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Análise molecular do gene receptor de andrógeno em pacientes e familiares com Síndrome de Insensibilidade aos Andrógenos

MARCELO SOUZA DE ANDRADE

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Tese apresentada ao Programa de Pós-Graduação da Rede Nordeste de Biotecnologia- RENORBIO na Área de Concentração em Biotecnologia e Saúde

Orientadora: Emygdia Rosa Leal Mesquita

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia - RENORBIO na Área de Biotecnologia e Saúde – Diagnósticos moleculares.

A Banca Examinadora da Defesa da Tese de Doutorado considerou o candidato aprovado em 25/04/2012

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“Somente se aproxima da perfeição quem a procura com constância, sabedoria e, sobretudo humildade. Saber cada dia um pouco mais e usar este conhecimento todos os dias para o bem, esse é o caminho dos verdadeiros cidadãos”

Jigoro Kano

RESUMO

Introdução. A Síndrome de Insensibilidade Androgênica (AIS) é uma doença rara (1:20.000 a 1:64.000), de transmissão ligada ao cromossomo X, que gera um distúrbio da diferenciação sexual do feto masculino (XY) com um espectro de fenótipo que varia desde o feminino completo (CAIS) até um fenótipo masculino com discretos sinais de insensibilidade androgênica. Um número crescente de mutações tem sido catalogadas e quase 500 mutações já foram relacionadas à CAIS e cerca de 1000 ao gene do receptor androgênico. O gene *AR* localiza-se em Xq11-12, com 8 exons, com cerca de 919 aminoácidos. **Objetivo.** Caracterizar as mutações no gene *AR* em famílias da região do "Bico do Papagaio", no sudoeste do Estado do Maranhão. **Metodologia.** Foram utilizadas técnicas de biologia molecular como extração de DNA, PCR, Eletroforese, Purificação de produtos de PCR e Sequenciamento automático. Além disso, foram analisados o quadro clínico e hormonal de 14 pacientes e de seus familiares. **Resultados.** Em uma das famílias (com duas gêmeas afetadas), foi encontrada a mutação R753X, sendo o terceiro diagnóstico molecular de CAIS em gêmeas descrito no Mundo. Em outra família, com 12 pacientes, foi a identificada uma mutação nova no exon 8, descrita como P893A, na proteína AR. **Conclusão.** Este trabalho possibilitou a aplicação de técnicas moleculares para o diagnóstico preciso de AIS, aconselhamento genético aos familiares das pacientes afetadas, além de contribuir para a formação de recursos humanos mais qualificados, visando o desenvolvimento da biotecnologia no Estado do Maranhão.

Palavras chaves: Gene *AR*, Andrógenos, CAIS, Mutação, Sequenciamento

ABSTRACT

Introduction. The androgen insensitivity syndrome (AIS) is a rare disease (1:20,000 to 1:64.000)-linked X chromosome, which generates a disorder of sexual differentiation of the male fetus (XY) with a spectrum of phenotypes ranging from females complete (CAIS) to a male phenotype with discrete signs of androgen insensitivity. An increasing number of mutations have been cataloged and nearly 500 mutations have been related to CAIS and 1000 to the androgen receptor gene. The *AR* gene is located on Xq11-12, with eight exons, about 919 amino acids. **Objective**. To characterize the mutations in the *AR* gene families in the region of the "Bico do Papagaio" in the southwestern state of Maranhao. **Methodology**. We used molecular biology techniques such as DNA extraction, PCR, electrophoresis, purification of PCR products and sequencing. In addition, we analyzed the clinical and hormonal characteristics of 14 patients and their families. **Results**. In one family (with two twin affected), we found the mutation R753X and the third molecular diagnosis of CAIS in twins described in the World. In another family, with 12 patients, was identified a new mutation in exon 8, as described P893A, protein AR. **Conclusion**. This work enabled the application of molecular techniques for the accurate diagnosis of AIS, genetic counseling for relatives of affected patients, and contribute to the formation of more qualified human resources, aiming at the development of biotechnology in the state of Maranhão.

Keywords: *AR* Gene, Androgens, CAIS, Mutation, Sequencing

SUMÁRIO

LISTA DE SIGLAS E ABREVIAÇÕES

AIS	*Androgen Insensitivity Syndrome* – Síndrome de Insensibilidade aos Androgênios
AR	*Human Androgen Receptor* - Receptor de Androgênios Humano
AR	Gene que codifica o Receptor de Andrógenos
ARE	*Androgen Response Element* **–** Elemento de Resposta aos Andrógenos
CAIS	*Complete Androgen Insensitivity Syndrome* - Síndrome de Insensibilidade Completa aos Androgênios
cDNA	*complementary DNA* - DNA complementar
DBD	*DNA Binding Domain* - Domínio de ligação ao DNA
DSD	*Disorders of Sex Development* – Distúrbios de diferenciação sexual
DHT	*Dihydrotestosterone* - Dihidrotestosterona
HAM	*Anti-Müllerian Hormone* - Hormônio anti-Mülleriano
Hinge	Região de dobradiça do receptor de andrógenos
LBD	*Ligand Binding Domain* - Domínio de ligação aos androgênios
LH	*Luteinizant Hormone* - Hormônio luteinizante
MAIS	*Mild Androgen Insensitivity Syndrome* - Síndrome de Insensibilidade discreta aos Androgênios
missense	Mutação de substituição que muda o aminoácido
NTD	*N-terminal domain* - Domínio amino-terminal
PAIS	*Parcial Androgen Insensitivity Syndrome*- Síndrome de Insensibilidade Parcial aos Androgênios
Pro893Ala	Mutação de substituição, na qual o aminoácido prolina é substituído por uma alanina no códon 893 da proteína do receptor de andrógenos

LISTA DE FIGURAS

1 INTRODUÇÃO

A síndrome de insensibilidade aos andrógenos foi inicialmente relatada por Morris et al. (1953) como síndrome de feminilização testicular após analisar 82 casos de mulheres inférteis que apresentavam fisionomia e perfil psicológico de mulher, denominada de Síndrome dos Testículos Feminilizantes devido à presença de características sexuais bem desenvolvidas como genitálias externa feminina, sem pêlos sexuais; o corpo apresentava silueta tipicamente feminina e desenvolvimento mamário normal.

O mecanismo que leva ao aparecimento da doença foi mais bem esclarecido, provando que o responsável clínico é uma resistência ao andrógeno e não uma deficiência androgênica como se acreditava, alterando o nome para Síndrome de Insensibilidade aos Andrógenos (AIS) (OAKES et al., 2008).

No complexo processo de diferenciação sexual estão envolvidos um grande número de hormônios, receptores hormonais, enzimas e fatores de transcrição. Alterações nos genes que codificam quaisquer destas substâncias culminam em comprometimento da diferenciação sexual natural em graus variados (DOMENICE et al., 2002).

As modificações que conduzem aos distúrbios de desenvolvimento sexual (DSD) referem-se a diversas condições congênitas em que há diferenciação sexual atípica, o que ocorre em diversas formas: gonadal, anatômica, gênica ou cromossômica (MENDONÇA et al., 2009).

Alterações no gene do receptor de andrógenos (*AR)* resultam em falha da virilização de um embrião XY, sendo tal condição rara, com incidência estimada entre 1:40.000 e 1:99.000, o que pode conduzir os afetados à síndrome de insensibilidade aos andrógenos (SCOTT et al., 2006; TADOKORO et al., 2009).

A ação dos andrógenos é indispensável para a expressão do fenótipo masculino. Os dois andrógenos mais importantes e que têm papéis específicos durante a diferenciação sexual: a testosterona que age na diferenciação dos ductos de Wolff em epidídimo, ducto deferente e vesícula seminal, enquanto a dihidrotestosterona (DHT)

conduz à diferenciação dos genitais externos, agindo sobre o tubérculo genital e seio urogenital. Ambos se ligam ao mesmo receptor que, adequadamente ativado, auxilia a ação hormonal (BRINKMANN, 2001).

Como os andrógenos são responsáveis pelo fenótipo masculino, os indivíduos com mutações neste gene apresentam um amplo espectro de fenótipos, variando desde o fenótipo feminino sem sinais de virilização promovendo resistência completa aos andrógenos (CAIS) a fenótipos com graus variáveis de virilização (PAIS), ou mesmo apenas esterilidade (ELHAJI et al., 2004). Mais recentemente, uma nova classificação foi proposta para casos discretos de sinais de falha da ação androgênica que se denomina MAIS (PETROLI et al., 2011; SCOTT et al., 2006; TADOKORO et al., 2009).

O Receptor de Andrógenos (AR) faz parte de uma família de fatores de transcrição nuclear, que estão agrupados não apenas pela sequência homóloga, mas também pela capacidade destes receptores ativarem a transcrição de genes-alvo através do mesmo elemento de resposta hormonal (QUIGLEY et al., 1995; MELO et al., 2005). Assim como outros receptores desta família, o AR, após a formação do complexo hormônio receptor, interage diretamente com os genes-alvo para regular a transcrição dos mesmos (MELO et al., 2005).

Alterações neste receptor oriundas de mutações nas repetições de nucleotídeos correspondentes ao domínio N-terminal, e também estão associadas à atrofia muscular bulbar espinhal, câncer de próstata, câncer de mama, câncer de endométrio, câncer colo retal e, possivelmente, a alguns casos de infertilidade masculina (GOTTLIEB et al., 2004a; MESQUITA, 2009).

O gene do receptor de andrógenos (*AR)* faz parte de uma família de fatores de transcrição nuclear, que estão agrupados não apenas pela sequência homóloga, mas também pela capacidade destes receptores ativarem a transcrição de genes-alvo através do mesmo elemento de resposta hormonal (QUIGLEY et al., 1995; MELO et al., 2005). Assim como outros receptores desta família, o receptor de andrógenos (AR), após a formação do complexo hormônio receptor, interage diretamente com os genes-alvo para regular a transcrição dos mesmos (MELO et al., 2005).

Cerca de 750 mutações no gene *AR* já foram descritas na literatura e podem ser divididas em duas categorias principais (OAKES et al., 2008). A primeira compreende as mutações que interrompem ou alteram substancialmente a sequência primária da proteína e podem ser causadas pela introdução de códons de parada prematura, por inserções ou deleções de nucleotídeos que conduzem a alteração no quadro de leitura, por alterações no mecanismo de *splicing* do RNA, ou ainda por deleções de grandes segmentos do gene. A segunda categoria, mais comum, compreende as mutações que levam a substituição de aminoácidos isolados na proteína receptora (AHMED et al., 2000; GUERRA; HACKEL, 2002; OAKES et al., 2008).

Segundo o banco de dados *The Androgen Receptor Gene Mutations Database. McGill*, o mais utilizado em relação ao gene *AR* (ARDB *on line),* já existem mais 1000 mutações relacionas ao gene *AR*, incluindo aqui alterações intrônicas e mutações que geram câncer de próstata, atrofia muscular bulbar espinhal, infertilidade e AIS (ARDB *on line;* GOTTLIEB et al., 2004b; GOTTLIEB et al., 2012*).*

Entre os diversos relatos de CAIS, são poucos os registros na literatura de vários casos em uma mesma família. O estudo de 33 casos de 21 famílias, onze casos de CAIS em 9 famílias e 22 casos de PAIS em 12 famílias (MELO et al., 2005), é a publicação nacional com o maior número de casos e descreveu sete novas mutações no gene AR, além de nove já conhecidas. Posteriormente, uma família com seis casos foi estudada na África do Sul (SCOTT et al., 2006).

Em relação aos trabalhos com gêmeas afetadas por CAIS, encontram-se na literatura, incluindo Andrade-Reis (2010), apenas três trabalhos. Morgan et al. (2002) encontraram duas mutações *missense* no exon 7, F856L e S865P, sendo a F856L uma mutação silenciosa. Correa et al. (2005) descreveram no Brasil, duas gêmeas com 20 anos de idade, com desenvolvimento neuropsicomotor normal, desenvolvimento sexual secundário feminino, com presença de gônadas palpáveis bilaterais na região inguinal e uma mutação *missense* P766A, no exon 5. Ambos os estudos encontraram as mutações na região de *Hot Spots* do gene *AR* (RAJENDER et al., 2007).

No Maranhão, Andrade-Reis (2010), descreveu a mutação R753X em gêmeas com CAIS, resultado que representa o capítulo II desta Tese e que também faz parte de

um projeto maior “Estudo Molecular do Gene do Receptor de Andrógeno em Famílias Brasileiras com a Síndrome de Insensibilidade aos Andrógenos (AIS)”.

O objetivo deste estudo foi caracterizar as mutações no gene *AR* em Pacientes e familiares diagnosticados com AIS através de técnicas de biologia molecular.

Este trabalho está dividido nas seguintes partes: Introdução, Revisão de Literatura, Referências, Resultados, os quais deram origem a três artigos, que são apresentados como capítulos e Considerações Finais.

CAPÍTULO I – Artigo submetido “New mutation P893A in exon 8 of the *AR* gene leads to androgen insensitivity syndrome in a large family in Maranhao, Northeast Brazil”.

CAPÍTULO II – Artigo submetido “An R753X mutation in the androgen receptor gene in monozygotic female twins with complete androgen insensitivity syndrome**”**

CAPÍTULO III – Artigo submetido “Androgen Insensitivity Syndrome Clinical, Hormonal and Molecular Analyses of Twelve Patients with Complete Androgen Insensitivity Syndrome from a Single Family in Brazil ”.

2 REVISÃO DE LITERATURA

2.1 ANDROGÊNIOS E ALTERAÇÕES DE DIFERENCIAÇÃO SEXUAL

Os andrógenos são sintetizados pelas células de Leydig que se encontram nos testículos. Para que a espermatogênese se processe é obrigatória à associação dos andrógenos ao receptor de androgênios, membro da superfamília de receptores nucleares (MESQUITA, 2009).

O complexo receptor androgênico desloca-se para o núcleo, onde reconhece uma sequência específica de DNA cromossômico e os elementos responsivos (ARE – *androgen response element*) e se une a coativadores que facilitam a transcrição de genes regulados por andrógenos. O receptor de andrógenos (AR) tem a capacidade de induzir a expressão gênica e promover a progressão do ciclo celular (YONG et al., 2003; DEEB et al., 2008).

Estes hormônios desempenham um papel crucial no controle da diferenciação sexual masculina e o desenvolvimento e na manutenção da função reprodutiva normal, além do seu papel no surgimento e manutenção dos caracteres sexuais secundários, e durante a iniciação e manutenção da espermatogênese (RONG et al., 2009).

Mutações no gene *AR* podem levar a não produção ou síntese do receptor de andrógenos não funcional, o que promove alterações no processo de espermatogênese e consequentemente causam a infertilidade masculina (MESQUITA, 2009).

Diversas desordens genéticas resultam em anomalia da diferenciação sexual 46, XY, como distúrbios da determinação gonadal, distúrbios da função testicular, distúrbios dos tecidos-alvo dependentes de androgênios (deficiência da 5α-redutase tipo 2 e a síndrome de insensibilidade aos androgênios) ou causa idiopática (DAMIANI; GUERRA-JÚNIOR, 2007).

Na puberdade, os pacientes com AIS apresentam elevação dos níveis de testosterona e LH e também elevação do estradiol proveniente da conversão periférica

de testosterona, induzindo o desenvolvimento mamário (QUIGLEY et al., 1995; AHMED et al., 2000).

Para desempenharem suas funções fisiológicas, tanto a testosterona como a dihidrotestosterona ligam-se ao mesmo receptor no núcleo das células-alvo, denominado receptor de androgênios. O gene *AR*, após a formação do complexo hormônio-receptor, sofre uma mudança conformacional que leva à dimerização, transporte nuclear, ligação à sequência específica do DNA em várias regiões promotoras ou reguladoras do gene, e consequente regulação da transcrição dos genes-alvo de resposta aos androgênios (ZUCCARELLO et al., 2008; NICHOLS et al., 2009).

