REGINALDO JOSÉ PETROLI

# *"EFFECT OF NOVEL MUTATIONS IN ANDROGEN RECEPTOR UPON MOLECULAR MECHANISMS"*

# "EFEITOS DE NOVAS MUTAÇÕES NO RECEPTOR DE ANDRÓGENOS SOBRE OS MECANISMOS MOLECULARES"

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Genética e Biologia Molecular na Área de Genética Animal e Evolução.

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**ABSTRACT**

The androgen receptor (AR) is a transcription factor that belongs to the superfamily of nuclear receptors activated by phosphorylation and dimerization by hormone binding. Several functions are attributed for AR, like male sex development, regulation of gene expression and cell differentiation in target tissues. The aim of this study was to analyze the effect of mutations p.Pro695Ser, p.Ser759Tre, p.Leu768Val, p.Cys806Phe+ p.Gln798Glu, p.Leu830Phe, p.Ile898Phe and p.Pro904Arg upon AR transactivation activity. All mutations studied here are located in the hormone-binding domain and were identified in patients with different degrees of androgen insensitivity syndrome (AIS) by *AR* gene sequencing. Mutations in this domain can result in the impairment of androgen ligation, but there are cases that it does not affect the binding but interfere with the interaction between the amino and carboxi-terminal domains (N/C terminal), important step for receptor-binding stabilization. Thus, both functions have been studied in this work. To evaluate the ability of AR transactivation of mutant proteins, the site-directed mutagenesis assay was performed on full-length cDNA, followed by transfection and expression in mammalian cells. The analysis of transactivation of a reporter gene with different dihydrotestosterone (DHT) concentrations was performed. The analysis of N/C-terminal interactions for each mutant AR was performed by two- hybrid mammalian assay. The mutation p.Pro695Ser reveled transactivation activities of 85% and 82% in transactivation assays with the full-length cDNA and two hybrid assay, respectively, at DHT physiological values (approximately 1 nM). The activities reached normal values at high DHT concentrations, indicating a low effect on gonadal activity. However, in low DHT concentrations, the transactivation activity decays to less than 50% in both experiments, which may affect AR functions in non-gonadal tissues. This mutation was considered "mild" and corresponds perfectly to the male phenotype of the patient who presented with gynecomastia, but with preserved fertility. With 1 nM hormone, the p.Ser759Tre, p.Leu830Phe, p.Ile898Phe mutations showed transactivation activity higher than 20%, the response increased with higher DHT concentrations. However, in N/C interaction assays, those mutations showed different results. The p.Ile898Phe revealed a complete disruption in N/C interaction at all hormone concentrations; the p.Ser759Tre and p.Leu830Phe showed positive response with the increasing in DHT concentrations and reached 50% and 250% of the transcriptional

activity of wild type, respectively. Such results indicate a partial AIS phenotype (PAIS) as functional effect for p.Ser759Tre and p.Leu830Phe. In these cases there was a positive correlation with the phenotypes of patients that presented different degrees of PAIS. For the p.Ile898Phe, the expected phenotype based on functional analysis would be the complete form of AIS (CAIS), but the patients with this mutation had variable degrees of genital ambiguity consistent to PAIS. This indicates that other factors must influence the phenotypic manifestation. The p.Leu768Val revealed a complete disruption at 1 nM DHT in both experiments, typical of CAIS. The p.Gln798Glu and p.Cys806Phe mutations studied separately revealed responses to the induction of DHT similar to Mild and Partial phenotypes, respectively. However, when analyzed together, the transactivation activity of 1 nM was lower than 10%, increasing in high ligand concentration, which is consistent to CAIS phenotype. Finally, p.Pro904Arg, although showed residual transcriptional activity around 20% of the wild type in the experiment with the full-length cDNA, it abolished the transcriptional activity when N/C terminal interaction was tested indicating a CAIS phenotype, as observed in the patient. Functional analysis of the AR performed here could elucidate some molecular mechanisms associated with each mutation, and may provide a basis for response to treatment with DHT in each particular case.

