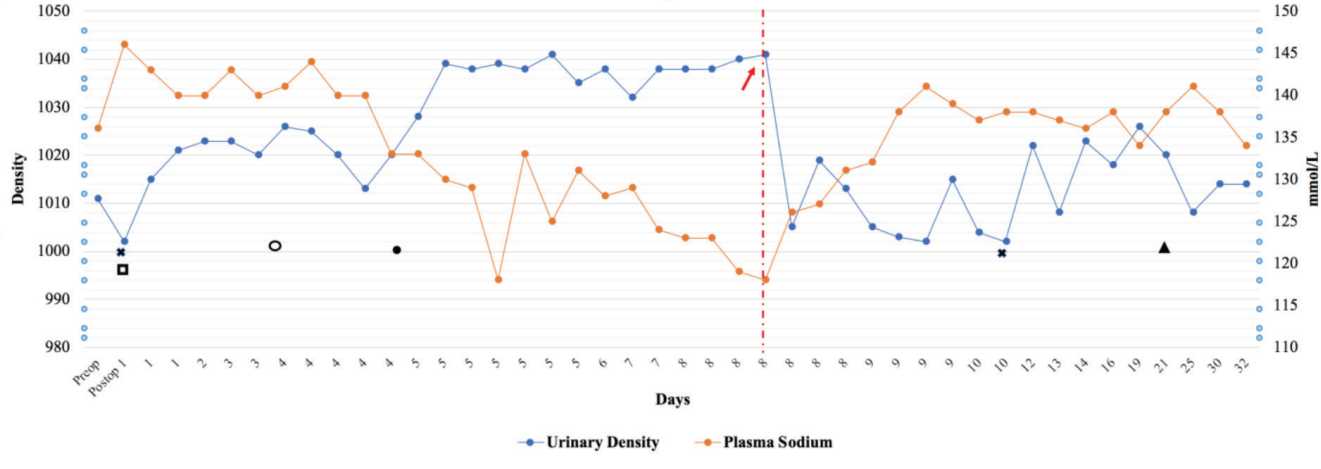

Figure 1. Plasma sodium and urinary output of the patient. Therapy is indicated by the following symbols on the chart: Cross-initiation of desmopressin; open square-initiation of dexamethasone; open circle-initiation of levothyroxine; point-desmopressin cessation; arrow-tolvaptan treatment; triangle-switching from dexamethasone to hydrocortisone

of the infants and for 13 months in the other, without any problems. In our patient, a single dose of tolvaptan at a dose of 0.13 mg/kg/day was sufficient, and in our patient, even desmopressin was started due to the development of hypernatremia and conversion of the condition to DI. In another article, tolvaptan treatment was given to three SIADH patients aged between four and seven years, at a dose of 0.05-0.3 mg/kg/day, and low dose tolvaptan treatment was continued for as long as 3-4 years without complications (5). Long-term therapy such as this can be given with confidence as the reported incidence of adverse effects is extremely low.

However, although the SIADH in our patient was very likely temporary and would return to DI, the final phase, spontaneously, her clinical condition was too severe to wait. Therefore, we accelerated the passage to the third phase with tolvaptan therapy. Finally, 42 hours after tolvaptan, desmopressin treatment for DI was started. No side effects were observed in our patient which could be attributed to tolvaptan.

For tolvaptan, time of onset of action for aquaretic and sodium increasing effects is two to four hours with a peak effect at between four and eight hours (23). Willemsen et al (3) suggested starting at a low dose to avoid rapid correction of hyponatremia. Furthermore, Peters et al (10) noted the correction of hyponatremia on the first day after conivaptan was given. In our patient, the aqauretic effect of tolvaptan began after only one hour following ingestion, despite the low dose. This was a fast and immediate effect after a single dose tolvaptan treatment. Therefore, patients need to be closely followed from the first hour in terms of both urine output and increase in serum sodium levels.

The use of tolvaptan or conivaptan therapy in childhood has still not been approved by FDA or the European Medicines Agency (EMA). The most important reason for this is that there is not enough clinical experience in terms of safety and effectiveness in children. We observed a successful treatment result with tolvaptan in a pediatric patient who suffered from SIADH. We report that only one low dose of tolvaptan in the triphasic episode was remarkably effective in correcting a serious clinical situation. This report of vaptan use, together with future cases, will increase the clinical evidence base in order for the FDA and EMA to decide the licensing status of this type of therapy.

Ethics

Informed Consent: A written consent form was obtained from the parents for the use of tolvaptan.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Fatih Gürbüz, Mehmet Taştan, İhsan Turan, Bilgin Yüksel, Concept: Fatih Gürbüz, Bilgin Yüksel, Design: Fatih Gürbüz, Bilgin Yüksel, Data Collection or Processing: Fatih Gürbüz, Mehmet Taştan, İhsan Turan, Bilgin Yüksel, Analysis or Interpretation: Fatih Gürbüz, Bilgin Yüksel, Literature Search: Fatih Gürbüz, Writing: Fatih Gürbüz, Bilgin Yüksel.

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CASE REPORT

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A Child with Prostaglandin I_2-associated Thyrotoxicosis: Case Report

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What is already known on this topic?

Continuous intravenous injection of epoprostenol prostaglandin I_2 (PGI_2) is an effective medication for patients with severe cardiac failure due to pulmonary artery hypertension. PGI_2 may cause the life-threatening side effect of hyperthyroidism with an incidence rate of 6.7%.

What this study adds?

We report the first pediatric case with portosystemic venous shunt syndrome, a patient who developed thyrotoxicosis after 10 years of prostaglandin I_2 (PGI_2) treatment. Prophylactic monitoring of thyroid function is mandatory for pediatric pulmonary artery hypertension patients undergoing PGI_2 treatment.