Com a ausência de andrógenos funcionais ou com resistência a estes hormônios o AR não forma o complexo hormônio-receptor adequado, assim surge a Síndrome de Insensibilidade aos Andrógenos (AIS) (MENDONÇA et al.,2009; NICHOLS et al., 2009).

2.2 O RECEPTOR DE ANDRÓGENOS (AR)

O AR é um ligante ativado pelo fator de transcrição nuclear que, inclui a diferenciação, a homeostase, a morfogênese e crescimento, onde a síndrome de insensibilidade aos andrógenos (AIS) é uma doença com herança ligada ao cromossomo X que afeta pacientes com cariótipo 46, XY, nos quais há prejuízo total ou parcial do processo de virilização intra-útero devido à alteração funcional deste receptor (MELO et al., 2003; MELO et al., 2005; BARBARO et al., 2007).

Receptores androgênicos defeituosos estão diretamente envolvidos comumente em três situações patológicas: (a) síndrome da insensibilidade aos andrógenos (AIS); (b) atrofia muscular bulbar espinhal (SBMA) e (c) câncer de próstata (MELO et al., 2005).

Adachi et al. (2000) sugeriram que uma outra forma de síndrome de insensibilidade androgênica poderia ser causada pala falta de um coativador crucial AF-1 fisiologicamente indispensável para o funcionamento do receptor de andrógeno.

2.3 SÍNDROME DE INSENSIBILIDADE AOS ANDROGÊNIOS (AIS)

A AIS é causada por uma alteração em um gene que bloqueia a resposta aos hormônios masculinos durante o desenvolvimento fetal e após o nascimento, tornando o indivíduo insensível à presença de androgênios. O desenvolvimento masculino que deveria ocorrer na presença de um gene normal é impossível e o corpo pode responder aos hormônios feminilizantes (WARNE, 1997; MELO et al., 2003; RADPOUR et al., 2007; RADPOUR et al., 2009).

Os distúrbios ocorridos na função do AR podem causar AIS com, algumas formas de apresentação. Assim, a AIS pode ser classificada como completa (CAIS), parcial (PAIS) ou discreta (MAIS), dependendo do local da mutação e do fenótipo apresentado pelo individuo afetado (MELO et al., 2003; MESQUITA, 2009).

CAIS é caracterizada por um fenótipo externo completamente feminino, exceto pela redução ou ausência de pêlos pubianos e axilares. PAIS apresenta uma gama de fenótipos que variam desde um fenótipo externo feminino com algum grau de virilização, como cliteromegalia ou fusão labial, até um fenótipo externo masculino com variadas anormalidades na genitália externa, o qual inclui hipospadias, microfalo e criptorquidismo. Já a forma MAIS apresenta virilização e fertilidade reduzidas, mas possui um fenótipo externo masculino normal (MELO et al., 2003; RAJENDER et al., 2008; TADOKORO et al., 2009).

2.3.1 **Síndrome de insensibilidade completa aos androgênios (CAIS)**

O fenótipo CAIS, inicialmente descrito como síndrome dos testículos feminilizantes, caracteriza-se por hábito feminino, genitália externa feminina com ausência ou rarefação de pêlos pubianos, vagina curta e em fundo cego, genitália interna sem os derivados de Wolff e de Muller e desenvolvimento mamário normal na puberdade. É comum a presença de hérnia inguinal representada pelos testículos, mas estes podem estar na cavidade abdominal ou mais raramente nos grandes lábios (BOEHMER et al., 2001; MELO et al., 2005; CORRÊA et al., 2005).

A CAIS resulta das mutações que ocasionam um comprometimento grave da função do AR. Nas famílias com CAIS as mutações correspondem geralmente ao

mesmo fenótipo. Esta concordância é pouco observada nos casos de PAIS, nos quais se observa a mesma mutação resultando em diferentes fenótipos, ainda que na mesma família. Possivelmente essa variação está relacionada a fatores transcricionais que interagem com o AR (BOEHMER et al., 2001; DEEB et al., 2005; GOTTLIEB et al., 2004a).

A CAIS é uma condição rara, com uma incidência estimada entre 1:20.000 e 1:64.000 nascidos do sexo cromossômico masculino (AUDI et al., 2010; GALANI. et al., 2008). Apesar do padrão de herança ser recessivo ligado ao cromossomo X cerca de 30% das mutações são esporádicas outras são mutações *de novo (*SCOTT et al., 2006; OAKES et al., 2008; MENDONÇA et al., 2009).

2.4 CLONAGEM DO GENE *AR* E ESTRUTURA DA PROTEÍNA AR

Ao ser realizado uma análise por hibridização de células somáticas, o *locus* do receptor de andrógenos foi descoberto em 1981 e está localizado entre Xq13 e Xp11, este relato representa um dos primeiros ao nível molecular sobre o gene *AR* (MIGEON et al., 1981).

O DNA complementar (cDNA) do gene *AR* foi clonado em 1988. O gene receptor androgênico (AR) localiza-se no braço longo do cromossomo X, mais precisamente Xq11-q12. Possui aproximadamente 90 Kb, com 8 exons, codificando uma proteína de 110 KD com cerca de 919 aminoácidos (LUBAHN, 1988; LUBAHN, 1989; SCOTT et al., 2006). A proteína AR é composta por três domínios: N-terminal, modulador da transcrição; domínio de ligação ao DNA (DBD) com dedos de zinco proximal e distal; região *hinge* e domínio de ligação ao andrógeno (LBD) (QUIGLEY et al., 1995; BRINKMANN et al., 2000; RAJENDER et al., 2007) (Figura 1).

Após a clonagem do gene *AR*, foi possível estudar as alterações moleculares que levam à Síndrome de Insensibilidade Androgênica (AIS) em humanos, sendo as mutações no *AR*, a causa mais frequente de AIS, uma doença polimórfica com uma ampla gama de fenótipos. Grande parte das alterações encontradas no gene *AR* são

mutações pontuais e estão localizadas no domínio de ligação (MENDONÇA et al., 2009).

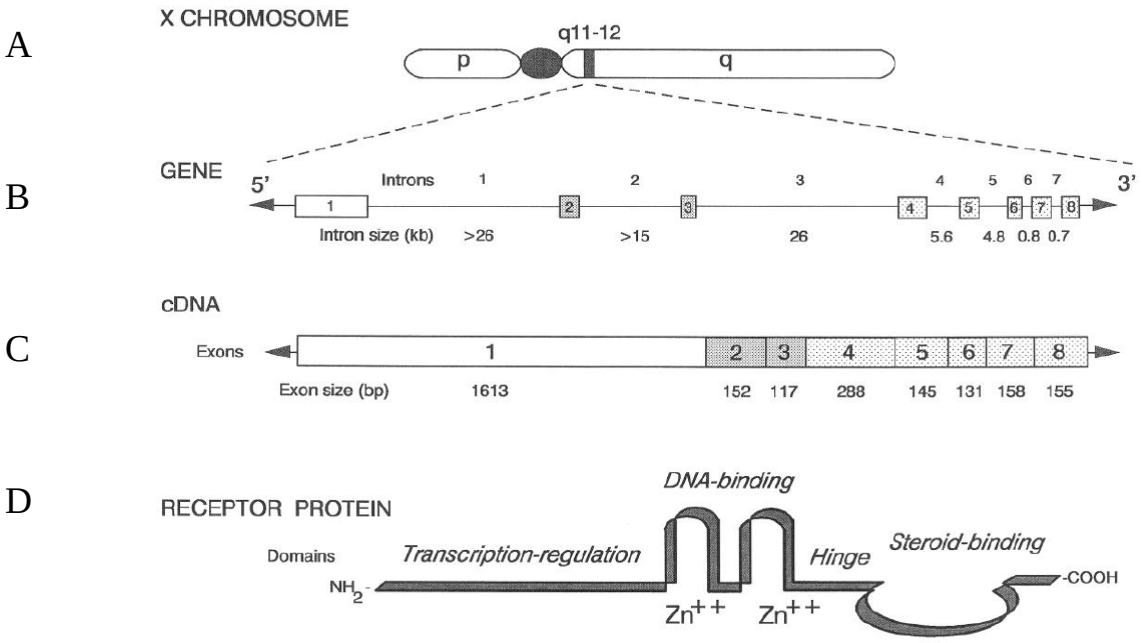

Figura 1: A- localização cromossômica; B- gene *AR;* C- cDNA; D- as regiões da proteína do receptor de andrógeno (adaptado de QUIGLEY et al., 1995).

A porção N-terminal, parte codificada pelo exon 1, é uma grande região variável de 529 aminoácidos envolvidos na ativação da transcrição. Uma região de 66 aminoácidos, codificadas pelos exons 2 e 3, é altamente conservada e representa o domínio de ligação ao DNA (DBD) contendo 2 dedos de zinco. A região da dobradiça, codificada por parte dos exons 3 e 4, representa a transferência de AR do citoplasma para o núcleo, e na região C-terminal de 252 aminoácidos, codificados pelos exons 4-8, é o domínio ligante de ligação (LBD), que também está envolvido na dimerização e ativação da transcrição (MENDONÇA et al., 2009; DEEB et al., 2008) (Figuras 1 e 2).

A região da dobradiça tem sido considerada um forma de ligação flexível entre os DBD e LBD na proteína do AR. Mais recentemente, no entanto, esta região mostrou-se envolvidas na ligação com DNA, assim como, a dimerização da proteína AR, sugerido que a região da dobradiça também atue para atenuar a atividade transcricional do gene AR (DEEB et al., 2008 e MELO et al., 2011)

A maioria das mutações ocorridas no gene *AR* é identificada nos LBD (entre os exons 4 -8), com cerca de 60% das mutações, sendo que grande parte ocorre nos exons 5 e 7 deste gene, e DBD com 20% das mutações (RONG et al., 2009). Embora o domínio amino-terminal seja o maior entre os três domínios, somente 15% das mutações ocorrem nessa região, onde quase sempre ocorrem mutações pontuais, com substituições imediatas do aminoácido. Mutações responsáveis pela existência dos diferentes fenótipos da AIS estão espalhados em toda extensão do gene *AR* (MESQUITA 2009; RONG et a.l, 2009).

A modificação funcional observada em mutações no gene *AR* depende do *locus* exato na sequência do gene. Mutações no NTD (exon 1 do gene) não ocorrem frequentemente e a grande maioria resulta em *stop codon* ou terminação prematura devido à alteração da matriz de leitura por uma inserção ou deleção de nucleotídeos. O LBD (exon 4-8), o qual é codificado por menos da metade do gene *AR*, é considerado o *hot-spot* das mutações, as quais em geral são substituições simples de bases (GALANI et al., 2008) (Figura 2).

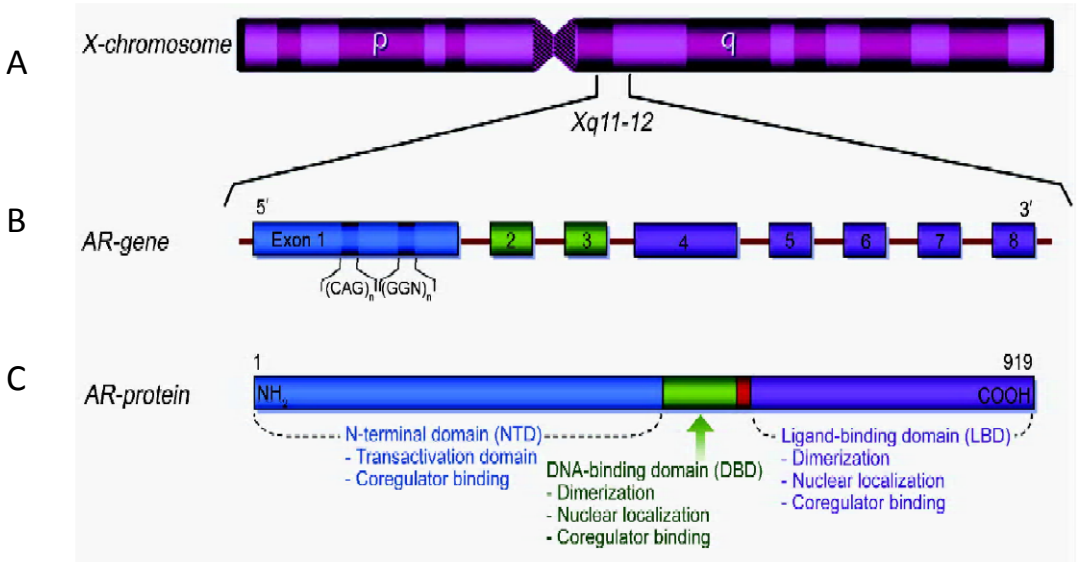

Figura 2: Estrutura organizacional do gene *AR*. A - Localização no cromossomo X; B- Gene *AR* e C- regiões de domínios da proteína do receptor de andrógenos (RAJENDER et al., 2007).

2.5 MUTAÇÕES NO GENE *AR*

Diversos tipos de mutações podem ocasionar AIS: mutações de ponto com substituições de aminoácidos ou códons de terminação prematuros (*stop codons*), inserções ou deleções de nucleotídeos ocasionando uma alteração no quadro de leitura (*frameshift)* e terminação prematura da proteína, deleções gênicas parciais ou mais raramente deleções completas do gene e mutações intrônicas. Tais mutações ocorrem em todo o gene, mas se concentram em pontos específicos chamados *hot spots* (BRINKMANN, 2001; SCOTT et al., 2006).

A substituição de um único nucleotídeo é muito mais frequente, quando comparada à frequência de deleções e inserções no gene *AR*. Quando estas mutações resultam em alteração do *splicing* do RNAm e códon de parada (*stop codon*) do AR, ocasionam grandes alterações na estrutura do receptor e são sempre responsáveis por CAIS (MCPHAUL, 1998, 2002).

O principal efeito da maioria das mutações no *AR* não está relacionado com a quantidade de receptor, mas com a sua função. Por este motivo, estudos têm sido realizados para avaliação dos níveis de expressão e função de receptores androgênicos normais e mutados. Entretanto, as mutações que originam proteínas truncadas (códon de parada prematura ou alterações do *splicing* do RNAm do *AR*) são exceções para esta regra (MCPHAUL et al., 2002)

As mutações no *AR* estão ligadas ao X, sendo 90% dos defeitos moleculares mutações de base única, principalmente mutações *missense* (o codon codifica para um aminoácido diferente). Existem cerca de 750 mutações conhecidas associadas ao *AR* resultando em vários fenótipos da AIS (OAKES et al., 2008; RONG et al., 2009). Para estabelecer correlações genótipo-fenótipo na AIS, é necessário caracterizar a localização das mutações que afetam o gene ligado ao X do *AR* (RONG et al., 2009).

A mutação mais comum é a do tipo sentido trocado (*missense*), em torno de 80%. As deleções completas do gene receptor de androgênios são encontradas em apenas 1% dos pacientes e deleções parciais em 7%. Anormalidades em *splicings* e terminação prematura de codons foram relatadas em 2% e 7% respectivamente. A

maioria das mutações ocorre nos exons 2-8, com um grande número de mutações relatadas nos exons 5 e 7, pouquíssimas no exon 1, e nenhuma na região *hinge* (NICHOLS et al., 2009).

A descoberta de novas mutações está em processo de crescimento, principalmente na última década, por exemplo, quando o número de mutações que conduzem a CAIS quase que dobrou. (GOTTLIEB et al., 2012; JÄÄSKELÄINEN et al.,2006) (Figura 3).