**RESUMO**

O receptor de andrógenos, do inglês *androgen receptor* (AR) é um fator de transcrição pertencente à superfamília de receptores nucleares o qual é ativado por fosforilação e dimerização sob a ligação ao hormônio. Várias funções são atribuídas a este receptor, sendo a principal delas o desenvolvimento e manutenção das características sexuais masculinas, atua na regulação da expressão gênica e diferenciação celular em tecidos alvos. O presente trabalho teve por objetivo principal a análise do efeito das mutações p.Pro695Ser, p.Ser759Tre, p.Leu768Val, p.Cis806Fen+p.Gln798Glu, p.Leu830Fen, p.Ile898Fen e p.Pro904Arg sobre a função do AR. As mutações acima citadas, localizadas no domínio de ligação ao hormônio, foram identificadas por sequenciamento direto do gene do *AR* de pacientes 46,XY com diferentes graus da Síndrome da Insensibilidade Androgênica (AIS). Mutações nesse domínio geralmente rompem a ligação aos andrógenos naturais, porém há algumas que não afetam essa ligação, mas interferem na interação entre os domínios amino e carboxi-terminal (N/C terminal), importante para a estabilização receptor-ligante. Assim, ambas funções foram investigadas. Para se avaliar a capacidade de transativação das proteínas AR mutantes, foi realizada a técnica de mutagênese sítio dirigida no cDNA completo, seguida de transfecção e expressão em células de mamíferos e análise de transativação induzida por concentrações crescentes de diidrotestosterona (DHT) utilizando-se um gene repórter. A análise das interações N/C-terminal para cada AR mutante foi realizada pela técnica de duplo-híbrido em células de mamíferos. A mutação p.Pro695Ser apresentou atividades de transativação de 85% e 82% nos ensaios transativação com o cDNA completo e no duplo-híbrido, respectivamente, em valores de DHT fisiológicos (cerca de 1 nM). As atividades atingiram valores normais em concentrações elevadas de DHT indicando um baixo efeito sobre a atividade gonadal. No entanto, em concentrações de DHT inferiores a atividade de transativação decai para menos de 50% nos dois experimentos, podendo afetar as funções do AR em tecidos não gonadais. Esta mutação foi considerada “branda” e corresponde perfeitamente ao fenótipo masculino do paciente que se apresentava com ginecomastia, mas com fertilidade preservada. Com 1 nM de hormônio, as mutações p.Ser759Tre, p.Leu830Fen, p.Ile898Fen apresentaram atividade de transativação superior a 20%, havendo um aumento de resposta com concentrações crescentes. No entanto, o comportamento de cada uma no experimento de

interação N/C diferiu sendo que a p.Ile898Fen não apresentou atividade em nenhuma concentração de ligante; as p.Ser759Tre e p.Leu830Fen responderam positivamente ao aumento da concentração de DHT atingindo 50% e 250% da atividade transcricional do receptor selvagem, respectivamente. Esses resultados indicam um fenótipo parcial de AIS (PAIS) para os portadores das mutações p.Ser759Tre e p.Leu830Fen. Nesses casos verificou-se uma boa correlação dos achados funcionais com os fenótipos dos pacientes que apresentavam graus variados de PAIS. Já para a mutação p.Ile898Fen o fenótipo esperado baseando-se nos resultados funcionais seria o de AIS na forma completa (CAIS), porém os pacientes portadores desta mutação apresentavam graus variados de ambiguidade genital compatíveis com o fenótipo PAIS. Isto indica que outros fatores devem estar influenciando a manifestação fenotípica nesse caso. A mutação p.Leu768Val apresentou atividade transcricional nula em 1 nM de DHT nos dois experimentos, um perfil típico do fenótipo CAIS apresentado pelo portador desta mutação. As mutações p.Gln798Glu e p.Cis806Fen estudadas separadamente apresentaram respostas à indução de DHT semelhantes às de mutações "brandas" e PAIS, respectivamente. No entanto, quando estudadas em conjunto, a atividade de transativação com 1 nM foi inferior a 10%, aumentando com o aumento da concentração de ligante, comportamento compatível com mutações mais graves resultando no fenótipo CAIS observado nesse caso. Por último, a mutação p.Pro904Arg, embora tenha reduzido a atividade transcricional para cerca de 20% da selvagem no experimento com o cDNA completo, no experimento com duplo híbrido a atividade foi nula indicando uma ação mais grave compatível com a forma CAIS observada. A análise funcional do AR aqui realizada pode elucidar alguns mecanismos moleculares associados a cada mutação, bem como pode fornecer subsídios para a resposta ao tratamento com DHT em cada caso em particular.