Abstract

Prostaglandin I_2 (PGI_2) causes hyperthyroidism, a critical complication in patients with pulmonary arterial hypertension (PAH). However, it remains unknown whether PGI_2 may have unfavorable effects on thyroid function in children with congenital portosystemic venous shunt syndrome (CPSVS). We present a boy with CPSVS who developed PAH at seven years of age. During ongoing PGI_2 therapy, he experienced thyrotoxicosis at 17 years of age. The literature review showed that the reported 12 patients with PAH (median 11 years of age) developed hyperthyroidism during between one and 11 years of PGI_2 treatment. Only one patient survived the acute PAH crisis due to hyperthyroidism. These data provide evidence that prophylactic intervention for hyperthyroidism is indicated for children with CPSVS during PGI_2 treatment.

Keywords: Prostaglandin I_2, pulmonary arterial hypertension, congenital portosystemic venous shunt syndrome, hyperthyroidism

Introduction

Pulmonary arterial hypertension (PAH) is a rare vascular disorder that has an annual incidence of 5 to 8 per million children under the age of 18 (1). With the advances in pharmacological management, the 5-year survival for PAH has risen to 60% over the past decades (2). Continuous intravenous injection of epoprostenol prostaglandin I_2 (PGI_2) has been used in patients with severe PAH (2). This medication has contributed to improving the prognosis of primary PAH. However, PGI_2 may cause a side effect of hyperthyroidism in 6.7% of the subjects (3). Thus, establishing the safest treatment strategies for PAH remains a challenge.

Here we report a 17-year-old boy with congenital portosystemic venous shunt syndrome (CPSVS), who developed severe hyperthyroidism during PGI_2 treatment. We also describe the demographic features of previously reported cases with PGI_2-associated hyperthyroidism by collecting their profiles from the literature.

Case Report

A 20-day-old male infant was referred to our hospital because of hypergalactosemia detected during neonatal mass screening test. He was diagnosed with congenital

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portal vein hypoplasia and CPSVS. At seven years of age, PAH was found on regular checkup using echocardiography. Continuous intravenous PGI_2 (47.2 ng/kg/min) was initiated at nine years of age. The administration of bosentan hydrate (62.5 mg/day) was added at age 10 years. The treatment strategy for his cardiac status was based on World Health Organization (WHO) functional class 2. The right ventricular systolic pressure, estimated from the moderate tricuspid regurgitation, was 80 mmHg on echocardiography. He underwent an assessment of thyroid function once at 16 years of age. The test results showed a low thyroid stimulating hormone (TSH) of 0.04 μU/mL, [reference range (rr): 0.27-4.20] and normal free T4 concentration of 1.42 ng/dL, (rr: 1.00-1.80).

At age 17 years, the patient was admitted to our hospital because of dyspnea, general fatigue and chest pain (WHO class 4). The body temperature was 37.5 ˚C and the heart rate was 120 bpm. On admission, his height was 162.4 cm [-1.1 standard deviation (SD)] and body weight was 44.1 kg (-1.8 SD) resulting in a body mass index of 16.4. Goiter was noted and the liver was palpable at 4.0 cm below the costal margin. Intensified pulmonic sounds with regurgitant systolic murmur was remarkable at the left sternal border. Cardiomegaly was evident on chest radiography. Echocardiography revealed severe tricuspid regurgitation with elevated right ventricular systolic pressure (120 mmHg). A unilateral enlargement of the thyroid gland was detected on ultrasonography with increased blood flow and the estimated thyroid weight was calculated as 3.1 g (right) and 16.7 g (left). Laboratory tests showed a C-reactive protein concentration of 1.8 mg/dL. Brain-type natriuretic peptide was 601.1 pg/mL (cut-off ≤18.4), TSH < 0.01 μIU/mL, free T4 at 6.35 ng/dL (rr: 1.00-1.80), thyroid stimulating antibody (TSAb) elevated to 2691% (rr: < 180%), TSH receptor antibody (TRAb) level was 10.7 U/L (rr: < 1.0 U/L) and thyroglobulin antibody level 1349.7 U/mL (rr: < 45 U/L).

Maximum doses of oral thiamazole, potassium iodide and intravenous hydrocortisone treatment failed to control the raging storm of hyperthyroidism. High-dose methylprednisolone therapy and destructive radioiodine (RI) (RI in Table 1) therapy were concurrently initiated on the 88th day of admission. Hyperthyroidism gradually improved after the combined therapy. PGI_2 was continued throughout the period of intensive care because PAH had been severe. When PAH started to improve, the estimated right ventricular pressure declined to 70 mmHg. The patient was discharged 132 days after admission (Figure 1). PAH has been controlled with euthyroid status thereafter. The patient has not received antithyroid therapy for more than four years although TSAb, TRAb and anti-thyroglobulin

Table 1. Clinical characteristics of pediatric pulmonary artery hypertension patients complicated with hyperthyroidism during PGI_2 treatment

Patient number	Sex	Age at diagnosis of PAH (years)	Age at diagnosis of	PAH severity (WHO functional class) at diagnosis of hyperthyroidism	Treatment of hyperthyroidism	Outcome	Reference
1	F	4	12	ND	MMI	Alive	Satoh et al (4)
2	F	4	15	ND	MMI	Alive	Satoh et al (4)
3	F	11	15	ND	MMI	Alive	Satoh et al (4)
4	M	11	19	ND	Observation	Alive	Satoh et al (4)
5	F	2	6	2	MMI	Alive	Trapp et al (5)
6	F	4	9	2	MMI	Alive	Trapp et al (5)
7	F	6	11	2	MMI	Alive	Trapp et al (5)
8	F	11	15	4	PTU, esmolol	Dead	Trapp et al (5)
9	F	11	15	4	PTU, esmolol, CS	Dead	Trapp et al (5)
10	F	14	18	4	Esmolol	Dead	Trapp et al (5)
11	F	16	17	4	PTU/MMI, SSKI, CS	Dead	Trapp et al (5)
12	F	17	19	4	PTU/MMI, SSKI, CS, esmolol, thyroidectomy	Alive	Trapp et al (5)
13	M	7	17	4	MMI, SSKI, CS, RI	Alive	The present case
Mean		9.2	14.7				
Median (range)		11 (2-17)	15.8 (6-19)				

F: female, M: male, WHO: World Health Organization, PAH: pulmonary artery hypertension, ND: not described in the literature, MMI: thiamazole or methimazole, CS: corticosteroids, PTU: propylthiouracil, RI: radioiodine, SSKI: saturated solution of potassium iodide