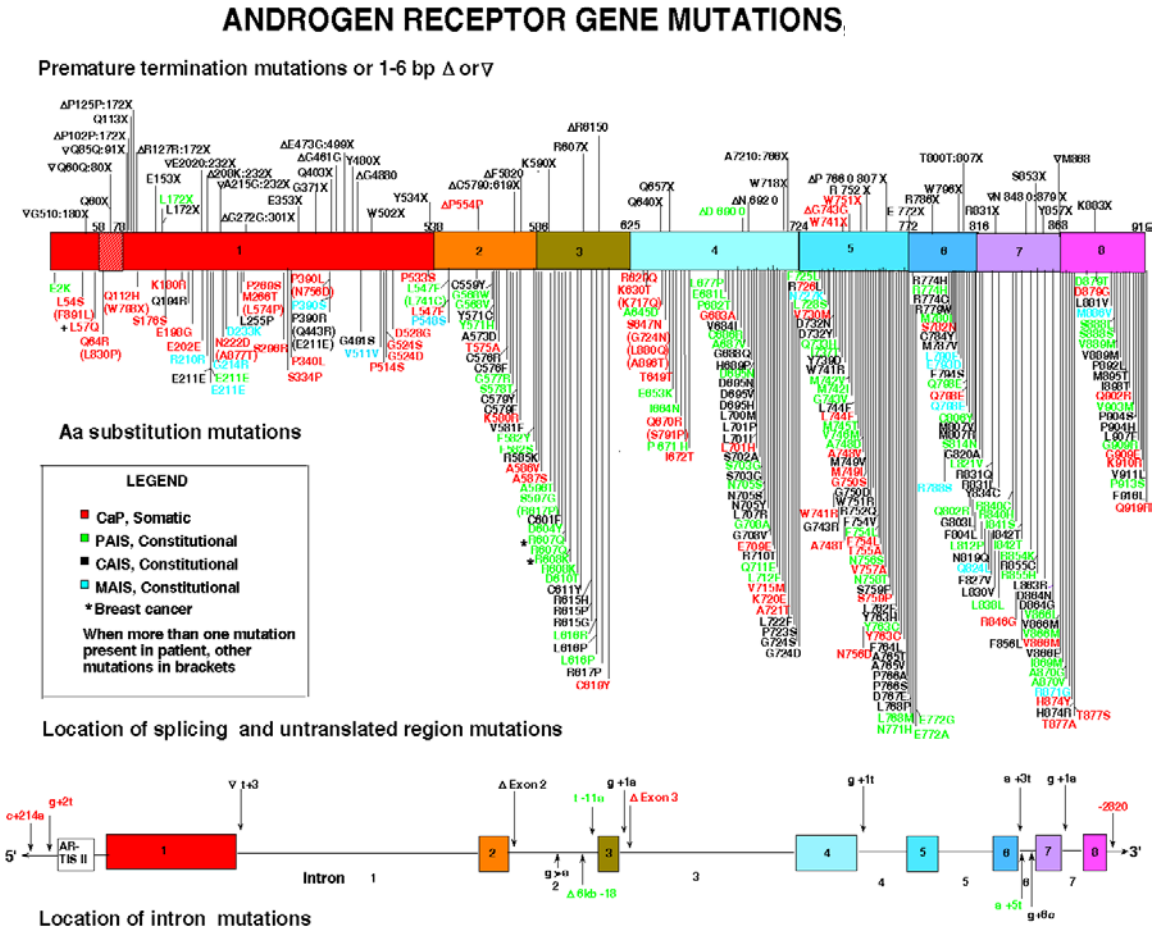

Figura 3: Banco de dados do Gene *AR* (ARDB *on line*)- última atualização em 24/10/2009).

2.6 GENÉTICA DE POPULAÇÕES DO GENE *AR*

Usando principalmente dados sobre a frequência de hérnia inguinal no sexo feminino, Jagiello e Atwell (1962) estimaram a frequência de feminização testicular como sendo cerca de 1 em 65.000 homens. Edwards et al., (1992) demonstraram que a distribuição do número de repetições CAG no exon 1 do gene *AR* foi menor em Afro-americanos, intermediário em brancos não-hispânicos, e maior em asiáticos, ao analisarem o equilíbrio de *Hardy –Weinberg*, apenas em Afro- americanos houve um desvio de *Hardy-Weinberg*.

Irvine et al. (1995) estudaram a distribuição da CAG e repetição GC (microssatélites) no exon 1 do gene *AR* em Afro-americanos, brancos não-hispânicos e asiáticos (japoneses e chineses) e confirmou as conclusões de Edwards et al. (1992).

Uma das funções essenciais do produto do gene *AR* é ativar a expressão de genes-alvo. Esta atividade de transativação reside no domínio N-terminal da proteína que é codificada no exon 1, que contém as repetições polimórficas. Dentro desse contexto, segundo Irvine et al.(1995), nos três grupos populacionais acima citados, a frequência de câncer de próstata é inversamente proporcional ao comprimento das repetições. Quanto menor o tamanho da repetição CAG, maior o nível de função de transativação do receptor, assim, possivelmente resultando em um maior risco de câncer de próstata.

Ao relacionarem em uma revisão, a heterogenidade de mutações no *AR*, no que concernem as sequências repetidas de CAG em várias populações (Afro-americanos, Americanos caucasóides, europeus – caucasóides e asiáticos), acreditam ser prematuro, apesar da variabilidade, fazer conclusões sobre a associação do gene *AR* com as seguintes desordens: infertilidade, câncer de próstata, de mama e de endométrio (RAJENDER et al., 2007).

A frequência de deleções e inserções no gene *AR* de portadores de AIS é de aproximadamente 5-10%. Estas mutações possuem tamanhos variáveis, desde a deleção de um único ou múltiplos nucleotídeos até a deleção de todo o gene. Os portadores deste tipo de mutação apresentam a forma completa da síndrome, uma vez que há uma alteração na leitura do *AR*, não existindo a expressão de uma proteína íntegra, e consequentemente, ausência de ligação aos andrógenos (MELO et al., 2005).

2.7 CITOGENÉTICA E GENE *AR*

Muller et al. (1990) descreveram uma mulher com 11 anos de idade, com AIS e 47, XXY síndrome de Klinefelter ao analisar marcadores de DNA no cromossomo, demonstraram que o X supranumerário resultaram da não-disjunção materna durante a meiose II. O erro de não disjunção, nesta fase, forneceu a base para homozigose da mutação no *locus* do *AR*, é possível compreender que pode-se ter AIS correlacionado com outras alterações cromossômicas.

Ao realizar uma cirurgia para retirada de hérnia inguinal, observada desde o nascimento, em uma menina de três meses de idade, o diagnóstico clínico foi caracterizado com CAIS (XU et al., 2003). Posteriormente, foi confirmado o cariótipo de 46, XY com inversão do cromossomo X, com um intervalo interrompendo o gene *AR*. A mãe desta paciente era fenotipicamente normal com 46, XX, mas apresentava a inversão em um cromossomo X, havia ainda, uma tia materna com CAIS e um cariótipo de 46, XY, que aos cinco anos de idade fez a cirurgia para retirada de hérnia inguinal (testículos foram identificados), sendo submetida mais tarde a gonadectomia devido potencial de malignidade (XU et al. 2003).

2.8 ASPECTOS CLÍNICOS DA AIS

O risco de desenvolvimento de tumores de células germinativas é um importante fator a ser considerado na abordagem dos pacientes com distúrbios do desenvolvimento sexual. No entanto, este risco é normalmente difícil de ser predito, progressos marcantes foram alcançados no que diz respeito à identificação de marcadores relacionados ao desenvolvimento de tumores de células germinativas e ao reconhecimento de alterações precoces nestas células (atraso na maturação; lesões pré-neoplásicas e neoplasia *in situ*) (AHMED et al., 2000).

Segundo Rosa et al. (2008), os indivíduos afetados por AIS, apesar de apresentarem, frequentemente, anormalidades somáticas decorrentes do

desenvolvimento anormal genital ao nascerem, acabam sendo diagnosticado somente na adolescência ou na idade adulta, devido a alterações menstruais e falta de desenvolvimento dos caracteres sexuais secundários.

Os distúrbios de desenvolvimento sexual (DDS) podem estar associados a problemas na estrutura de alguns indivíduos, no crescimento normal do osso e na massa óssea em decorrência da secreção ou ação anormal de esteróides sexuais (BERTELLONI et al., 2010). Em pacientes com AIS há vários relatos de redução da densidade mineral óssea (DMO). DMO reduzida é comum nos pacientes não gonadectomizados, mas é mais intensa nos pacientes gonadectomizados, principalmente quando a terapia de reposição hormonal não é garantida (BERTELLONI et al., 2010).

Um tecido gonadal indiferenciado, parece ser um padrão de diferenciação que comporta um alto risco de desenvolvimento de gonadoblastoma (COOLS et al., 2005; COOLS et al., 2006). Espera-se que a combinação destes achados permitirá que seja estimado o risco de desenvolvimento tumoral em cada paciente individualmente. O consenso atual sobre abordagem dos distúrbios relacionados ao intersexo recomenda gonadectomia no momento do diagnóstico, para as pacientes com PAIS, e após a puberdade, nas pacientes com CAIS dada à facilidade da reposição hormonal (COOLS et al., 2005; COOLS et al., 2006; LEE et al., 2006; CHEIKHELARD et al., 2008).

Em pacientes com CAIS, o receptor de androgênios está completamente defeituoso e ocorre falha no desenvolvimento da genitália tanto interna como externamente (NICHOLS et al., 2009).

Sobel et al. (2006) estudaram indivíduos com CAIS e deficiência de 5-alfa-redutase- tipo 2 para determinar o efeito direto dos androgênios sobre a densidade mineral óssea (DMO). Em indivíduos com CAIS, a DMO foi significativamente reduzida na coluna e quadril, enquanto indivíduos com deficiência 5-alfa-redutase-2 tinha valores de DMO normal. Sobel et al. (2006) concluíram que os andrógenos são de importância direta no desenvolvimento e/ou manutenção da densidade mineral óssea e que a testosterona e/ou baixos níveis de dihidrotestosterona parecem ser suficientes para o desenvolvimento ou manutenção DMO.

Para investigar a interação de andrógenos com o sistema IGF, Elmlinger et al. (2001), realizaram a comparação da expressão de fator de crescimento semelhante à insulina (IGFs) e Fatores de crescimmento de fibroblastos IGFBPs em culturas de fibroblastos da pele genital de 9 pacientes com a síndrome de insensibilidade androgênica completa, com os fibroblastos da região genital de 10 homens normalmente virilizados.

Linhagens de fibroblastos da pele genital de pacientes CAIS produziram significativamente menor IGF2 e mRNA de IGF2 que o controla em células de fibroblastos da região genital. A produção de IGFBP2 também foi diminuída em fibroblastos da pele genital de pacientes com CAIS, enquanto a de IGFBP3 não diferiu. Os autores concluíram que, além das ações endócrinas do IGF1, IGF2 e IGFBP2, eles também estão envolvidos como fatores de crescimento locais na mediação de andrógeno e ação do crescimento de tecidos genital (ELMLINGER et al., 2001).

Boehmer et al. (2001) analisaram a relação genótipo-fenótipo na AIS e a ocorrência de possíveis causas de variação fenotípica em famílias com múltiplos casos afetados. De 49 casos índices com AIS identificados, 59% tinham familiares afetados. Um total de 17 famílias foi estudado, sete famílias com CAIS (18 pacientes), nove famílias com PAIS (24 pacientes), e uma família com fenótipos pré-púberes do sexo feminino (duas pacientes). Nenhuma variação fenotípica foi observada nas famílias com CAIS. Entretanto, a variação fenotípica foi observada em uma de três famílias com PAIS resultando em sexo diferente de criação e as diferenças na exigência de cirurgia reconstrutiva (BOEHMER et al., 2001)

Houve um crescente aumento na determinação de novas mutações no gene *AR* de 2008 a 2012, o número registrado no banco de dados *The Androgen Receptor Gene Mutations Database*, o mais utilizado em relação ao gene *AR*, aumentou de 300 para quase 500 tipos de mutações relacionadas à CAIS e de 750 para mais de 1000 alterações no gene *AR*. É válido destacar que este banco de dados corresponde à sequência de referência NM-000044 do *AR* no National Center for Biotechnology Information- NCBI (GOTTLIEB et al., 2012).

A identificação e confirmação genético molecular de afetados e de portadores de mutações no gene *AR* é de importância clínica para o aconselhamento genético eficiente para os indivíduos afetados e seus familiares, o que vem sendo National Center for Biotechnology Informationrealizado com as famílias que participaram deste trabalho.

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3 RESULTADOS

3.1 CAPÍTULO I

Artigo Submetido à revista Molecular and Cellular Endocrinology – Fator de Impacto 4,119 (A2), em 17/04/2012

New mutation P893A in exon 8 of the *AR* gene leads to androgen insensitivity syndrome in a large family in Maranhao, Northeast Brazil.

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Andrade et al. 2012

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ABSTRACT

Androgen insensitivity syndrome (AIS) is a rare disorder, with the incidence between 1:20,000 and 1:64,000 live male births. Transmission is X-linked, and the syndrome is caused by mutations in the androgen receptor (*AR*) gene. This study included twelve patients and their relatives spanning six generations from a single Brazilian family. We have investigated mutations in the *AR* gene in affected individuals and characterized the mutations in close relatives of the affected patients, especially in prepubescent girls and women with amenorrhea. In all patients and in the heterozygous carriers, there was a C->G substitution at nucleotide position 3792 in exon 8 of the *AR* gene, changing proline to alanine at amino acid 893. Research such as this can reveal the molecular and hereditary mechanisms underlying disorders for patients and families. This study extends current understanding of *AR* mutations associated with AIS.

KEYWORDS: androgen receptor; androgen insensitivity syndrome; AIS; mutation

1 INTRODUCTION

Androgen insensitivity syndrome (AIS) is an X-linked disorder characterized by end-organ resistance to testosterone and dihydrotestosterone (DHT). It typically results from mutations in the androgen receptor (*AR*) gene in patients with typical male 46, XY karyotypes, which results in abnormal development of internal and external genitalia (Brown et al. 1988; Li et al. 2011; Lubahn et al. 1988).

In general, subjects with AIS are characterized by a 46, XY karyotype, presence of testes, normal or high levels of androgens in the blood, and decreased usual response to androgens associated with various aberrations of male differentiation and virilization. The phenotypic variants range from males with low fertility to phenotypic females (Peters et al. 1999).

Mutations that severely impair AR protein function cause complete androgen insensitivity syndrome (CAIS). The incidence of this disorder is between 1:20,000 and 1:64,000 live male births (Galani et al. 2008). CAIS is caused by a complete inability of AR to bind the steroids testosterone and dihydrotestosterone and a consequent inability of the target cells to respond to androgen (Rong et al. 2009; Scott et al. 2006).

The AR protein is an intracellular transcription factor that binds to androgen as a hormone receptor complex. The *AR* gene is located on the X-chromosome and is composed of eight exons. It is comprised of 4 functional domains: an N-terminal domain, encoded by exon 1 (DBD); a DNA-binding domain, encoded by exons 2 and 3 (DBD); a “hinge” region, encoded by exon 4; and an androgen-binding domain, encoded by exons 4-8 (LDB) (Jaaskelainen et al. 2006; Melo et al. 2003; Quigley et al. 1995).

In addition to ligand binding, the LBD is involved in receptor dimerization, nuclear localization, and interaction with heat shock proteins, and it contains a ligand dependent transactivation function (AF-2) (Mooney et al. 2003; Peters et al. 1999).

Missense mutations affecting the hinge region in the AIS are rare relative to those located in the DBD or LBD. Mutations in the LBD impair receptor function due to defective between N-terminus domains and C-terminus domains, N/C interaction (Deeb et al. 2008).

The total number of reported mutations in *AR* gene has risen from 605 to 1,029 between 2004 and 2012, and the reported number of both AR coregulators and interacting proteins has substantially increased from 70 to 311 during the same time period. However, it is important to note that in a number of cases, either the interacting protein's properties (10.0%) or its AR interaction domain (45.7%) or both have not yet been characterized (Gottieb et al. 2012).

This study included twelve patients and relatives from a single Brazilian family with six generations investigated. We have investigated mutations in the *AR* gene in affected individuals, characterized the mutations in close relatives of affected patients, especially in prepubescent girls and women with amenorrhea, and, finally, we analyzed the genotype–phenotype relationship.

2 MATERIAL AND METHODS

2.1 Subjects

The study was approved by the Scientific Committee (COMIC) and Ethics Committee in Research (CEP) with Human Beings at the University Hospital of the Federal University of Maranhão (HUUFMA) (protocol: 001421/2008 -10 and CEP054/11). All the patients and family members were invited to sign an informed consent. It is also important to highlight that the patients and relatives that are described in the present report are from the Bico do Papagaio region. This region is extremely impoverished and is located between the north and northeast regions of Brazil, far from large urban areas, in the pre-Amazon region.