**SUMÁRIO**

**A MINHA ESPOSA PRISCILA**

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# 1. Introdução

A expressão do fenótipo masculino normal é decorrente da ação dos andrógenos, hormônios esteroides importantes que apresentam funções cruciais na diferenciação sexual masculina. Os principais andrógenos circulantes em mamíferos são a testosterona (T) e a dihidrotestosterona (DHT). Esses hormônios esteroides são produzidos pelas glândulas adrenais e pelas gônadas, e desempenham um papel vital na reprodução, especialmente na diferenciação do sexo masculino durante o desenvolvimento fetal e na diferenciação sexual secundária masculina e feminina (Hughes, 2001).

## 1.1 O Receptor de Andrógenos

O receptor de andrógenos [AR; Online Mendelian Inheritance in Man 313700], cuja função essencial é o desenvolvimento do sexo masculino, é um membro da superfamília de fatores de transcrição nucleares ligantes-dependentes, que inclui os receptores para progesterona, glicocorticoides, estrógenos, vitamina D, hormônio tireoidiano e ácido retinóico (Quigley *et al.*, 1995).

A ativação dos andrógenos é mediada pelos receptores acima mencionados, que por sua vez medeiam a diferenciação sexual masculina *in utero* e são responsáveis pelo desenvolvimento e manutenção das características sexuais masculinas (Ghali *et al.*, 2003).

A sequência de DNA complementar (cDNA) que codifica o receptor de andrógenos em humanos foi elucidada em 1988 por diversos grupos quase que simultaneamente (Chang *et al.*, 1988; Lubahn *et al.*, 1988; Trapman *et al.*, 1988; Tilley *et al.*, 1989). Os autores mapearam o gene *AR* no cromossomo X, especificamente na região Xq11-13, porém, sua localização foi redefinida em 1993 por Brown e colaboradores localizando-o na região Xq11-12.

O gene *AR* em humanos é composto por oito *exons* (Figura 1), separados por *introns* de até 26 kb. A região codificante do gene apresenta aproximadamente 2.757 pares de bases, que codificam uma proteína de aproximadamente 920 aminoácidos cujo peso molecular é de aproximadamente 110 kDa (Lubahn *et al.*, 1988). O número de aminoácidos na proteína

varia e está diretamente relacionado ao número de repetições de glicinas e glutaminas encontrados em duas regiões polimórficas no domínio N-terminal, como será mostrado mais adiante.