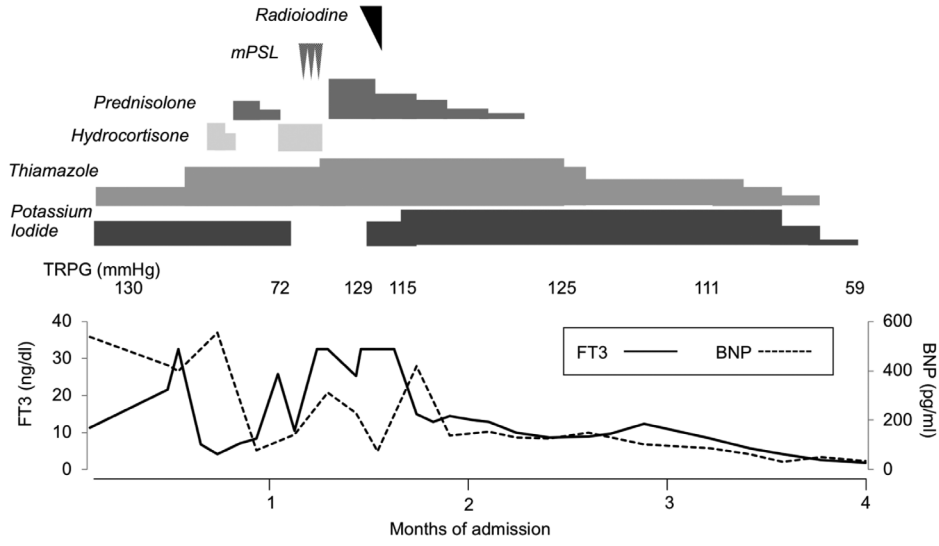

Figure 1. Treatment course of the present case after admission. Applied medications (italics) and their duration of treatment (blocks) are shown at the top. Radioiodine (410 MBq), methylprednisolone (1 g/day for three days), prednisolone (10-45 mg/kg/day), oral administration of thiamazole (15-75 mg/day) and potassium iodide (200-300 mg/day) were used to control the thyrotoxicosis. Echocardiography-based measurements of tricuspid regurgitation peak gradient are shown in the middle. Line charts at the bottom indicate the declining concentrations of free-T3 (reference range: 2.2-4.4 pg/mL) and brain natriuretic peptide (reference range: ≤18.4 pg/mL) over four months of intensive care for the present case

mPSL: methylprednisolone, PSL: prednisolone, TRPG: tricuspid regurgitation peak gradient, FT3: free-T3, BNP: brain natriuretic peptide

antibody levels continue to be abnormal. None of his family members were affected by autoimmune thyroiditis. He had no past history of other autoimmune disorders. He had never experienced hypoglycemia, hyperandrogenism or other metabolic attacks before and after this episode.

Written informed consent was obtained from the patient and his parents for the publication of this report.

Literature Review

We performed a literature search for patients under the age of 20 years who presented with hyperthyroidism during treatment with PGI_2. We found that 12 such cases had been reported in the years from 2010 to 2017 (4,5). Table 1 summarizes the clinical profiles of these 12 cases and compares with data from our patient (case 13 in Table 1). The median (range) age at diagnosis of PAH was 11 (2-17) years, while the hyperthyroidism developed at a median (range) age of 15.8 (6-19) years. Thus, duration to the development of PGI_2-associated thyroiditis varied widely from 1 to 11 years after the diagnosis of PAH. Four patients (31%) died of complications including cardiopulmonary dysfunction. We found that six (cases 8-13) among the 13 cases had severe cardiac dysfunction (WHO class 4). Although these six patients underwent thyroidectomy, propylthiouracil or RI therapies, only two (case 12 and the present case) survived the critical period.

Discussion

We described a case with exacerbated PAH during PGI_2 treatment. The literature review for the reported cases under 20 years of age indicated a high mortality rate (31%) for PAH patients when complicated by hyperthyroidism. Unfavorable prognosis of PAH was likely to be associated with the severity of cardiac dysfunction at the onset of hyperthyroidism.

PGI_2 regulates both innate and adaptive immune responses. Recent studies showed evidence that it accelerates the differentiation of naïve T cells into Th17 cells and enhances Th17 cell functions (4,6,7,8). The Th17-interleukin (IL)-17 axis may thus explain the mechanisms of PGI_2-associated hyperthyroidism and thyroiditis. Considering that the earlier 12 cases presented with hyperthyroidism years after the diagnosis of PAH, the pathogenic mechanisms were less likely to involve acute reactions to PGI_2. We speculate that deregulation of the physiological immune system by persistent exposure to PGI_2 in PAH patients might be one of the causes augmenting the pathogenesis of hyperthyroidism. Although we have not analyzed the population of Th17 cells or IL-17 in peripheral blood in our patient, serial immunological studies may detect the prodromal signs of hyperthyroidism in PAH patients.

Experimental studies demonstrated that PGI_2 regulates both innate and adaptive immune systems (9). PGI_2 analogs were also shown to inhibit proinflammatory responses to lipopolysaccharides in monocyte and macrophage populations (10). Notably, inflammatory macrophage populations were reported to be expanded in the lungs a mouse model of PAH (11). Thus, delineating the downstream signals to PGI_2 in the lung macrophage will be the key to understand its deleterious effects on thyroid functions. Among them, monocyte chemoattractant protein-1 (MCP-1/CCL2) is known as a downstream molecule following prostaglandin stimulation (12). Paradoxical effects of PGI_2 on thyroid functions might therefore result from differential MCP-1 synthesis in each tissue as a result of long-term treatments.

We considered that the exacerbation of PAH was a consequence not only of the increased cardiac outputs with hyperthyroidism, but also from the direct effect of thyroid hormone on proliferative vascular endothelial cells (13). Together with our case report, the literature review also supports the necessity of prophylactic monitoring and management of thyroid function for PAH patients undergoing PGI_2 treatment. Earlier intervention may prevent PAH patients from the progressive worsening of cardiac dysfunction. In this regard, prophylactic therapy might have been helpful if initiated in our patient at age 16 years, when he showed a low TSH concentration on thyroid testing. Future studies will clarify whether this alternative strategy might have changed the unfavorable outcomes of these patients.