The proband was a teenager with primary amenorrhea who had undergone inguinal hernia surgery whose histology revealed testicular tissue. Through interviews with the parents of this patient, other women were identified in the family with a history of primary amenorrhea with or without reports of previous surgery for inguinal hernia repair. The prepubescent females were screened by ultrasound to identify those without a uterus.

For the molecular study, the samples were collected and analyzed at the Laboratory of Genomic and Histocompatibility Studies (LEGH). A total of 31 subjects were recruited, including patients (12) and relatives of the same family. The patients were subjected to physical and clinical evaluations, and family histories of all the

patients were recorded for elaboration of the pedigrees (Figure 1). This study was conducted in a private clinic that specializes in endocrinology and at the LEGH. Upon complete clinical examination, phenotypes that were diagnosed as CAIS were referred to the LEGH for molecular analysis. The assignment of androgen insensitivity was based upon the presence of a 46, XY karyotype, abdominal gonads, when they had not been removed, testicular tissue in gonads, and the lack of uterus and tubes as determined by B-ultrasound. The phenotypically female patient expressed satisfaction with her sexual life.

It should be noted that in those patients who did not undergo gonadectomy, testosterone was produced at the same level as in normal males (241-827 ng/100 ml). The phenotypically female patients had elevated luteinizing hormone and estrogen compared to normal men, which is unusual for normal women. Estrogen levels, despite being lower than in women without CAIS, were enough to induce mammary development, as seen in the literature.

2.2 Identification of the *AR* gene mutations

The genomic DNA of the patients and their families was extracted from peripheral blood using the commercial EZ-DNA kit (Biological Industries, Beit Haemek, Israel). Following the extraction, the DNA was amplified by PCR to allow for the identification of mutations in exons 1-8 of the *AR* gene. This procedure was performed as previously described, with modifications (Radpour et al. 2007). The PCR reactions had a final volume of 25 µl and contained 100 ng of DNA, 5 U/µl GoTaq Flexi DNA (Promega, Madison, Wisconsin, United States), 0.8 mM dNTPs (Amresco, Sólon, Ohio, United States), and 0.8 pmol of each primer (Invitrogen, São Paulo, Brazil).

The PCR products were purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, North Rhine-Westphalia, Germany).

The sequencing reaction was performed using a BigDye Terminator v. 1.1 and v. 3.1 Kit (Applied Biosystems, Warrington, United Kingdom, England). The DNA was precipitated with isopropanol/ethanol, and the samples were subsequently resuspended in formamide and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied

Biosystems, Foster City, California, United States). The electropherograms were analyzed with SeqScape v. 2.5 software (Applied Biosystems, Foster City, California, United States), and the standard sequence of the *AR* gene (AR; OMIM: 313700) was compared to the results that were obtained from the sequencing analyses. A part of the sequences were analyzed by BioEdit software version 7.1.3. (Hall, 2011).

3 RESULTS AND DISCUSSION

We analyzed 12 patients clinically diagnosed with CAIS, which showed the base substitution mutation of guanine for cytosine, P893A. The mutation in heterozygous carriers and 12 (mostly mothers of patients) was also confirmed by sequencing. The characterization of the mutation in the patients' mothers excluded the possibility of *de novo* mutation (Figures 1 and 2).

The mutation is a substitution of cytosine (C) for Guanine (G) at nucleotide position 3792 in exon 8 of the *AR* gene and generates a change in amino acid 893 of the AR protein, replacing proline (GCC) with alanine (GCG). According to the McGill University database (ARDB online) there is no record of this missense mutation in the literature. In the same locus there is only one reported substitution of cytosine to thymine, which results in an exchange from proline to serine (Peters et al. 1999).

[Insert figure 1 here]

Figure 1. Great Family Bico do Papagaio region-MA, showing 6 generations and 13 patients affected with CAIS (III-3, III-8, III-11, IV-09, IV-14, IV-20, IV-21, IV-22, IV-23, V-32, V-34, IV-32 (deceased) e 12 heterozygous carriers to mutation P893A (II-2, II-3, III-5, III-7, III-10, IV-11, IV-15, IV-25, IV-28, IV-31, V-32 and V-36).

Mutations in the androgen receptor gene has been reported to be the most frequent causes of disorders of sex development of 46, XY individuals and are associated with a variety of phenotypes (Audi et al. 2010).

Normal male sexual development requires both the synthesis of testosterone and DHT by the testes and the ability to form an active complex between AR protein and circulating androgens (Rong et al. 2009). Individuals with AIS are androgen resistant and have disorders of male sexual differentiation ranging from CAIS, with a normal female phenotype in the presence of an XY genotype, to phenotypic males who present with infertility problems (Li et al. 2011).

The P893A mutation is located in the direct vicinity of the proposed C-terminal helix (helix 12) of the LBD of AR, which contains the AF-2 transcriptional activating function core (Peters et al. 1999). This finding directly confirms the genotype-phenotype relationship because when the P893A mutation is present, individuals develop resistance to androgen as a clinical sign.

Sequencing may also be used for the detection of mutations in carriers (heterozygous females), thus allowing a more accurate diagnosis and genetic counseling for relatives of early patients with AIS (Figure 2).

[Insert figure 2 here]

Figure 2. Electropherogram of the patients and relatives with mutation P893A, indicating the substitution of a cytosine for guanine in exon 8 of the *AR* gene. A - The reference sequence corresponds to a normal individual; B - individual heterozygous for the mutation; C - affected individuals for the mutation.

Many different mutations in AR are associated with CAIS, including mutations that interfere with the dimerization of the receptor with androgens, hormone binding, and the association of the receptor complex with DNA, which can interfere with the function of transcriptional activation of the androgen receptor (Melo et al. 2003; Peters et al. 1999).

The presence of families with more than one affected patient is possibly due to the absence of a prior diagnosis. Early diagnosis is important for the correct and appropriate genetic counseling and for the use of an appropriate therapy (Melo et al. 2005; Scott et al. 2006; Solari et al. 2008). Pedigree analysis along with a molecular study of the androgen receptor gene in affected families facilitates genetic counseling provided to family members (Li et al. 2011).

This study obtained data from six generations from which the pedigree was prepared, showing the distribution of CAIS in this large family, and genetic counseling was performed. Thus, our methods could be readily adapted to prenatal screening and genetic counseling for families harboring this rare disorder (Li et al. 2011).

Genetic counseling of families is performed to better understanding the risks of recurrence of genetic disorders (Scott et al. 2006). However, in Brazil, there are few genetic services that can provide care to all newborns and their families with a genetic

syndrome. Most centers that perform this type of care are located in large cities, especially the Southeast. Thus, studies of this nature are useful to minimize these regional differences.

In summary, research such as this can elucidate an underlying molecular mechanism for hereditary disorders afflicting patients and their families and can contribute to a valuable collection of data on AIS. This research can introduce different diagnostic tests and therapies enabling a treatment more tailored to the genetic profile.

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3.2 CAPÍTULO II

Artigo Submetido à revista Twin Research and Human Genetics – Fator de Impacto 1,58 (B2), em 17/11/2011 e Reenviado em 25/03/2012.

AN R753X MUTATION IN THE ANDROGEN RECEPTOR GENE IN MONOZYGOTIC FEMALE TWINS WITH COMPLETE ANDROGEN INSENSITIVITY SYNDROME

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ARTIGO II

AN R753X MUTATION IN THE ANDROGEN RECEPTOR GENE IN MONOZYGOTIC FEMALE TWINS WITH COMPLETE ANDROGEN INSENSITIVITY SYNDROME

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Running Title: R753X mutation in the *AR* gene in twins with CAIS

Abstract

Complete androgen insensitivity syndrome (CAIS) is an X-linked recessive disorder that affects patients with a 46, XY karyotype. This condition is caused by mutations in the androgen receptor (*AR*) gene. Mutations in this gene prevent the response to male hormones during fetal development and postnatal life, rendering the individual insensitive to androgens. The aim of this study was to investigate the presence of *AR* mutations and to correlate the *AR* genotype and phenotype in patients who have been clinically diagnosed with CAIS. This study was conducted in a private clinic that specializes in endocrinology and in a Laboratory of Genomics Studies and Histocompatibility. We examined two seven-year-old monozygotic twin sisters with a history of inguinal hernia. The patients exhibited a 46, XY karyotype, female external genitalia and an absence of Müllerian duct derived structures. The molecular analysis identified a nonsense mutation at codon 753 of AR, which encodes an amino acid in the ligand-binding domain (LBD). This mutation changed an arginine (CAG) codon to a stop codon (TAG). Mutations that interfere in *AR* gene transcription, such as the premature termination codon described in this study, give rise to truncated proteins that cannot bind to AR, resulting in CAIS. This is the third reported case of CAIS in monozygotic twins for which a molecular investigation was performed.

Androgens play a crucial role in both normal male fetal development and pubertal maturation. These hormones act on their target cells via an interaction with the androgen receptor (AR), resulting in the direct regulation of gene expression (Brinkmann et al., 1995). Defects in the *AR* gene cause androgen insensitivity syndrome (AIS), an X-linked disorder in 46, XY individuals with normal androgen production. This condition is the result of *AR* mutations and causes a wide variety of phenotypic abnormalities in affected males (Quigley, 1995). The cloning of the human *AR* complementary DNA allowed for the characterization of the molecular defects that are responsible for AIS. AR is encoded by a single gene that consists of eight exons and is located on the X chromosome (Xq11-12) (Brown et al., 1988; Lubahn et al., 1988).

Mutations that severely impair AR protein function cause complete androgen insensitivity syndrome (CAIS) (Quigley, 1995). The incidence of this disorder is between 1:20,000 and 1:64,000 live male births (Galani et al., 2008). CAIS is caused by a complete inability of the AR to bind the steroids testosterone and dihydrotestosterone and a consequent inability of the target cells to respond to androgen (Nichols et al., 2009). CAIS is characterized by a failure of both Wolffian duct development and male external genitalia differentiation. In general, the primary phenotypic characteristics of individuals with CAIS include the following: female external genitalia; a short and blind-ending vagina with no cervix or fallopian tubes; the absence of Wolffian duct-derived structures, such as the epididymides, vas deferens, and seminal vesicles; the absence of a prostate; the development of gynecomastia; and the absence of pubic and axillary hair (Quigley, 1995). Occasionally, in approximately one-third of CAIS patients, residual Mullerian stuctures exist due to the possibility of a common link between the androgen insensitivity and defective action of the anti-Mullerian hormone (Nichols et al., 2009). The gender identity is that of a normal female; however, the

patients present with testes that are located either in the abdomen or the inguinal area, and the uterus is absent (Kawate et al., 2005).

Because of the genetic heterogeneity of CAIS, every study and documentation of a mutation in a patient with AIS provides important information regarding the function of a specific amino acid residue. It is also important to highlight that the patients that are described in the present report are from the Bico do Papagaio region (Figure 1). This region is extremely impoverished and is located between the north and northeast regions of Brazil, far from large urban areas. The aims of this study were to investigate the presence of *AR* mutations and to correlate the genotype and phenotype in patients who have been clinically diagnosed with CAIS.

[Insert fig 1 here]

Figure 1 – Study setting: Bico do Papagaio, in the pre-Amazon region. Adapted from the

http://www.integração.gov.br/progamas/programasregionais/papagaio/abrangência.asp

Materials and Methods

Subjects

The patients who were examined are monozygotic twins with non-consanguineous parents. The patients have two older brothers, who do not exhibit sexual development disturbances, and there is no suggestive familial history. The patients were born by cesarean delivery at full term. The children exhibited normal female external genitalia, and the initial physical examination revealed normal stature, weight and psychomotor development. When the patients were two years and eight months of age, the mother noticed a protuberance in the inguinal region of one of the girls, who complained of local pain. She was then taken to the pediatrician for a physical examination, which revealed a bilateral hernia inguinal. A cytogenetic analysis revealed a 46, XY karyotype

in this patient, and it was indicated that the same investigation be performed for the sister. In both twins, the ultrasound analyses revealed gonads that were positioned in the inguinal channel. The other intrabdominal structures were normal, with no signs of internal sexual structures. A biopsy performed by the pediatrician confirmed the presence of testes in the two girls.

Three months following the initial diagnosis, the family sought a specialized evaluation in the southeastern region of Brazil. The patient evaluations revealed basal testosterone (T) dosages that were initially normal (patients 1 and 2: T <11 ng/dl; reference values for pre-pubertal females older than one year: <19 ng/dl), and the testosterone levels were elevated following the HCG stimulation test (patient 1: T = 175 ng/dl and patient 2: T = 194 ng/dl; reference values for pre-pubertal females older than one year: <14 ng/dl). These findings excluded the possibility of defective testosterone synthesis and suggested CAIS. During this period, the two patients underwent a bilateral gonadectomy. Five months following the surgery, the family sought specialized evaluation in their home state. At this time, the patients and their family members were included in this study, with the intent to molecularly characterize the mutation in question. For the molecular study, the samples were collected and analyzed in the Laboratory of Genomics Studies and Histocompatibility at the Federal University of Maranhão. This study was approved by the Ethics Committee for Research with Human Beings at the University Hospital of the Federal University of Maranhão.

Identification of the **AR** *gene mutations*

The genomic DNA of the patients and their mother was extracted from peripheral blood samples using the EZ-DNA commercial kit (Biological Industries, Beit Haemek, Israel). Following the extraction, the DNA was amplified to allow for the identification of mutations in exons 1, 2, 4, 5, 6, 7, and 8 of the *AR* gene using a polymerase chain

reaction (PCR). This procedure was performed as previously described, with modifications (Radpour et al., 2007). The PCR reactions had a final volume of 25 µl and contained 100 ng of DNA, 5 U/µl GoTaq Flexi DNA (Promega, Madison, Wisconsin, United States), 0.8 mM dNTPs (Amresco, Sólon, Ohio, United States), and 0.8 pmol of each primer (Invitrogen, São Paulo, Brazil). The temperature cycles consisted of 95ºC for 5 minutes, followed by 35 cycles of 94ºC for 1 minute; annealing steps of 57ºC for 1 minute (for exons 2, 5 and 6), 58ºC (for exons 4, and 7) or 60ºC (for exons 1 and 8); and an extension step of 1 minute at 72ºC. These cycles were followed by a final extension at 72ºC for 1 additional minute.

The PCR products were purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, North Rhine-Westphalia, Germany). Following this step, the products were diluted from an initial concentration of 100 ng to a final concentration of 50 ng/ml. The sequencing reaction was performed using a BigDye Terminator v. 1.1, v. 3.1 Kit (Applied Biosystems, Warrington, United Kingdom, England). The DNA was precipitated with isopropanol/ethanol, and the samples were subsequently resuspended in formamide and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, United States). The electropherograms were analyzed with SeqScape v. 2.5 software (Applied Biosystems, Foster City, California, United States), and the standard sequence of the *AR* gene (*AR*; OMIM: 313700) was compared to the results that were obtained from the sequencing analyses.

Results

The sequencing analysis revealed a T-to-C substitution in exon 5 of *AR*, which changed an arginine codon to a premature stop codon at codon 753, confirming the clinical diagnosis of CAIS (Figure 2).

The characterization of the mutation in the patients' mother excluded the possibility of a *de novo* mutation.

[Insert fig 2 here]

Figure 2- Electropherogram indicating a T-to-C substitution in exon 5 corresponding to position 753 of AR protein. A – Reference sequence. B – Sequence of a normal individual. C e D – Twin's affected sequences.