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Ethics

Informed Consent: Written informed consent was obtained from the patient and his parents for the publication of this report.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Yuri Sonoda, Kenichiro Yamamura, Kanako Ishii, Kazuhirro Ohkubo, Kenji Ihara, Concept: Yuri Sonoda, Kenichiro Yamamura, Kenji Ihara, Design: Yuri Sonoda, Kenichiro Yamamura, Yasunari Sakai, Data Collection or Processing: Yuri Sonoda, Kenichiro Yamamura, Analysis or Interpretation: Yuri Sonoda, Kanako Ishii, Kazuhiro Ohkubo, Kenji Ihara, Shouichi Ohga, Literature Search: Yuri Sonoda, Kenichiro Yamamura, Yasunari Sakai, Writing: Yuri Sonoda, Kenichiro Yamamura, Kanako Ishii, Kazuhiro Ohkubo, Kenji Ihara, Yasunari Sakai, Shouichi Ohga.

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CASE REPORT

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Novel Compound Heterozygous Variants in the *LHCGR* Gene in a Genetically Male Patient with Female External Genitalia

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What is already known on this topic?

Both loss and gain of function mutations of the *LHCGR* gene can cause human diseases. Inactive *LHCGR* variant causes type 1 Leydig cell hypoplasia, which is characterized by the complete absence of male differentiation. To date, 77 variants have been reported, including 49 missense, 11 nonsense, five gross deletions, four small insertions, four small deletions, three splicing variants and one gross insertion.

What this study adds?

In this study, we identified two novel heterozygous variants in the *LHCGR* gene (c.349G > A, p.Gly117Arg and c.878C > A, p.Ser293*) causing type 1 Leydig cell hypoplasia in a 2.75 year old patient presenting with female external genitalia and bilateral testis tissue in the inguinal region.

Abstract

The *LHCGR* gene encodes a G-protein coupled receptor that plays a pivotal role in sexual differentiation in males, ovarian development in females and in fertility via its interaction with luteinizing hormone and chorionic gonadotropin. Inactive variants of the *LHCGR* gene cause Leydig cell hypoplasia (LCH), which is a rare disease and one of the causes of disorder of sexual differentiation (DSD) in males. The aim of this work was to clarify the clinical and molecular characteristics of a 2.75 year old patient with type 1 LCH. Whole exome sequencing was performed for the patient family and variants in the *LHCGR* gene were validated by Sanger sequencing. Pathogenicity of the missense variant was evaluated by multiple *in silico* tools. Our Chinese patient, who exhibited DSD, had female external genitalia (normal labia majora and minora, external opening of urethra under the clitoris and blind-ended vagina) and bilateral testis tissues in the inguinal region. Genetic sequencing revealed compound heterozygous variants in the *LHCGR* gene in the patient, including a novel missense variant in exon 4 (c.349G > A, p.Gly117Arg) and a novel nonsense variant in exon 10 (c.878C > A, p.Ser293*). The missense variant is in the first leucine-rich repeat domain of the *LHCGR* protein, which is predicted to affect ligand recognition and binding affinity and thus protein function. The patient is molecularly and clinically diagnosed with type 1 LCH, which is caused by novel, compound heterozygous variants of the *LHCGR* gene. We believe this report will serve to expand the genotypic spectrum of *LHCGR* variants.
Keywords: Disorder of sexual differentiation, Leydig cell hypoplasia, *LHCGR* gene, novel variants

Introduction

The human luteinizing hormone (LH)/chorionic gonadotropin (CG) receptor (*LHCGR*; OMIM #52790) gene belongs to the G-protein coupled receptor 1 family. The *LHCGR* gene encodes a shared receptor for both LH and CG and the receptor plays a critical role in male sexual differentiation, female ovarian development and fertility (1). *LHCGR* is located on chromosome 2p21 and contains 12 exons. The *LHCGR* gene encodes a 699 amino acid protein that consists of an N-terminal cysteine-rich region, a tandem leucine-rich repeats (LRRs) region and a C-terminal cysteine-rich region (2,3). In males, the N-terminal region and the LRR1-LRR7 repeats are essential for the high affinity binding of human CG (hCG), which stimulates the production of testosterone and maturation of fetal Leydig cells during early embryogenesis. In addition, the interaction between LH and *LHCGR* maintains a postnatal testosterone level

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that is required for male secondary sex characteristics and spermatogenesis during puberty (4,5).

Both loss and gain of function mutations of the *LHCGR* gene can cause human diseases. In males, germline activation of *LHCGR* is associated with inherited, autosomal dominant precocious puberty (OMIM#152790). Biallelic inactivation of the *LHCGR* causes Leydig cell hypoplasia (LCH, OMIM#238320) that leads to male disorders of sexual differentiation (DSD). Constitutively inactive *LHCGR* variant causes type 1 LCH, which is characterized by the complete absence of male differentiation. Partially inactive *LHCGR* variants result in type 2 LCH that features hypogonadal phenotypes with variable severity (6,7). In females, inactivated *LHCGR* gene has no effect on the primary and secondary sex characteristics, but it causes amenorrhoea and infertility due to aberrant follicular maturation and ovulation (8).

In this study, we report a rare pediatric patient of type 1 LCH due to novel, compound heterozygous mutations in the *LHCGR* gene. Our findings expanded the spectrum of genotype-phenotype correlation in the *LHCGR* variants.

Case Report

The proband was a 2.75 year old child whose social gender was female. The child was taken to our hospital due to absence of vagina. The patient was born full term by spontaneous delivery, and she is the second child of healthy parents of non-consanguineous marriage. Her birth weight was 3,900 g. Her weight at presentation was 17 kg (96.8th percentile) and her height was 97 cm (73.5th percentile). Physical examination showed that the patient exhibited predominantly female external genitalia, with normal bilateral labia majora, bilateral labia minora and external opening of urethra under the clitoris. However, she had a blind-ended vagina without external opening. The patient showed absence of scrotum and penis. Abdominopelvic ultrasound examination detected bilateral testis tissues in the inguinal region (left 2.0 cm × 0.7 cm × 0.9 cm; right 1.7 cm × 0.7 cm × 0.9 cm). Uterus or other Mullerian structures were not observed. Laboratory results showed that the patient had extremely low serum testosterone and dihydrotestosterone levels (0.01 nmol/L), which could not be stimulated by hCG. Serum levels of LH and follicle stimulating hormone were within the normal ranges (3.84 IU/L and 9.09 IU/L, respectively) and both of were hyper-responsive (24.48 IU/L and 22.33 IU/L, respectively) to stimulation with 2.5 μg/kg of LH releasing hormone. Thyroid hormones, estradiol, prolactin, blood chemistry and complete blood count were all normal. Primary genetic analysis revealed that the patient's karyotype was 46, XY and no pathogenic variant was identified in the *SRY* gene. The patient was primarily diagnosed as a case of male pseudohermaphroditism.

All procedures followed were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000, and the protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no: XJMU-FAHIRB-2017005). Informed consent was obtained from the patient's family.

Genetic Sequencing

To obtain a rapid and accurate clinical genetic diagnosis, trio-whole exome sequencing (WES) was used to screen for causal variants. Briefly, a total of 3 μg of genomic DNA was sheared to obtain DNA fragments with sizes between 150 bp and 200 bp. The capture library was prepared using SureSelect Human All Exon V6 kit (Agilent Technologies Inc., Santa Clara, CA, US) following the manufacturer's protocol. Next, clusters were generated by isothermal bridge amplification with an Illumina cBot station and sequencing was performed by an Illumina X10 System (Illumina, CA, USA). Alignment of sequence reads to the reference human genome (Human 37.3, SNP135) was performed using the NextGENe® software (SoftGenetics, PA, USA). All single nucleotide variants (SNVs) and indels were saved in a VCF format file, which was then uploaded to Ingenuity® Variant Analysis™ (Ingenuity Systems, CA, USA) for biological analysis and interpretation. The variants were validated by Sanger sequencing using the ABI3730XL sequencer (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the forward and reverse primers. The potential pathogenicity of the missense variant was analyzed by using MultAlin (http://multalin.toulouse.inra.fr/multalin/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/), and MutationTaster (http://www.mutationtaster.org/).

Identification of the Causal Variants

For the patient, WES yielded a total of 103,509,228 reads, and the mean target coverage was 133 reads with 95.52% having 20× coverage and 99.83% having 1× coverage. The candidate variants were first filtered by the following parameters: (1) minor allele frequency (MAF) under 1% in genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/); (2) the benign variants, including synonymous and harmless missenses predicted by

Ingenuity and those predicted to have no impact on splicing by MaxEntScan. Subsequently, clinical symptoms of male pseudohermaphroditism were used as filtering indexes to analyze the candidate variants. As a result, we identified a compound alteration with two heterozygous variants within the *LHCGR* gene, which we believe to have contributed to the patient's condition. Of the two variants, one is a novel missense variant in exon 4 (c.349G>A, p.Gly117Arg), and the other was a novel nonsense variant in exon 10 (c.878C>A, p.Ser293*). We have further confirmed the compound heterozygous variants by Sanger sequencing. The patient's father was heterozygous for the nonsense variant and the patient's mother was heterozygous for the missense variant (Figure 1A).

Pathogenicity Predictions for c.349G>A (p.Gly117Arg)

To evaluate the pathogenicity of the novel variant c.349G>A, we first analyzed the conservation of Gly117 using MultAlin software. As shown in Figure 1B, results from MultAlin show that the amino acid glycine at codon 117 is highly evolutionarily conserved. Next, we used three *in silico* prediction software analyses to evaluate the impact of the variant on protein function. The PolyPhen-2 score of the variant is 0.96, indicating that the variant is probably damaging. The MutationTaster score is 1, which implies that the variant is likely disease causing. The CADD score is 25.4, which suggests that the variant can be damaging. To better understand the missense variant, the WT and variant amino acid at codon 117 were modeled into the three-dimensional structure of the *LHCGR* protein (9) (Figure 2). Based on the structure (9) and domain information of the *LHCGR* wild-type protein obtained from Uniprot (http://www.uniprot.org/), the amino acid substitution at the 117th position *(p.Gly117Arg)* was predicted to disrupt the first LRR domain, which may affect recognition and binding affinity of *LHCGR* to hCG and/or other ligands. Taken together, our analysis results indicate that the *c.349G>A (p.Gly117Arg)* variant is likely harmful to the protein function.

Discussion

In the current study, we report a socially defined female, Chinese patient presenting with a DSD. The patient had normal labia majora and minora, external opening of urethra under the clitoris, but a blind-ended vagina, a karyotype of