Discussion

Prior to the gonadectomy, the patients presented with bilateral inguinal gonads, which were determined to be testes. The incidence of CAIS in children with inguinal pre-menarche hernia is 1.1%. Inguinal hernias are observed in 80–90% of CAIS patients. The histological analyses of the testicles of CAIS patients revealed characteristics of cryptorchidism without spermatogenesis (Correa et al., 2005; Quigley, 1995). Many different mutations in *AR* are associated with CAIS, including mutations that interfere with the dimerization of the receptor with androgens, hormone binding and the association of the receptor complex with DNA (Mongan et al., 2002). The functional impact of *AR* mutations depends on the precise location of the mutation in the gene's sequence. AR contains four domains: the N-terminal domain (NTD), the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD) (Mooney et al., 2003; Zhu et al., 1999).

The large NTD is encoded by exon 1 and is the least conserved region among the steroid receptors. The NTD is involved in the transcriptional activation of the target genes. The DBD is encoded by exon 2 and 3 and contains two zinc finger motifs; this domain is the most highly conserved region among steroid receptors. The carboxyl-terminal of AR contains the LDB and is encoded by a portion of exon 4 and exons 5–8. This domain is responsible for specific high-affinity ligand binding. Between the DBD

and the LDB is the hinge region, which is encoded by the remainder of exon 4 and contains the nuclear translocation signal (Mooney et al., 2003; Zhu et al., 1999).

In 70% of CAIS cases, the cause is a germline mutation that is transmitted in an X-linked manner by the carrier mothers; in approximately 30% of cases, the mutation occurs *de novo* (Kohler et al., 2005). With respect to the presence of AIS in monozygotic twin sisters, only two clinical cases for which molecular investigations were performed have been reported.

Mongan et al. (2002) described two recurrent missense mutations in monozygotic twin sisters with CAIS, F856L and S865P, both of which are located in exon 7 of *AR*. The F856L mutation results in a protein that is similar to the wild type receptor, without influencing either DNA-binding or transcriptional activation. However, the S865P mutation completely abolishes DNA-binding and *AR*-induced transcriptional activation. This result implies a diagnosis of the complete form of AIS in patients with the S865P mutation. Correa et al. (2005) described 20-year-old twin sisters who reported thelarche at the age of 13 and puberty at the age of 16. Normal neuro-psychomotor development was reported, as was secondary female sexual development. Bilateral palpable gonads were present in the inguinal region, and a P776A missense mutation was detected in exon 5 of *AR*.

Far more than 500 *AR* mutations have been identified worldwide in patients with AIS (AR database at http://www.androgendb.mcgill.ca/) Nevertheless, only five cases with the same mutation that was identified here have been previously described. Pinsky et al. (1992) first described the R753X mutation, followed by Brinkman et al. (1995). Yaegashi et al. (1999) described the R753X mutation in a 12-year-old patient with karyotype 46, XY, normal female external genitalia and a testicle in the inguinal region. Melo et al. (2003) described the R753X mutation in a 14-year-old patient with positive family history, primary amenorrhea, well-developed breasts, and testicles in the

abdominal region (Melo et al., 2003). Ledig et al. (2005) described a patient suffering from CAIS who was raised as a female and a negative family history.

Only two substitutions of Arg 753 have been described previously. A premature stop codon was reported in five families, and a glycine substitution was reported in seven other families (AR database at http://www.mcgill.ca/androgendb/). In all of these cases, the reported clinical form is CAIS, suggesting an important role of Arg 753 in the function of AR. Matias et al.(2000) described the specific mechanism of the interaction between the binding domain of AR and human androgen, reporting that a binding between the linking A-ring and Arg 753 is responsible for stabilizing AR.

Arg 753 is located in the protein domain that corresponds to androgenic binding and is both responsible for the androgenic-receptor interaction and fundamental for androgen signaling. These facts are in agreement with the serious compromise of androgenic function in the patients described herein. Mutations that result in alterations in the splicing of *AR* mRNA or the premature truncation of *AR* mRNA cause significant alterations in the structure of the receptor and always cause the gravest form of the disease (Melo et al., 2005).

Twenty distinct mutations in *AR* have been described in 33 Brazilian patients with AIS. Although mutations in this gene are very frequent, this is only the third reported case of a molecular diagnosis in twin sisters with CAIS and the second in Brazil. Furthermore, this is the sixth family that has been described as carrying this specific mutation. CAIS is considered to be a rare disease, and its study will contribute to the treatment and genetic advising of patients and their families. In summary, two twin sisters were examined with CAIS and were identified as carrying the R753X nonsense mutation in the androgen-binding domain of *AR*, consistent with the clinical diagnosis of CAIS.

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CAPÍTULO III

Artigo submetido à revista American Journal of Medical Genetics – Fator de Impacto B1

Marques et al.

Androgen Insensitivity Syndrome Clinical, Hormonal and Molecular Analyses of Twelve Patients with Complete Androgen Insensitivity Syndrome from a Single Family in Brazil

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ABSTRACT

Androgen insensitivity syndrome is a rare disease that is transmitted through an X-linked gene related to mutations in the androgen receptor (AR) and leads to the partial or complete impairment of virilization of fetuses with an XY karyotype. This study included twelve patients from a single Brazilian family. Evidence of X-linked inheritance and the results from clinical, laboratory and imaging studies led to the clinical diagnosis of Complete Androgen Insensitivity Syndrome (CAIS). All patients presented with a normal female phenotype without signs of virilization, with XY karyotype, showing normal development of adult female external genitalia and normal breasts. However, they did present with sparse pubic or axillary hair. A study of the genetic sequence found that the androgen gene (*AR*) was present in all patients with a mutation that is not cataloged *The Androgen Receptor Gene Mutations Database World Wide Web Server (http://androgendb.mcgill.ca/)* and is characterized by a substitution of a cytosine (C) for a guanine (G) at nucleotide position 3792 in exon 8 of the androgen receptor (AR).

KEYWORDS: Androgen Insensitivity Syndrome (AIS); androgen receptor (AR); disorders of sexual development (ADS)

INTRODUCTION

cDNA cloning of the gene responsible for encoding the androgen receptor (AR) has made it possible to elucidate the molecular basis of genetic disorders found in carriers of this gene [LUBAHN et al., 1988]. The AR gene consists of eight exons and is located on the X chromosome (Xq11-q12) [BROWN 1989]. Mutations in this gene give rise to, among other diseases, Androgen Insensitivity Syndrome (AIS), which is a rare disease (1:20,000 to 64,000 live births) with X-linked inheritance that leads to changes in the sexual differentiation of the male (XY) fetus with variable deficits in virilization. The clinical presentation varies from a female phenotype with no signs of virilization (Complete Androgen Resistance - CAIS) to varying degrees of genital ambiguity (partial resistance to androgens - PAIS) to a male phenotype with infertility [BRINKMANN, 2001; GOTTLIEB, 2004]. The family in this study is from a region known as Bico do Papagaio, which is located at a border point between the three Brazilian states of Maranhão, Pará and Tocantins. The region is located far from major centers of clinical research (Figure 1).

Figure 1 the region of study (Mesorregião do Bico do Papagaio, Brasil) Source http://www.integracao.gov.br/pt/resultado

OBJECTIVES

The objectives of this study were to characterize the clinical, hormonal and molecular characteristics of the patients affected with CAIS from a single family and to inform families about the diagnosis, advise them about the risk of malignancy and educate the patients about the need for gonadectomy and hormone replacement estrogen.

MATERIALS AND METHODS

The study was approved by the Scientific Committee (COMIC) and Ethics Committee (CEP) of HUUFMA (protocol: 001421/2008 -10 and CEP054/11). The proband was a teenager with primary amenorrhea who had undergone inguinal hernia surgery whose histology revealed testicular tissue . Through interviews with the parents of this patient, other women were identified in the family with a history of primary amenorrhea with or without reports of previous surgery for inguinal hernia repair. The prepubescent females were screened by ultrasound to identify those without a uterus.

Adult patients with primary amenorrhea and prepubertal females without a uterus were the criteria used to make the cytogenetic diagnosis. Several interviews were conducted with the patients and their families to assemble the pedigree.

Twelve patients underwent a clinical evaluation that included their history and a physical examination, with information about amenorrhea, the time of menarche, the presence of an inguinal hernia with or without previous surgery, pubertal stage (staging Tanner) and anthropometric measurements.

During the interviews, the patients were asked about their knowledge regarding the diagnosis and other related questions. Information on sexual activity, discomfort during intercourse, libido and other medical complaints were reported.

The laboratory tests included measurements of testosterone, luteinizing hormone **(**LH), follicle stimulating hormone FSH and estradiol and a cytogenetic evaluation to determine the karyotype. Imaging studies included pelvic ultrasound, bone densitometry and MRI.

Sequencing was used to detect genetic mutations in the *AR* gene. The genomic DNA of the patients' peripheral blood was extracted using the commercial EZ-DNA kit (Biological Industries). The DNA was amplified so that mutations in exon 1 to 8 of the AR gene could be identified using the Polymerase Chain Reaction (PCR) as previously described with modifications [RADPOUR et al., 2007]. PCR reactions were performed in a final volume of 25 μl that contained 100 ng of DNA, GoTaq Flexi DNA (Promega), 0.8 mM dNTPs (Amresco) and 0.8 pmol of each primer (Invitrogen). The PCR products were purified using the NucleoSpin Extract II Kit (Macherey-Nagel). Subsequently, the product was diluted to an initial concentration of 100 ηg and a final concentration of 50 ηg. The sequencing reaction was performed using the Big Dye Terminator v1.1, v3.1 Kit (Applied Biosystems). The DNA was precipitated with isopropanol/ethanol, and the samples were resuspended in formamide and sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The electropherograms were analyzed with the SeqScape v2.5 program (Applied Biosystems), and the standard AR gene sequence was compared to the results obtained from sequencing.

RESULTS

Nine additional women in the family with a history of primary amenorrhea were identified through interviews with the parents of the proband and other family members. Six of these women, including the proband, had a history of surgery for inguinal hernia repair, and four had a history of amenorrhea but no surgery. A cytogenetic XY karyotype was confirmed for all of the patients.

A pelvic ultrasound screening method confirmed the absence of a uterus or remnants of structures derived from the Wolffian or Müllerian ducts in all patients and identified two prepubescent girls who were then included in the study. The cytogenetic study revealed a XY karyotype in both. The study included twelve patients from the family, which is the highest number of cases from a single family reported in the Brazilian literature.

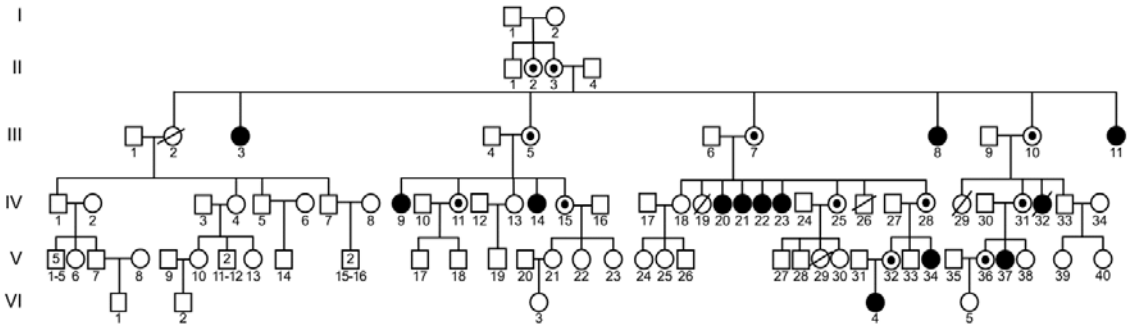

Figure 2 Great Family of the do Bico do Papagaio-MA , showing 06 generations and 13 patients affected with CAIS (III-3, III-8, III-11, IV-09, IV-14, IV-20, IV-21, IV-22, IV-23, V-32, V-34 e IV-32 (deceased) and 09 heterozygous for the AR gene (II-2, II-3, III-5, III-7, III-10, IV-15, IV-28, IV-31 e V-32).

Five adults and one teen patient had undergone ingnal hernia repair. Among the patients evaluated, three patients who were between 53 and 65 years of age had undergone a gonadectomy over 20 years ago and had not received hormone replacement for many years. These patients did not have the results from laboratory tests or pathology reports but informed that the gonadectomy was related to an ingnal hernia.

Two patients were prepubertal, and the remaining patients were adults with primary amenorrhea and no history of previous surgery (Table I). The four adult patients were not sisters of the patients who had undergone the gonadectomies and had no palpable inguinal hernias. The pedigree assembled from interviews with family members was characteristic of X-linked inheritance.

Table 1 the bone mineral density values of studied.

	Current age in years	Age at gonadectomy in years	Bone mineral density-femur **SD**	Bone mineral density-spine **SD**
	26	**	-0.7	-1.3
	29	**	-0.6	-2.0
	31	**	0.1	-1.2
	34	**	1.1	-0.4
	30	19	-2.6	-4.4
	34	17	-0.9	-1.8
	53	18	0.4	-3.7
	58	26	0.7	-3.1
	65	30	-2.2	-5.8

* SD: standard deviation

** No surgery was performed to remove the gonads

For postmenopausal women and men over 50 years it is used the standard deviation relative to the young adult (T-score):

- Normal: greater than or equal to –1.0 SD
- Osteopenia: between –1.0 and –2.5 SDs
- Osteoporosis: less than or equal to –2.5 SDs

For the premenopausal women, men under 50 years and children, we use the standard deviation relative to the same age (Z-score):

- bone mineral density below the expected range for age < or = -2.0
- bone mineral density within the limit for the age group > -2.0

Physical examination revealed that all patients were of average adult height, which was slightly higher than the other women with no family history of amenorrhea, and sparse pubic and axillary hair; furthermore, these patients demonstrated no signs of virilization. The first signs of puberty in the patients presented between 14 and 15 years old, whereas the other women of the family entered puberty at approximately 10 years of age and menarche between 12 and 13 years of age.

The bone mineral density (BMD) of the older patients (sisters aged 52, 58 and 65 years) who underwent gonadectomies between the ages of 22 and 30 years indicated osteoporosis in the lumbar spine of between -3.1 and -5.8 standard deviations (SD). The older sister presented with osteopenia in the femur of -2.2 SD. The other two sisters, who were 52 and 58 years old, did not exhibit a low BMD in the femur. Among the young adults, two sisters were gonadectomized at 16 and 18 years of age, and one received estrogen hormone replacement therapy (EHRT) irregularly and had a bone mineral density (BMD) that was within the normal range for her age but also had spinal osteopenia of -1.8 SD. The other sister, who had not received estrogen hormone replacement therapy on any occasion, had osteoporosis at the lumbar spine -4.4 SD . The four sisters not submitted to gonadectomy had normal BMD for women of their age with a tendency toward a slight reduction in the BMD of the lumbar spine (Table II). Laboratory evaluation revealed elevated levels of testosterone, LH and estradiol in four patients who had not undergone gonadectomies and, unexpectedly, the teenager who had a gonadectomy during infancy. Furthermore, the LH, FSH, testosterone and estradiol levels were found to be significantly below the normal range for the relevant age at baseline.

Table 2 Clinical and laboratory characterization of patients with 46 XY karyotype, female phenotype and no uterus detectable by pelvic ultrasound.

Current Age	Previous gonadectomy	Stage of pubertal development	T (ng/dl)	E (pg/ml)	Lh (Ul/ml)	Fsh (mUl/ml)
1	No	No pubic hair No axillary hair No breast development	0.1	0.31	0.07	0.8
12	No	No pubic hair No axillary hair No breast development	2.00	8.0	0.5	1.2
16	Yes	No pubic hair No axillary hair Areola enlargement	91.5	62.9	4.21	0.78
26	No	Minimal pubic hair No axillary hair Adult breast	312.0	29.5	17.5	2.8
29	No	Minimal pubic hair No axillary hair Adult breast	293.4	24.9	16.5	2.03
30	Yes	Minimal pubic hair No axillary hair Adult breast	29.0	20.0	16.4	59.48
31	No	Minimal pubic hair No axillary hair Adult breast	258.0	22.6	15.5	2.1
34	No	Minimal pubic hair No axillary hair Adult breast	483.0	31.5	20.6	4.07
34	Yes	Minimal pubic hair No axillary hair Adult breast	15.4	16.2	18.6	62.3
53	Yes	Minimal pubic hair No axillary hair Adult breast	3.0	15.5	7.8	35.7
58	Yes	Minimal pubic hair No axillary hair Adult breast	13.6	21.9	19.6	52.2
65	Yes	Minimal pubic hair No axillary hair Adult breast	<2.0	12.8	16.6	34.9

T: testosterone E: estradiol Lh: Luteinizing hormone Fsh: Follicle stimulating hormone

Gonadal tissue was subsequently confirmed by intra-abdominal and unilateral imaging. Hormone therapy that was begun early to induce puberty and preserve bone mass was suspended in the context of the current findings.