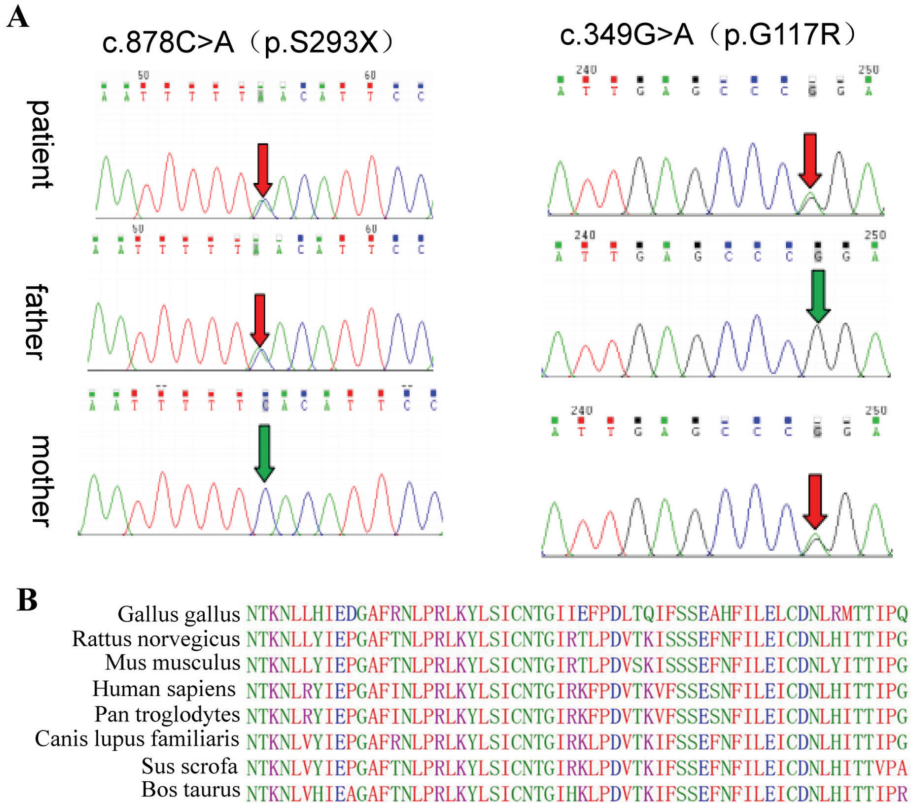

Figure 1. Genetic sequencing of the *LHCGR* gene. (A) Sanger sequencing confirmed a novel heterozygous missense variant in exon 4 (c.349G>A, p.Gly117Arg) and a novel heterozygous nonsense variant in exon 10 (c.878C>A, p.Ser293*) in the patient, which were inherited from the parents. (B) The referred amino acid of codon 117 (Gly) is highly evolutionarily conserved across species

Table 1. A summary of the inactivating variants of *LHCGR* gene

Patient	Variant	AF	Position	Phenotype	Reference
1	c.340A > T; p.I114F (het)*	0	Exon 4	Pseudohermaphroditism (46, XY)	11
2	c.391T > C; p.C131R (hom)	0	Exon 5	Pseudohermaphroditism (46, XY)	11
3	c.430G > T; p.V144F (hom)	0.0012 %	Exon 5	Pseudohermaphroditism (46, XY)	11
4	c.455T > C; p.I152T (het)	0	Exon 5	Pseudohermaphroditism (46, XY)	11
	c.537-3C > A (het)	0	Intron 6		11
5	c.508C > T; p.Q170* (hom)	0	Exon 6	Primary amenorrhea	12
6	c.562G > T; p.E188* (hom)	0	Exon 7	Female external genitalia	13
7	c.580T > G; p.F194V (hom)	0.00041 %	Exon 7	Pseudohermaphroditism (46, XY)	11
8	c.774dupA; p.L259Ifs25* (hom)	0.0012 %	Exon 9	46, XY disorder of sex development	14
9	c.907C > T; p.Gln303Ter (hom)	0	Exon 10	Pseudohermaphroditism (46, XY)	15
10	c.948-1G > A (hom)	0.00041 %	Intron 10	Micro penis (46, XY)	11
11	c.1027T > A; p.C343S (het)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
	c.1627T > C; p.C543R (het)	0	Exon 11		11
12	c.1060G > A; p.E354K (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
13	c.1121T > C; p.I374T (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
	c.1175C > T; p.T392I (hom)	0.00041 %	Exon 11		11
14	c.1199A > G; p.N400S (hom)	0	Exon 11	Empty follicle syndrome	11
15	c.1244T > C; p.I415T (het)	0	Exon 11	Micro penis (46, XY)	16
	c.580 T > C (het); affecting splicing	0	Exon 7		16
16	c.1395G > A; p.W465* (hom)	0	Exon 11	Primary amenorrhea	17
17	c.1435C > T; p.R479* (hom)	0.00041 %	Exon 11	Primary amenorrhea	12
18	c.1448C > A; p.A483D (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	18
19	c.1473G > A; p.W491* (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	6
20	c.1505T > C; p.L502P (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
21	c.1573C > T; p.Q525* (hom)	0	Exon 11	Primary amenorrhea	12
22	c.1582_1585del; p.I528* (het)	0	Exon 11		5
	c.681-1G > A (het)	0	Intron 8		5
23	c.1635C > A; p.C545* (hom)	0.0011 %	Exon 11	Leydig cell hypoplasia	19
24	c.1660C > T; p.R554* (hom)	0.00041 %	Exon 11	Luteinizing hormone resistance	1
25	c.1757_1758del;p.S586Ffs*19 (het)	0	Exon 11	Infertility	20
	c.34_60; p.K12_P20del (het)	0.00080 %	Exon 1		20
26	c.1764dupT; p.A589Cfs*17 (hom)	0.00041 %	Exon 11	Pseudohermaphroditism (46, XY)	21
27	c.1777G > C; p.A593P (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
28	c.1824_1829del; p.V609_L610del (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
29	c.1836T > G; p.Y612* (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	11
30	c.1847C > A; p.S616Y (hom)	0.0020 %	Exon 11	Micro penis (46, XY)	12
31	c.1850delG; p.C617Lfs*22 (hom)	0	Exon 11	Pseudohermaphroditism (46, XY)	8
32	c.1874T > A; p.I625K (hom)	0	Exon 11	Micro penis (46, XY)	11
34	Del Exon10 (hom)	0		Pubertal development, small testicles, and delayed bone maturation	22
35	c.349G > A; p.G117R (het)	0	4	Pseudohermaphroditism (46, XY)	This study
	c.878C > A; p.S293* (het)	0	10		This study

AF: allele frequency in gnomAD (http://gnomad.broadinstitute.org/), het: heterozygous, hom: homozygous, del: deletion

*Only heterozygous p.I114F was identified in the patient, there should be another heterozygous variant in the patient, such as exon deletion.

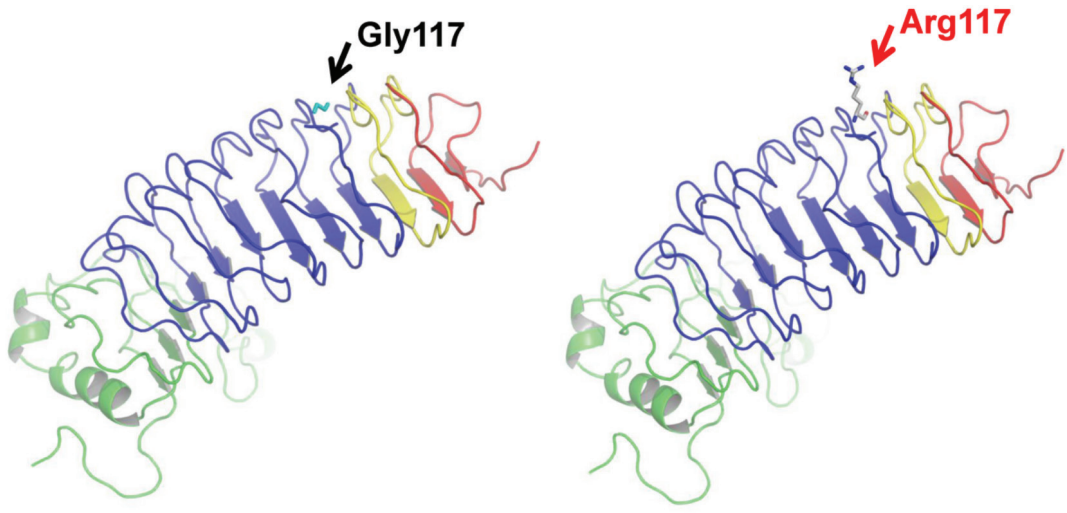

Figure 2. Three-dimensional structure model of the LHCGR protein. The indicated amino acid (p.117, colored arrow: black, wild-type; red, variant) is located in the first leucine-rich repeat domain of the LHCGR protein

46, XY and bilateral testis tissues in the inguinal region. By performing WES, we identified a compound heterozygous variant in the patient, with a novel missense variant (c.349G > A, p.Gly117Arg) and a novel nonsense variant (c.878C > A, p.Ser293*) in her *LHCGR* gene that contributed to the patient's condition. The missense and nonsense variants were inherited from the unaffected heterozygous father and mother, respectively. According to the variant interpretation guidelines from the American College of Medical Genetics and Genomics/the Association for Molecular Pathology (10), the nonsense variant is classified to be pathogenic (PVS1 + PM2 + PP4), and the missense variant is also likely to be pathogenic (PM2 + PM3 + PP3 + PP4). Therefore, the patient was molecularly and clinically diagnosed with type 1 LCH.

Gender assignment for LCH patients is influenced by genital appearance, surgical options, fertility potential and the views of the family, and can be difficult (11). Timing of gender assignment can also be controversial, especially when the psychological age is taken into consideration. The social gender of our patient was female. We assessed that the patient's psychological gender was also female. Physically, the patient's abnormal testis tissues showed no function in the provocation test using hCG. Based on medical advice from experts and discussions with the parents, the patient underwent bilateral orchidectomy. Testicular histology revealed that the seminiferous tubules were lined only by a few Sertoli cells. The interstitial region appeared to have only a few fusiform cells that appeared to be immature Leydig cells (Figure 3), a finding which is consistent with LCH phenotypes and confirmed the diagnosis of LCH. Interestingly, several previously reported cases showed that delayed orchidectomy after adolescence might result in primary amenorrhea and breast underdevelopment (12,13). For these reasons, orchidectomy was performed in our patient right after the gender assignment, and normal development of secondary female characteristic is expected in the future.

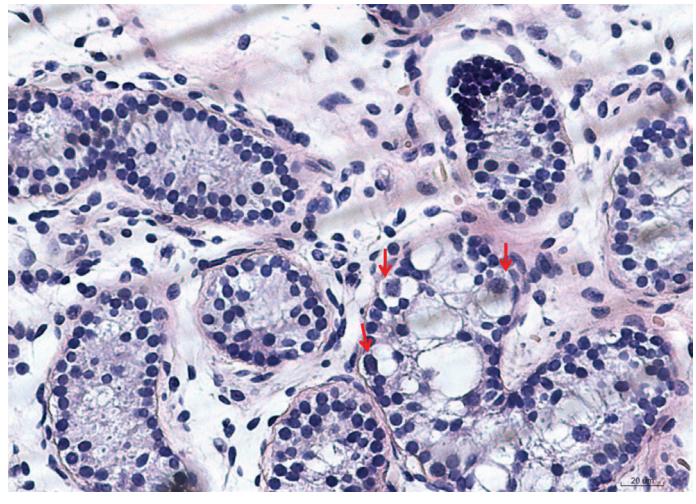

Figure 3. Histologic analysis of the surgical testicular tissue samples (400x). Hematoxylin and eosin staining revealed that the seminiferous tubules were lined only by a few Sertoli cells, and the interstitial tissue appeared to have only a few fusiform cells that might be immature Leydig cells

To date, a total of 77 variants have been identified in the *LHCGR* gene (Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/), including 49 missenses, 11 nonsenses, five gross deletions, four small insertions, four small deletions, three splicing variants and one gross insertion. In contrast to the infrequency of activating variants, inactivating homozygous and compound heterozygous variants that

alter structure of the *LHCGR* protein and subsequently its function is more common. As shown in Table 1, we summarized the inactivating variants from the reports in the literature (14,15,16,17,18,19,20,21,22,23,24,25). Interestingly, although the frequency of these variants is extremely low in gnomAD database (most of them are 0), homozygous variants account for most gene variations in the patients. Variants occurring more frequently in exon 11 may be simply explained by the fact that it is the largest exon of the *LHCGR* gene.

In our case, the nonsense variant (p.Ser293*) is a loss of function mutation, and our analysis shows that the missense variant (p.Gly117Arg) is mostly likely also a loss of function mutation, which lead to the inactivation of *LHCGR*. However, the functional analysis is lacking and this should be performed in a future study.

We report a 46, XY, DSD Chinese Uyghur patient with type 1 LCH with novel heterozygous compound variants in the *LHCGR* gene. Her clinical features correlated with the molecular diagnosis. She was treated after choosing her social gender to be female. This is one of only a few LCH cases that underwent gender assignment and treatment following molecular confirmation of clinical diagnosis.