The AR gene was sequenced, and a genetic mutation characterized by a substitution of a cytosine (C) for a guanine (G) at nucleotide position 3792 in exon 8 of the AR gene was found. This mutation generates a change at amino acid 893 of the AR protein by changing a proline to an alanine. There is no previous record of this mutation in the online MCGILL database (ABDM, http://www.androgendb.mcgill.ca). The mutation was observed in all affected patients, and they all presented with the same phenotype. The mutation was confirmed in the heterozygous carriers and was absent in the other women of the family who were tested.

When asked about their diagnoses, none of the patients could describe them with clarity, and all demonstrated uncertainties when referring to the terms used by health professionals in their diagnoses of CAIS, such as "hermaphrodite" or "male-female."

The patients referred to all of the CAIS-related symptoms they had experienced as "problems" that accompanied some relatives from birth. One of the patients, who did not initially agree to participate in the study but was interested in participating later, reported negative experiences related to how the diagnosis was addressed by health professionals when she underwent the gonadectomy.

Although none of the patients felt in any way masculine, doubts lingered about how the differences in their physiology would manifest as male characteristics.

All patients were heterosexual, with female sexual identities. All adult patients were sexually active. The patients who did not undergo a gonadectomy did not have significant complaints about discomfort during sexual intercourse. The gonadectomized patients who did not receive hormone replacement complained of decreased libido and vaginal dryness. The married patients had adopted one or more children, including some

who had adopted nephews or children of unaffected sisters. The patients were satisfied with motherhood but reported that they had never talked about the reason for their infertility with their spouses.

When asked about the gonadectomy, the primary reason given for undergoing the procedure was cosmetic change and discomfort caused by the gonads in the inguinal region.

Complaints of decreased libido and weight gain were associated with gonadectomy, which discouraged other patients from undergoing the procedure, even after they were informed about the risk of malignant transformation and the possibility of hormone replacement.

DISCUSSION

In the Brazilian literature to date, the present report contains the greatest number of CAIS cases in one family. A previous study that described eleven cases of CAIS from nine families and 22 cases of parents from 12 families contains the highest number of cases and described seven new mutations in the AR gene and nine known mutations [MELO et al., 2005]. CORREA et al., [2005] described the mutation P766A in a case of CAIS in identical twins. PETROLI et al., [2011] studied molecular changes the androgen receptor gene, in individuals of a Brazilian family with PAIS and characterized a new mutation in five patients affected with different phenotypes.

IMPERATO-McGINLEY et al., [1990] described a family with seventeen individuals who were affected by CAIS. The study excluded large deletions in the gene and suggested a small insertion or deletion as the likely cause of the syndrome in affected individuals.

Mutations in CAIS can cause severe impairment in the function of AR [BRINKMANN, 2001; GOTTLIEB et al., 2004]. In families with CAIS, mutations generally correspond to the same phenotype in the studied family. This pattern is consistent with the findings of some cases in Brazil, in which we observed that the same mutation resulted in different phenotypes even in the same family. This variation may be related to transcriptional factors that interact with *AR* [BOEHMER et al., 2001; DEEB et al., 2005; GOTTLIEB et al., 2004].

The following criteria are used to indicate that a novel mutation is pathogenic: 1) the mutation changes a highly conserved base or disrupts a conserved base pair, 2) the mutation is absent in the controls, 3) the mutation has been reported in several pedigrees with similar phenotypes and 4) there is a correlation between the levels of mutated DNA and the severity of symptoms [RADPOUR et al , 2008]. In the family studied in the present report, the new mutation found in exon 8, a point mutation, likely resulted in the impairment of receptor binding to the hormone.T he mutation was observed in affected patients who all had the same phenotypic presentation; the mutation was confirmed in heterozygous carriers and was absent in other women of the family who were tested.

The CAIS phenotype was initially described as a syndrome of feminizing testes characterized clinically by female external genitalia with an absence or thinning of pubic hair, a short blind ended vagina without the internal genitalia derived from Wolff or Müller and the development of normal breasts during puberty. Often, the testes are present in an inguinal hernia, but the testes can also be found in the abdominal cavity or, more rarely, in the labia majora. In patients without previous surgery, except for the one-year-old child, there were no gonads in the inguinal region or in the labia majora [BOEHMER et al., 2001].

The presentation of CAIS often occurs after the discovery of a hernia, which emphasizes the importance of considering CAIS in any female child with an inguinal hernia. Estimates of the incidence of AIS in these children range from 1-12%, which indicates that any female child with an inguinal hernia should have her karyotype determined [AHMED et al., 2000].

Among the 46 cases of CAIS studied by AUDI et al., [2010], two were aborted fetuses diagnosed due to disagreement of the genotype/phenotype, 22 (47.8%) were diagnosed during childhood because of inguinal hernias and 21 (45.6%) were diagnosed at puberty because of amenorrhea. Gonadectomy was performed before puberty in eight patients (18.2%) and after pubertal development in 20 patients (45.5%).

In a study by Ahmed et al. [2000], gonadectomy was performed before puberty in 66% of cases and after puberty in 29% of cases of CAIS.

The risk of developing germ cell tumors is an important factor to be considered. The current consensus on the recommended approach to intersex disorders is a gonadectomy at diagnosis for patients with PAIS and CAIS who have undergone puberty, given the ease of EHRT [COOLS et al., 2005; COOLS et al., 2006; LEE et al., 2006].

Although the previous practice was to remove the gonads at the time of diagnosis, it is now more common to keep the gonads until puberty is complete. The risk of malignancy is low at this age, and delaying the gonadectomy allows for spontaneous pubertal development without estrogen replacement. Furthermore, this delay allows the adolescent to be involved in the discussion about whether to undergo surgery [BERRA et al., 2010].

In a study by BERRA et al., [2010], a small group of adult women chose to keep their gonads despite the risk of malignancy. Some were reluctant to use hormone replacement therapy, and others were concerned about the reports of energy loss and

decreased libido. For similar reasons, four patients in our study had thus far preferred not to undergo gonadectomy.

All patients who underwent surgery, except the proband, had a gonadectomy after puberty when their breasts were in puberty stage M5. However, the decision was not based on breast development but on when they felt the need due to discomfort and embarrassment as a result of herniation in the region and when inguinal hernia surgery could be performed. The patients had LH and testosterone levels within the normal range for males (175-780 ng/dl). Estrogen levels, despite being lower than those of women without CAIS (follicular phase: 21.8 to 215.2 pg/ml; luteal phase: 21.8 to 231.5 pg/ml), were still sufficient to induce mammary development, as described in the literature [AHMED et al., 2000; QUIGLEY et al., 1995].

BOUVATTIER et al., [2002] sequentially measured plasma testosterone, LH and FSH during the first 3 months of life in 15 neonates with AIS causing mutation in AR. Patients were divided into two groups: those with partial or complete AIS (CAIS or PAIS). In patients with PAIS, testosterone levels were above normal at 30 and 60 days. In contrast, plasma testosterone levels were below the normal range in nine of the 10 patients with CAIS on days 30 and 60.

The authors concluded that the peak of LH and postnatal testosterone usually occurs in newborns with PAIS but is absent in those with CAIS. Our youngest patient to date, who is one year old , had her blood collected within 30 days of birth and had low LH rates and testosterone [BOUVATTIER et al., 2002].

In cases of CAIS, the reduced BMD is evident in patients who remained with the gonads and those who had their gonads removed, but this reduction is more pronounced in the latter group especially when hormone replacement was not administered. In patients with partial androgen insensitivity syndrome or with 5-alpha-reductase 2 deficiency, no BMD deficit is observed if the gonads are not removed or if

hormone replacement therapy is optimized. The ideal administration of estrogen appears to be important to preserve bone mass and to increase trabecular bone volume [BERTELLONI, 2010].

In a study including women with CAIS; gonadal dysgenesis (XY karyotype) and gonadal dysgenesis (XX karyotype), suggests that androgens may be directly involved in cortical bone mineralization based on the following observations: first, women with CAIS are completely resistant to androgens; second, these women demonstrate estrogen deficiency, as indicated by the inhibitory effect on stature in patients who receive early estrogen replacement. The lack of a relationship between the age at gonadectomy and the age at introduction of estrogen replacement and BMD in CAIS patients suggests that androgens exert their greatest effects on bone mineralization during the early periods of life (before gonadectomy) [HAN et al., 2008].

This observation is consistent with the results of adult patients who had not undergone gonadectomies developed and osteopenia in the spine. Patients who received hormone replacement therapy after a gonadectomy showed no spinal osteoporosis, and cases are more severe in older patients who remained for a long time without hormone replacement therapy [HAN et al., 2008].

The identification of the molecular and genetic causes of changes in sexual development, the increased awareness of ethical issues and patient advocacy concerns have necessitated a reassessment of the previous nomenclature. Terms such as "intersex," "pseudo hermaphroditism," "hermaphroditism," and "sex reversal" are particularly controversial. These terms are perceived as potentially pejorative by patients and can be confusing for professionals and parents. The authors of the Consensus Statement on Management of Intersex, which was published in 2006, proposed the term "disorders of sex development" (DSD), which is defined as a congenital condition in which the development of chromosomal sex, gonads, or

anatomy is atypical. Furthermore, the disorders were classified based on etiology [LEE et al., 2006].

DAMIANI and GUERRA-JUNIOR, [2007] emphasize the need to adopt more neutral terms to refer to disorders of sexual development (ADS). However, the final consensus still leaves room for stigma and assumes that patients ignore the meaning of the terms 46, XY; 46, XX or the term "ovotesticular", which itself denotes a fusion of the ovaries and testis. Such terms are unlikely to be accepted by patients and relatives.

WIESEMANN et al., [2010] used the term “differences in sexual development” to replace the term "disturbances." In patients with CAIS, gender identity is usually female, and both breast size and the shape of the body are normal. The absence of the uterus in these women affects body image and self-esteem, especially if combined with a degree of impaired sexual life due to vaginal hypoplasia [WISNIEWSKI et al., 2000]. Between our patients, decreased libido complaints were related gonadectomy, since remained without EHRT.

In the study by WISNIEWSKI et al., [2000], 57% of patients with CAIS did not know their karyotype or gonadal characteristics and did not understand the importance of estrogen replacement.

The scientific and geographic isolation of the region where the family in this study is from has allowed several generations of affected women to live for many years with doubts, questions and assumptions about their diagnosis. Moreover, the lack of information deprived four patients with gonadectomies or EHRT.

In 1997 Dr GL Warne, published the first booklet with explanations to patients and families, recently reissued, still has no Portuguese version which makes it inaccessible for most of our patients (WARNE, 2012).To alleviate this problem, a simplified booklet was prepared by our staff to provide information about the diagnosis to the patients and families and is available at www.insensibilidadeandrogenica.com.br

in addition to other links to related literature and community support. The diagnosis was reported for each patient or their parents by a health professional who was able to answer possible questions and provide information on the risk of germ cell tumors and the possibility of estrogen hormone replacement.

The intersex community has established two influential support groups, the AIS Support Group (which has branches in the UK, North America and Australia) and the Intersex Society of North America (ISNA). It is ironic that at a time when advances in biomedical science regarding AIS are a source of pride, these support groups are accusing the medical profession of having ignored the real needs of patients with AIS. Since the support groups are willing to assist the medical profession to develop better approaches to the management of intersex disorders, a collaborative approach is likely to be mutually beneficial for patients and physicians (WARNE 1997).

The way disorders of sex development (DSD) are viewed and managed in different cultures varies widely. They are complex conditions and even well-educated lay people find them difficult to understand, but when families are very poor and lacking in basic education, and the health system is starved of resources, traditional beliefs, folk remedies and prejudice combine to make the lives of children and adults with DSD extremely difficult. There is a need for standardized instruments that would allow a true comparison of the quality of outcomes from the patients' perspective. (WARNE; RAZA, 2008).

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4 CONSIDERAÇÕES FINAIS

Os dados obtidos no capítulo I são de extrema relevância científica, pois nunca havia sido descrita na literatura a mutação P893A. O que evidencia a quantidade de informação biológica que precisa ser investigada e publicada.

Com a caracterização molecular de CAIS tanto no artigo do capítulo II, quanto a nova mutação descrita no capítulo I, foi possível direcionar melhor a terapêutica das pacientes, além de assistência, esclarecimento e orientação para os familiares envolvidos sobre o potencial risco de malignização de gônadas disgenéticas e distribuição ao longo das gerações futuras.

Em relação ao capítulo II, só há registro de cinco trabalhos publicados na literatura com esta mutação, sendo apenas um no Brasil e, em nenhum deles, a mutação foi encontrada em gêmeas, o que demonstra a importância deste achado.

O registro de novos pacientes e suas prováveis novas mutações, incluindo aqui a encontrada no capítulo I, contribuirão com o crescente banco de dados sobre o gene *AR*. Isso pode influenciar em um diagnóstico mais preciso e proporcionar um melhor aconselhamento genético para os indivíduos afetados e seus familiares. Assim, este estudo fornecerá subsídios para trabalhos posteriores e também contribuirá para uma melhor caracterização desta síndrome.

A respeito do capítulo III a descrição do quadro clínico, principalmente hormonal das pacientes com CAIS, vem proporcionando as perspectivas de tratamento e as orientações aos familiares, propiciando uma contribuição importante entre duas áreas do conhecimento, a genética e a endocrinologia.

Em conclusão, os resultados obtidos confirmam a literatura quanto ao local mais freqüente de mutações, a região LDB (de parte do exon 4 até o exon 8 do gene *AR*), justamente onde foram encontradas as mutações R753X (exon 5) e P893A (exon 8).

Em relação às metas propostas inicialmente nesta pesquisa, vale ressaltar que mais do que dados científicos, foram fornecidas informações e acompanhamento de pacientes, aconselhamento, o que pode fornecer uma melhor perspectiva de vida para pacientes e familiares.

APÊNDICES

APÊNDICE A - Termo de Consentimento Livre e Esclarecido (TCLE)

UNIVERSIDADE FEDERAL O MARANHÃO

HOSPITAL UNIVERSITÁRIO

LABORATÓRIO DE ESTUDOS GENÔMICOS E DE HISTOCOMPATIBILIDADE - LEGH

Termo de Consentimento Livre e Esclarecido (TCLE)

Título: **Estudo Molecular do Gene do Receptor de Andrógeno em Famílias Brasileiras com a Síndrome de Insensibilidade aos Andrógenos (AIS).**

Coordenadora: Profª. Drª. Emygdia Rosa do Rêgo Barros Pires Leal Mesquita.

Pelo fato de você ou algum membro de sua família apresentar uma alteração genética chamada síndrome de insensibilidade aos andrógenos (AIS), que leva à infertilidade (incapacidade para engravidar), você está sendo convidado a participar de uma pesquisa para estudar quais as alterações genéticas que você e/ou seus familiares apresentam, e que são responsáveis por esta infertilidade.

Solicitamos que você doe um pouco de sangue (aproximadamente uma colher de sopa = 10cc). Este sangue será usado para fazer os exames de avaliação genética e investigar as possíveis modificações genéticas, que acometem vários membros da sua família. No dia da doação de sangue, você poderá ingerir sua dieta normalmente. Nós faremos a assepsia do seu braço com álcool e em seguida usaremos o torniquete. Nós coletaremos sangue do seu braço, utilizando técnica estéril/limpa e seringa descartável, portanto não precisa se preocupar.