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We are deeply grateful to the patient and the patient's family, for their participation in this study.

Ethics

Informed Consent: Obtained from the patient's family.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Mei Yan, Maimaiti Mireguli, Concept: Maimaiti Mireguli, Yiping Shen, Design: Maimaiti Mireguli, Yiping Shen, Data Collection or Processing: Mei Yan, Julaiti Dilihuma, Analysis or Interpretation: Mei Yan, Maimaiti Mireguli, Literature Search: Yanfei Luo, Baoerhan Reyilanmu, Writing: Mei Yan.

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Diazoxide Causality Assessment of a Pericardial Effusion in a Child with Kabuki Syndrome

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To the Editor,

A 15 month-old girl with KS (KDM6A mutation) was referred to our tertiary care paediatric cardiology centre for respiratory and hemodynamic distress. Her medical history involved congenital hypothyroidism treated from birth by levothyroxine in addition to congenital hyperinsulinism and renal malformations. She had been treated by diazoxide for 10 months in a dose of 10 mg/kg/d with a 5% maltose dextrin diet, under correct glycaemic monitoring. The pediatric cardiologist confirmed by echocardiography the absence of any congenital heart disease (CHD) or pulmonary hypertension (PH), but diagnosed a severe pericardial effusion. Ibuprofen and colchicine were started, successively, but had no effect. On day 12, a pericardial puncture was performed. The results of routine etiological assessment for pericarditis (brain natriuretic peptide, troponin, thyroid status, and common viruses) were negative. On day 16, the diazoxide was suspended and a continuous diet with 10% maltose dextrin was introduced. The pericardial effusion started to regress on day 25 and disappeared on day 28. On day 29, in order to preserve the patient's pancreatic function, diazoxide was reintroduced in a low dose (3 mg/kg/d) under close echocardiographic monitoring. On day 34 (5 days after diazoxide was reintroduced), a 3-mm pericardial effusion was diagnosed. The diazoxide was then increased to 4.5 mg/kg/d and food intake was decreased to 5% maltose dextrin. On day 36, the pericardial effusion was noted to be stable (3 mm). However, on day 47, e.g. 19 days after reintroduction of diazoxide, pericardial effusion significantly increased to 6 mm. Therefore, diazoxide was definitively stopped. On day 52, e.g. five days after diazoxide was stopped, the echocardiography showed a regression of the pericardial effusion. Currently, after a six-month follow-up since diazoxide was suspended, echocardiography assessments have revealed normal results.

We report a new case with an adverse event of diazoxide. This patient, a 15-month-old girl with Kabuki syndrome (KS), developed a severe pericardial effusion following diazoxide. We performed, for the first time, a causality assessment of this drug toxicity and found a high probability of a causal relationship between diazoxide and pericardial effusion. Indeed, after the treatment was suspended, the pericardial effusion regressed and reappeared when diazoxide was reintroduced. Using the Naranjo algorithm, the adverse drug reaction probability scale total score was rated at 10 (e.g. the reaction is considered definite if the score is 9 or higher) (1).

KS is a rare multiple congenital malformation syndrome, in which CHD have been described. However, pericardial effusion is not a common complication. Two cases of potential toxicity of diazoxide with pericardial effusion have been previously reported (2,3). A recent retrospective study on 295 children with congenital hyperinsulinism reported that 2.4% were diagnosed with PH after diazoxide initiation (4). Indeed, pericardial effusion may occur in severe PH, however, but our patient did not present with PH. Another drug with a similar action, the minoxidil, was suspected to be associated with pericardial effusion, that may be explain the cardiac toxicity of the drug (5).

We recommend monitoring patients with KS under diazoxide with echocardiography to detect pericardial effusion.

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Erişkinlerde: Çocukluk döneminde başlayan BHE: Üçten fazla hipofiz hormonu eksikliği olanlarda, tanımlanmış bir genetik sebebe, yapısal hipotalamo-hipofizer anomalilere, santral sinir sistemi tümörlerine veya yüksek doz kraniyal ışınlamaya bağlı şiddetli BHE olan kişilerde ya da hipotalamo-hipofizer hastalık veya yetmezliğine sekonder BHE'li kişilerde, eğer büyüme hormonu tedavisini bıraktıktan en az 4 hafta sonra IGF-I < -2 SSS ise test gerekli değildir. 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; gebelik ve emzirme; kalp cerrahisi ve abdominal cerrahiyi takiben akut yaşamsal hastalık komplikasyonları, kazaya bağlı çoklu travma, akut solunum yetmezliği veya benzer durumları; somatropine ya da bileşimindeki maddelerden herhangi birisine aşırı duyarlılık; kronik böbrek yetmezliği olan çocuklarda böbrek transplantasyonu; epifizleri kapanmış çocuklar. **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 eksikliği: 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 yaşına 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. 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 anne sütüne geçme olasılığı göz ardı edilemez. **Yan Etkiler/Advers Etkiler:** Erişkinlerde periferik ödem, baş ağrısı, parestezi, artraljieklem 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. 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 28 gün saklayınız. Işıktan koruyunuz. Dondurmayınız. Ürün, alternatif olarak, 25°C'nin altında maksimum 21 gün saklanabilir. **Ruhsat Sahibi:** Novo Nordisk Sağlık Ürünleri Tic. Ltd. Şti. Nispetiye Cad. Akmerkez E3 Blok Kat 7 34335 Etiler – İstanbul. Ruhsat Tarihi ve No: Norditropin® SimpleXx® 5mg; 07.01.2002-111/56, Norditropin® SimpleXx® 10mg; 25.12.2001-111/45, Norditropin® SimpleXx® 15mg; 25.12.2001-111/44 Yalnız reçete ile kullanılmalıdır. **Perakende satış fiyatı:** Ürünün güncel fiyatı için lütfen firmamıza başvurunuz. Kısa Ürün Bilgisi Yenilenme Tarihi: 09.10.2018. Norditropin®, SimpleXx® ve NordiPen® Novo Nordisk'in ticari markalarıdır. Daha geniş bilgi için firmamıza başvurunuz.

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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 girişim 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 girişim 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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