Você também será convidado (a) a responder um questionário e permitir a realização de exames, se necessário, como ultrassonografia, densitometria, óssea e tomografia computadorizada, deixando bem claro que todos são exames não invasivos e que não provocam qualquer tipo de dor.

RISCOS: Os riscos possíveis associados à participação neste estudo são relacionados à coleta de sangue e são sangramentos ou equimoses.

BENEFÍCIOS: Os benefícios em participar deste estudo é que os resultados desta pesquisa poderão ser úteis para as futuras gerações de sua família, ou de outras famílias com distúrbio semelhante, uma vez que os indivíduos poderão ser orientados e aconselhados quanto aos possíveis tratamentos. Se algum exame mostrar-se alterado, o resultado lhe será comunicado e será fornecida adequada orientação médica ou encaminhamento para assistência pelo Sistema Único de Saúde.

CONFIDENCIALIDADE DO ESTUDO: O registro da participação neste estudo será mantido confidencial, até o limite permitido pela lei. No entanto, agências regulamentadoras Federais no Brasil e o Comitê de Ética podem inspecionar e copiar registros pertinentes a

pesquisa e estes podem conter informações identificadoras. Os registros de cada indivíduo serão guardados e somente os pesquisadores membros da equipe terão acesso a estas informações. Cada indivíduo receberá um número para ser utilizado no laboratório. Se qualquer relatório ou publicação resultar deste trabalho, a identificação do paciente será preservada. Os resultados serão relatados de forma sumarizada e o indivíduo não será identificado. **Os dados genéticos coletados nesta pesquisa só serão utilizados para o proposto neste estudo sendo, portanto garantido a não utilização para outro fim sem o prévio consentimento do indivíduo doador ou seu representante legal, mediante a elaboração de um novo protocolo de pesquisa, com aprovação do Comitê de Ética e Pesquisa.**

PARTICIPAÇÃO VOLUNTÁRIA

Em primeiro lugar, é importante frisar que qualquer voluntário (a) poderá desistir deste estudo a qualquer momento. Não há penalidade para alguém que decida não participar neste estudo. Ninguém também será penalizado se decidir desistir de participar do estudo, em qualquer época. A sua assistência/tratamento /acompanhamento não será diferente caso você decida participar ou não desta pesquisa. Quanto aos exames você poderá decidir se quer saber os resultados ou não. **O participante pode ter acesso a seus dados genéticos, assim como tem o direito de retirá-los de banco onde se encontrem armazenados, a qualquer momento.**

Não haverá qualquer despesa para os (as) voluntários (as).

Em caso de dúvidas sobre a realização dos exames ou outros procedimentos referentes à pesquisa, entrar em contato com a pesquisadora responsável, Profª. Drª. Emygdia Rosa Leal Mesquita, na Rua dos Prazeres, 215 (Cobertura do Hospital Materno Infantil), Centro. CEP: 65020 – 090, Laboratório de Estudos Genômicos e de Histocompatibilidade – LEGH. Fone: (098) 2109 – 12 65 ou 12 58. Ou se tiver dúvidas referente aos aspectos éticos deverá fazer contato junto ao Coordenador do Comitê de Ètica em Pesquisa na Rua Barão de Itapary S/N – Centro (4º andar) HUUFMA – Unidade Presidente Dutra, Fone: (098) 2109 – 1223, a qualquer momento, pessoalmente ou através dos telefones.

Tomando conhecimento do estudo, declaro concordar em participar da pesquisa, assinando duas cópias desse termo de consentimento ficando uma em meu poder.

OBS. Os menores só participarão deste estudo após autorização dos pais ou responsáveis.

Data____/___/____

Participante/Responsável_____

Assinatura

Pesquisador:_____

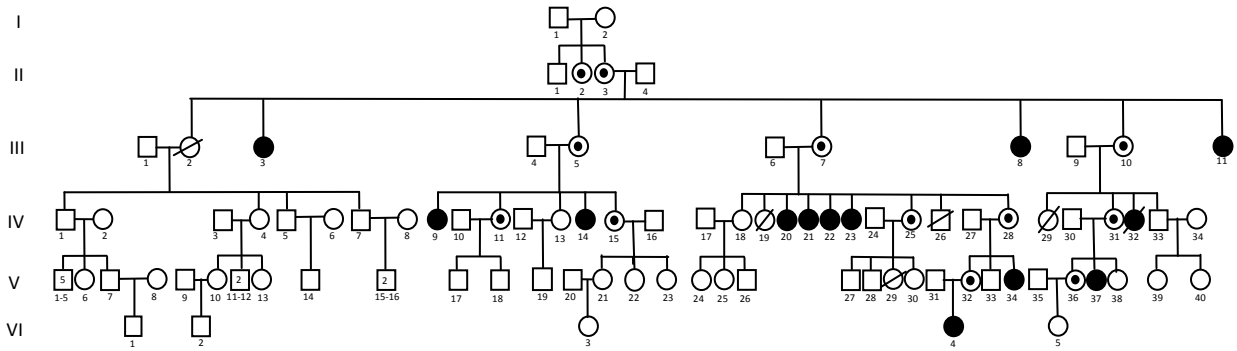
Assinatura

Pesquisador Responsável: Profª. Drª. Emygdia Rosa Leal Mesquita

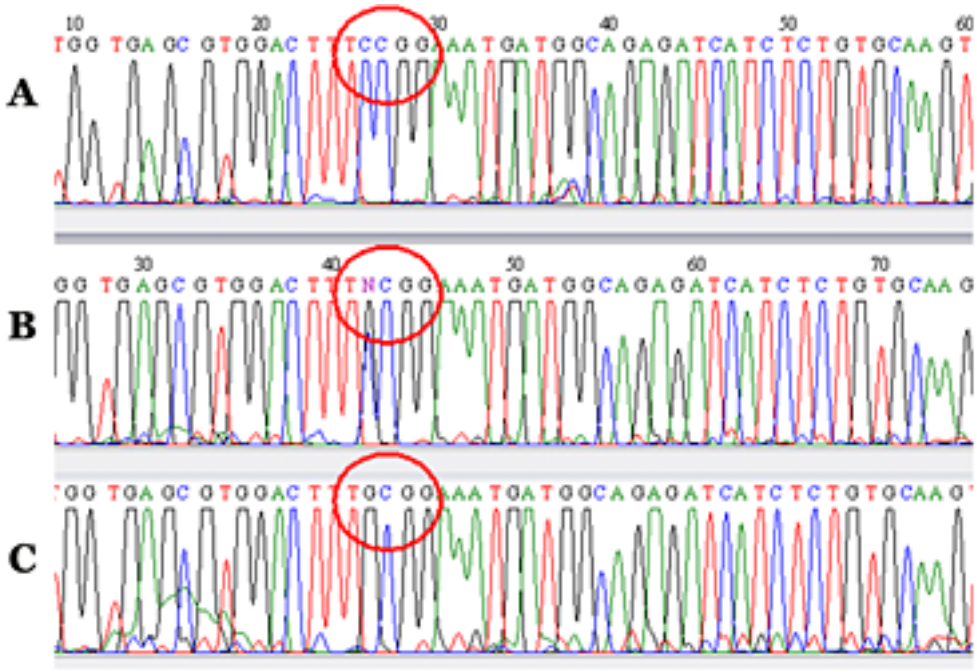
Rua dos Prazeres, 215 (Cobertura do Hospital Materno Infantil), Centro. CEP: 65020 - 090

Laboratório de Estudos Genômicos e de Histocompatibilidade – LEGH Fone: (098) 2109 – 12 65 ou 12 58.

APÊNDICE B – Heredograma representativo da grande família da região do Bico do Papagaio-MA, com 13 afetadas por CAIS e 12 portadoras do gene *AR*



APÊNDICE C – Figura 2 do Capítulo I: Eletroferograma das pacientes e familiares com a mutação P893A, indicando a substituição de uma citosina por uma guanina no exon 8 do gene *AR*. A- Sequencia referência, corresponde a um indivíduo normal; B – indivíduo heterozigoto para a mutação; C- indivíduo afetado para a mutação.



APÊNDICE D – Figura 2 do capítulo II. Electropherogram indicating a T-to-C substitution in exon 5 corresponding to position 753 of AR protein. A – Reference sequence. B – Sequence of a normal individual. C e D – Twin's affected sequences.

ANEXOS

ANEXO A - PARECER CONSUBSTANCIADO DO COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE FEDERAL DO MARANHÃO
HOSPITAL UNIVERSITÁRIO
DIRETORIA ADJUNTA DE ENSINO, PESQUISA E EXTENSÃO
COMITÊ DE ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO INICIAL	**Nº. do Parecer: 080/11** **Registro do CEP: 054/11** **Nº do Protocolo: 001629/2011-30** **Parecer: APROVADO**
PROJETO DE PESQUISA	

I - Identificação:

Título do projeto: **Estudo Molecular do Gene do Receptor de Andrógeno em Famílias Brasileiras com a Síndrome de Insensibilidade aos Andrógenos (AIS) SÃO LUÍS**	
Identificação do Pesquisador Responsável: Emygdia Rosa do Rêgo Barros Pires Leal Mesquita	
Identificação da Equipe executora: Dr. Manuel dos Santos Faria – Médico – CEPEC/HUUFMA, Ms. Marcelo Souza de Andrade – Biólogo/Geneticista e Doutorando do RENORBIO, Ana Lígia Barros Marques – Médica e Doutoranda do RENORBIO, Laura Moreira de Andrade – Mestre em Ciências da Saúde-UFMA, Danilo Santos de Azevedo – Médico Geriatra, Bruno de Almeida Nunes – Biólogo CEPEC/HUUFMA, Ana Paula Braga Garcez - Discente de Biologia UFMA, Rayanna Pereira Chaves - Discente de Biologia UFMA	
Instituição onde será realizado: Laboratório de Estudos Genômicos e de Histocompatibilidade (LEGH) do Hospital Universitário da Universidade Federal do Maranhão UFMA (HUUFMA)	
Área temática:	Multicêntrico: Não
Cooperação estrangeira	

II - Objetivos:

Geral: Estudar por meios de técnicas de biologia molecular o gene do receptor de andrógenos e as repercussões clínicas e hormonais de suas mutações em famílias brasileiras com AIS.

Específicos:

- Identificar mutações no gene do AR nos indivíduos afetados;
- Caracterizar mutações no gene do AR em parentes próximos dos pacientes afetados, em especial meninas pré-púberes, mulheres com amenorréia primária e homens inférteis;
- Analisar o quadro clínico dos pacientes afetados: sexo social, órgãos genitais internos e externos e densidade mineral óssea;
- Fazer a correlação genótipo-fenótipo;
- Determinar o perfil hormonal dos pacientes: testosterona, estradiol, FSH, LH;

III- Sumário do projeto:

A Síndrome de Insensibilidade Androgênica (AIS) é um distúrbio raro (1:40.800 a 1:99.000), de transmissão ligada ao cromossomo X, que cursa com distúrbio da diferenciação sexual do feto masculino (XY) com um espectro de apresentações que varia desde o fenótipo feminino completo (insensibilidade completa - CAIS) passando por diversos graus de ambigüidade genital (insensibilidade parcial - PAIS) até um fenótipo masculino com discretos sinais de insensibilidade androgênica (discreta insensibilidade - MAIS).
Estudo a ser realizado partindo de 2 famílias , onde o caso índice da família 1 é uma menina pré-pubere de 12 anos que, após submeter-se a herniografia inguinal, recebeu laudo histopatológico da peça cirúrgica compatível com tecido testicular. A anamnese do caso índice levou as outras nove mulheres na família com cirurgia prévia para hérnia inguinal e ausência de útero ou em amenorréia primária sem investigação. O caso índice da família 2 é uma mulher (fenótipo feminino) com amenorréia primária, ausência de útero e anexos, sem cirurgia prévia. As pacientes serão submetidas à avaliação clínica, ginecológica, radiológica e laboratorial. A

avaliação clínica e ginecológica constará de anamnese e exame físico, com informações sobre amenorréia, época da telarca, hérnia inguinal com ou sem cirurgia prévia, estadiamento de Turner, exame ginecológico, avaliação antropométrica e caracterização de quaisquer patologias concomitantes. A avaliação radiológica incluirá ultrassonografia pélvica e densitometria óssea, buscando caracterizar os órgãos genitais internos ou resquícios rudimentares e medida da densidade mineral óssea, uma vez que, as três pacientes afetadas na segunda geração realizaram gonadectomia há 20 anos, em média, e permanecem sem terapia de reposição estrogênica até então. A avaliação laboratorial constará de avaliação hormonal com dosagem de testosterona, LH, FSH e estradiol, além de avaliação genética para detectar mutações no gene. Para a obtenção do DNA genômico (avaliação genética) será extraído de sangue total (5 a 10 ml de sangue coletado a vácuo em tubos contendo EDTA como anticoagulante), para a realização das técnicas moleculares. A avaliação clínica, ginecologia e radiológica será realizada na região de origem das pacientes, (interior do estado do Maranhão) com deslocamento dos pesquisadores e recursos do Sistema Único de Saúde, considerando que os exames são de rotina de acordo com o protocolo assistencial. A avaliação genética será realizada no Laboratório de Estudos Genômicos e de Histocompatibilidade (LEGH) do Hospital Universitário da Universidade Federal do Maranhão UFMA (HUUFMA), que será responsável pelo desenvolvimento do estudo. O desenvolvimento do projeto contará com recursos próprios do Laboratório de Estudos Genômicos e de Histocompatibilidade (LEGH) do HUUFMA. O orçamento financeiro incluirá os materiais de consumo utilizado na rotina do LEGH, considerando ser estudo em andamento e de interesse do Centro de Pesquisa Clínica (CEPEC) do HUUFMA. Será de responsabilidade dos pesquisadores os deslocamentos para a avaliação clínica e ginecologia.

IV - Comentários do relator frente à resolução 196/96 e complementares:

O protocolo de pesquisa possui a seguinte estrutura: Folha de Rosto, Folhas de identificação, Sumário, Introdução, Fundamentação Teórica, Objetivos, Justificativas, Metodologia, Orçamento, Cronograma, Referência Bibliográficas, Curriculo Lattes e TCLE. Portanto, em conformidade com o estabelecido na Res. 196/96 CNS/MS.

V - Parecer Consubstanciado do CEP

Assim, mediante a importância social e cientifica que o projeto apresenta, a sua aplicabilidade e conformidade com os requisitos éticos, o referido projeto foi analisado e classificado como **APROVADO em 2008**, pois o mesmo atendeu aos requisitos fundamentais da Resolução 196/96 e suas complementares do Conselho Nacional de Saúde / MS.

Solicita-se ao (à) pesquisador (a) o envio a este CEP, dos relatórios parciais sempre quando houver alguma alteração no projeto, bem como o relatório final gravado em CD-ROM.

São Luís, 15 de abril de 2011

Prof. Dr. João Inácio Lima de Souza
Coordenador do CEP-HUUFMA
Ethica homini habitat est

ANEXO B – Submissão do artigo “New mutation P893A in exon 8 of the AR gene leads to androgen insensitivity syndrome in a large family in Maranhao, Northeast Brazil”

Elsevier Editorial System(tm) for Molecular and Cellular Endocrinology
Manuscript Draft

Manuscript Number: MCE-D-12-00166

Title: New mutation P893A in exon 8 of the AR gene leads to androgen insensitivity syndrome in a large family in Maranhao, Northeast Brazil.

Article Type: Research Paper

Keywords: KEYWORDS: androgen receptor; androgen insensitivity syndrome; AIS; mutation

Corresponding Author: Dr. MARCELO SOUZA ANDRADE, PhD

Corresponding Author's Institution: Universidade Federal do Maranhão
First Author: MARCELO S ANDRADE, MSc.

Order of Authors: MARCELO S ANDRADE, MSc.; MARCELO SOUZA ANDRADE, PhD; ANA LÍGIA B.MARQUES, B.S; LAURA M ANDRADE-REIS, MSc.; BRUNO A NUNES, MSc.; Anaregina S ARAUJO, MSc.; Semíramis J. H MONTE, PhD; Gilvan C NASCIMENTO, MSc; LUCIANA HELENA G VAZ, MSc.; MANUEL S FARIA, PhD; EMYGDIA ROSA R LEAL-MESQUITA, PhD

Manuscript Region of Origin: BRAZIL

Abstract: ABSTRACT
Androgen insensitivity syndrome (AIS) is a rare disorder, with the incidence between 1:20,000 and 1:64,000 live male births. Transmission is X-linked, and the syndrome is caused by mutations in the androgen receptor (AR) gene. This study included twelve patients and their relatives spanning six generations from a single Brazilian family. We have investigated mutations in the AR gene in affected individuals and characterized the mutations in close relatives of the affected patients, especially in prepubescent girls and women with amenorrhea. In all patients and in the heterozygous carriers, there was a C->G substitution at nucleotide position 3792 in exon 8 of the AR gene, changing proline to alanine at amino acid 893. Research such as this can reveal the molecular and hereditary mechanisms underlying disorders for patients and families. This study extends current understanding of AR mutations associated with AIS.

ANEXO C - Normas para autores da revista Molecular and Cellular Endocrinology

MOLECULAR AND CELLULAR ENDOCRINOLOGY

ISSN: 0303-7207

DESCRIPTION

Molecular and Cellular Endocrinology was established in 1974 to meet the demand for integrated publication on all aspects related to the biochemical effects, synthesis and secretions of extracellular signals (hormones, neurotransmitters, etc.) and to the understanding of cellular regulatory mechanisms involved in hormonal control.

The journal is fulfilling this aim by publishing full-length original research papers, rapid papers, invited reviews, At the Cutting Edge essays, and book reviews.

The scope encompasses all subjects related to biochemical and molecular aspects of endocrine research and cell regulation. These include: (1) mechanisms of action of extracellular signals (hormones, neurotransmitters, etc.), (2) interaction of these factors with receptors, (3) generation, action and role of intracellular signals such as cyclic nucleotides and calcium, (4) hormone-regulated gene expression, (5) structure and physicochemical properties of hormones, hormone receptors and other hormone-binding components, (6) synthesis, secretion, metabolism and inactivation of hormones, neurotransmitters, etc. (7) hormonal control of differentiation, (8) related control mechanisms in non-mammalian systems, (9) methodological and theoretical aspects related to hormonal control processes, (10) clinical studies as far as they throw new light on basic research in this field, (11) control of intermediary metabolism at the cellular level, (12) ultrastructural aspects related to hormone secretion and action.

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

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If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

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• NCT: ClinicalTrials.gov (NCT ID: NCT00222573)

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• PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)

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TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required. If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low; • Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

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Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version.

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Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

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Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;

2. *Two authors:* both authors' names and the year of publication;

3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;

List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;

CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

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with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions.

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Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

• Telephone and fax numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print

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When you use the DOI to create URL hyperlinks to documents on the web, the DOIs are guaranteed never to change.

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ANEXO D – Submissão do artigo " **An R753X Mutation in the androgen receptor gene of monozygotic female twins with complete androgen insensitivity syndrome (CAIS)"**

2011/11/29 ISTS <ists@qimr.edu.au>
Dear Laura

Many thanks for submitting " AN **R753X Mutation in the androgen receptor gene of monozygotic female twins with complete androgen insensitivity syndrome (CAIS)"** Author(s) Andrade-Reis, L. M.; **Andrade, M. S.;** Marques, A.L.B.; Nunes, B. A; Faria, M. dos S.; Leal-Mesquita, E.R.R.B.P.

to ***Twin Research and Human Genetics***.
For future correspondence, please note that your manuscript has been assigned the reference **number TR083/11.**

Kind regards,

Lorin Grey
Editorial Assistant, *Twin Research and Human Genetics*
Genetic Epidemiology
Queensland Institute of Medical Research
Locked Bag 2000
Post Office Royal Brisbane Hospital Q 4029
www.ists.qimr.edu.au

- RE: Submission of Manuscript TRO83/11

Documentos, Fotos |25/03/2012

To Lorin Grey,

Editorial Assistant

Good morning,

We are resubmitting the manuscript TRO83/11 with corrections listed below:

- All the manuscript was corrected by a native English teacher;

- The Abstract was structured;

- We agree and corrected that it is not true that AIS patients **never** present with female internal genitalia;

- The testis biopsy measurements was taken;

- We corrected that CAIS testicular structure is never normal for age;

- The testosterona has been measured at age of 3 year and the levels were elevated because of the HCG stimulation test;

- Later the submission the exon 1 was sequenced and included at the study;

- The *AR* gene does not dimerize, but AR protein did:

"Testosterone and dihydrotestosterone, secreted by the Leydig cells of the testes, bind to this intracellular protein and cause a conformacional change that leads to dimerization, nuclear transport, target DNA binding and eventually transcription" (Nichols et al., 2009) and others authors agree - Moura 2011; Melo et al. 2005; Reid et al., 2003; Dehm et al., 2007.

- "exact locus" was substitute by "exact location".

- Figures was fomated and it adheres to the journal's formatting requirements (80mm, 600dpi Tiffs). However, we are in doubt about the format and location of the captions that moreover were inserted in the text of the manuscript.

Cordiality,

Laura Reis

ANEXO E- Normas para autores da revista Twin Research and Human Genetics

Twin Research and Human Genetics

Scope

Twin Research and Human Genetics is the official journal of the International Society for Twin Studies. Twin Research and Human Genetics communicates the results of original research in human genetics with a special emphasis on multiple birth research. It also provides timely state-of-the-art reviews on all aspects of human genetics and twin studies. Topics covered include: genetic epidemiology, behavioral genetics, complex diseases, endocrinology, fetal pathology, medical genetics, obstetrics, pediatrics, psychiatric genetics and other areas of human genetics, with an emphasis on twin studies.

Editorial Policy

All contributions and general correspondence regarding editorial matters should be addressed to the Editor and sent to the Editorial Office. Manuscripts submitted to the journal must represent reports of original research. Manuscripts will be sent for anonymous review either by members of the editorial board, or by individuals of similar standing in the field. Authors are requested to submit up to three suggested reviewers for the manuscript. Authors will be notified of acceptance, rejection or the need for revision within 6 weeks. When a manuscript is returned to the corresponding author for revision, it should be returned to the Editors within 2 months, otherwise it may be considered withdrawn. Accepted manuscripts will appear in the journal within 3 months, whenever possible.

Preparation of the Manuscripts

All manuscripts should be in English presented electronically in Word or Word Perfect. All sections of the manuscript should be double-spaced with one inch margins on all four sides. * Please subdivide manuscripts into the following sequence of sections: Title page, Abstract, Introduction, Materials and methods, Results, Discussion, Acknowledgement, References, Tables, Figure legends. Number each page (title page is 1). Please indicate the position of each figure and table in the text using the words “Figure X about here”.

* Effective from October 2006, please note that manuscripts will be charged publication charges of:

ISTS MEMBERS (first/last author) - AUD100.00 per printed page for each page NON-ISTS MEMBERS (first/last author) – AUD150.00 per printed page for each page

Included in this publication cost as of 2007 is open access for all papers over one year old. Authors may purchase immediate online access at the time of publication for their papers for an additional AUD200. Please contact the editorial office for more information. In exceptional circumstances, the Editor may consider reducing or waiving these charges. Please contact Professor Nick Martin directly.

In general, the formatting requirements are those recommended by the American Psychological Association (APA), especially for references:

American Psychological Association. (2001). Publication manual of the American Psychological Association (5th ed.). Washington, DC: Author.

Title Page/keywords

The title should be as brief as possible with no abbreviations. Include each author's initials and surname and complete addresses in English, including department, institution, city with postal code and country (indicating clearly which author is at which address). A corresponding author should be indicated with telephone, fax numbers and e-mail address provided. Please also provide a running title of not more than 50 characters.

Abstract

The second page of the manuscript should contain only an abstract in a single paragraph of not more than 200–250 words. Abbreviations and reference citations should be avoided.

Introduction

The introduction should assume that the reader is knowledgeable in the field and should therefore be a brief as possible and should not exceed 1500 words. Do not use the heading "Introduction".

References

References should follow the general format advocated by the Publication Manual of the American Psychological Association (5th ed.). However, in the text, "et al." should be used for references with three or more authors, as follows:

One author: (Jones, 1981)

Two authors: (Jones & Smith, 1981)

Three or more authors: (Jones et al., 1981).

At the end of the manuscript references should be listed (double-spaced) in alphabetical order. All authors should be listed, and full periodical titles should be used. First and last page numbers for each reference should be provided. Abstracts and letters must be identified as such.

Abbreviations

These should be defined in parentheses after their first mention in the text, except for the use of accepted abbreviations, such as SI Symbols, which need not be defined. Use generic names when referring to drugs; trade names may be given in parentheses at first mention.

Tables

These should be typed on separate sheets and numbered consecutively with Arabic numerals. Tables should be self-explanatory and include a brief descriptive title. Footnotes to tables indicated by lower case letters are acceptable, but they should not include extensive experimental detail. Please indicate in the manuscript the most appropriate position for each table using the words “Table X about here”.

Illustrations, figures, photographs and mathematics

Electronic submission of artwork

To ensure optimum quality, please follow these guidelines when submitting artwork via e-mail or disk.

1. Photographs, graphs and figures should be prepared to the correct size (max. width 80mm single column or up to 160mm double column). Figures should be in black and white line art (artwork that has only text and lines, no shades of grey or

blocks of colour).

Each photograph, graph or figure should be:

■ supplied as an individual file, separate to the manuscript Word file with placement instructions included in the Word document, such as [insert Fig 1 here]

OR

■ if created in Microsoft Word, Excel or Powerpoint, embedded in the Word file at the end of the document and supplied ALSO as a PDF.

• Figures created in a drawing program such as Adobe Illustrator, CorelDRAW, Freehand, Microsoft Publisher or similar should be saved as EPS (encapsulated postscript) files and PDFs.

• Figures created in Photoshop or with other photographic software should be saved with a minimum resolution of 600 dpi and in TIF format. Minimum resolution for scanned graphics is 300 dpi for halftone work (e.g., photographs) and 600 dpi for line art, and these should also be in TIF format.

2. Manuscripts which contain equations created with LaTeX or similar specialist software need to be supplied as a PDF file as well as a Microsoft Word document.

3. Prior to sending artwork, the separate files of figures, graphs, illustrations, and so on, should be printed by the author to test that the fonts have been embedded correctly and there is no distortion in the artwork (e.g., lines and fonts reproduce

cleanly with no jagged lines or fuzzy edges), as any such faults cannot be corrected by the publisher.

4. Preferred media for delivery: e-mail as attachments, Macintosh or PC floppydisk, Macintosh or PC Zip disk, CD-ROM.

Reviews

Scholarly reviews of topics within the scope of Twin Research and Human Genetics will be considered for publication after paper review. Please send a one page letter of inquiry to the Editor before preparing your manuscript, to make sure that a similar review is not in press.

Proofs

Manuscripts will be scheduled for publication upon receipt of proofs. Extensive changes to the proofs will result in publication delay. Important new information that has become available between acceptance of the manuscript and receipt of the proofs may be inserted as an Addendum in proof with the permission of an Editor. Proofs must be checked immediately for typographical errors and returned to the publisher along with the copyright assignment form. Authors will receive a PDF offprint of their article with a licence to reproduce up to 100 copies free of charge.

ANEXO F – Figura 1 do capítulo II. – Study setting: Bico do Papagaio, in the pre-Amazon region. Adapted from the http://www.integração.gov.br/progamas/programasregionais/papagaio/abrangência.asp

ANEXO G- Submissão do artigo III "Androgen Insensitivity Syndrome Clinical, Hormonal and Molecular Analyses of Twelve Patients with Complete Androgen Insensitivity Syndrome from a Single Family in Brazil"

Date: Wed, 18 Apr 2012 22:17:02 -0400
> From: editor.ajmg@hsc.utah.edu
> To: lighiama@hotmail.com
> Subject: MS 12-0359 Submission to AJMG
>
> Dear Dr. Marques:
>
> The American Journal of Medical Genetics acknowledges receipt of your manuscript:
>
> Androgen Insensitivity Syndrome Clinical, Hormonal and Molecular Analyses of Twelve Patients with Complete Androgen Insensitivity Syndrome from a Single Family in Brazil
>
> which has been assigned the following number: MS 12-0359.
>
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> http://mc.manuscriptcentral.com/ajmg-a
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> and log in using your user ID and password.
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> Please remember in any future correspondence regarding your work to always include your manuscript ID number.
>
> We want to thank you for the opportunity to consider your work.
>
> Sincerely,
>
> John C. Carey, MD
> Editor-in-Chief
> American Journal of Medical Genetics

ANEXO H - Figura 1 Capítulo III. The region of study (Mesorregião do Bico do Papagaio, Brasil) Source http://www.integracao.gov.br/

ANEXO I - Normas para autores da revista American Journal of Medical Genetics – Parte A

Author Guidelines

The American Journal of Medical Genetics Part A welcomes the submission of your manuscript.We emphasize the importance of following these instructions carefully. Failure to do so will delay the processing of your manuscript. We also ask that you consult the Journal's mission statement and consider if your submission fits into our focus.

Manuscript Submission

Authors should carefully review and implement these instructions. We will return manuscripts that do not substantially conform to specifications. If questions remain, authors may contact the editorial office for guidance. When necessary, the editorial staff can refer unanswered questions to the Editor or Deputy Editor. Electronic mail is preferred for editorial correspondence.

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Please note: This journal does not accept Microsoft Word 2007 documents at this time. Please use Word's "Save As" option to save your document as an older (.doc) file type.

The American Journal of Medical Genetics Part A is a member of, and subscribes to the principles of, the Committee on Publication Ethics (COPE) (www.publicationethics.org).

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If the authors have no conflict of interest to declare, they must also state this at submission. It is the responsibility of the corresponding author to review this policy with all authors and to collectively list in the cover letter to the Editor-in-Chief, in the manuscript (under the Acknowledgments section), and in the online submission system ALL pertinent commercial and other relationships.

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Components of the Submission

Cover letter: Authors must submit a cover letter, in a separate file, stating that all contributors have read and approved the submission to the JOURNAL. Submission of a paper by a student, fellow, house-officer, or other kind of trainee implies that the first author has obtained, if necessary local approval of submission.

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Research Articles. The JOURNAL seeks papers reporting exciting, original research in medical genetics.

Clinical Reports. These reports should be concise and focused. They should address observations of patients or families that add substantially to the knowledge of the etiology, pathogenesis and delineation of the natural history or management of the condition described. Reports that involve cytogenetic observations should include molecular cytogenetic definition of the aberration. Reports should include an abstract and key words. These papers should be no more than 12 double spaced manuscript pages in length (including text, figures, tables, and references).

New Syndrome? This is for (non-cytogenetic and nonmetabolic) cases of possible new syndromes observed in sporadic and familial instances. Before submission, standard genetic databases and the literature should have been searched to exclude similar cases. The number of authors on such clinical reports should be kept to a minimum. Avoid duplication of text and legends within the paper. It is not appropriate to include a wideranging review of the literature with these reports.

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Correspondence. These are letters to the editor and generally comment on previously published work in the JOURNAL. These are kept brief and to the point. Like all other material published in the JOURNAL, correspondence is subject to editorial or peer review. The corresponding author of the original manuscript which is the subject of the submitted letter will be offered the opportunity to respond. If a response is provided, every effort will be made to publish these letters together. Only one round of comment is allowed. No key words need to be supplied, but on proofs, words should be circled for indexing purposes.

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Response to reviews. A paper judged to be of sufficient interest and quality, but needing changes prior to publication, will be revised by the authors in response to the reviewers' and editor's critiques. Include a cover letter with the revised manuscript that specifies in a point-by-point fashion each criticism/ comment from the editor(s) and peer reviewers with the appropriate response from the author.

Final proofreading. Before publication, all manuscripts will be copyedited for style, grammar, and content; the author will review such editing of the manuscript in page proofs. Authors are responsible for verification of references against original sources.

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Checklist for Authors: Follows the order of elements for a paper submitted to the JOURNAL

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