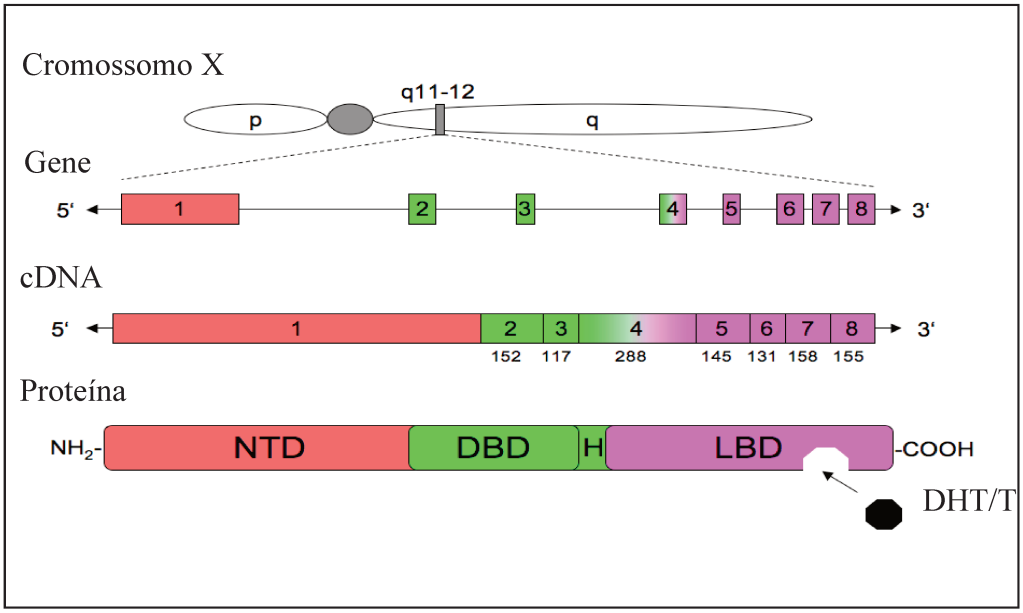


**Figura 1**: Esquema da localização e organização do gene e da proteína do receptor de andrógenos. Acima: cromossomo X, com destaque para a localização do gene *AR* na posição Xq11-12. Ao centro: o gene *AR* com 75-90 kb de comprimento, dividido em 8 *exons* separados por 7 *introns* de até 26 kb. Abaixo: o AR e os seus três domínios, o domínio N-terminal (NTD), o domínio de ligação ao DNA (DBD), região *hinge* (H) e o domínio de ligação ao ligante (LBD) com sulco para a ligação da testosterona (T) e diidrotestosterona (DHT). O *exon* 1 codifica o NTD (vermelho), os *exons* 2 e 3 codificam o DBD (verde). A região 5' do *exon* 4 codifica a região *hinge* (verde) e a parte restante do *exon* 4 juntamente com os *exons* 5-8 codificam o LBD (rosa) (Adaptado de Quigley *et al.*, 1995).

## 1.2 A Proteína do Receptor de Andrógenos

O AR, assim como outros receptores nucleares, apresenta três diferentes domínios funcionais (Figura 1). O primeiro domínio, chamado de domínio de regulação transcricional *NH2 terminal domain* (NTD), o segundo é o domínio de ligação ao DNA (DBD) e o terceiro corresponde ao domínio de ligação ao hormônio (LBD) (Ghali *et al.*, 2003; Tufan *et al.*, 2005; Wu *et al.*, 2010). Há também uma região de dobradiça entre o DBD e LBD denominada região *hinge,* que por conter o sinal de localização nuclear (NLS) é responsável pela translocação do complexo hormônio-receptor do citoplasma para o núcleo da célula (Quigley *et al.*, 1995).

### 1.2.1 Domínio Regulador Transcricional

O NTD que é codificado pelo *exon* 1 do gene *AR* é um extenso domínio ativador da transcrição. Sua extensão corresponde a mais da metade da proteína AR e é formado por cerca de 555 resíduos aminoacídicos, número este que pode variar. A variação neste número se deve à presença de repetições dos resíduos de glutaminas (CAG) e glicinas (GGC) a partir dos resíduos 57 e 450, respectivamente. Estas duas regiões polimórficas conferem uma variabilidade grande para a região. Assim, o número de repetições CAG pode variar, normalmente produzindo alelos com números de resíduos entre 11 e 31. Existem variações no número destas repetições entre diferentes grupos raciais. Na população negra, por exemplo, o alelo mais frequente apresenta 18 repetições, ao passo que em populações caucasianas o número mais frequente é 21 (Quigley *et al.*, 1995; Girardin *et al.*, 2009). Estudos *in vitro* com alelos *AR* com mais de 40 repetições CAG, mostraram uma redução na atividade transcricional em comparação com moléculas que apresentavam 20 a 25 repetições. Assim, pode-se deduzir que o número de repetições CAG esteja diretamente relacionado à atividade transcricional do gene *AR* (Hughes *et al.*, 2006; Rajender *et al.*, 2007). A expansão da região de poliglutaminas para 40 a 65 resíduos está associada à síndrome de Kennedy, doença rara associada à atrofia testicular, redução na produção de espermatozóides e infertilidade (Ferlin *et al.*, 2006; Rajender *et al.*, 2007).

O número de repetições da trinca de nucleotídeos GGC, que corresponde ao aminoácido glicina, varia entre 16 a 27 resíduos dentro da normalidade na população. Repetições acima desta faixa estão associadas ao câncer de próstata e infertilidade masculina (Ferlin *et al.*, 2006). Por outro lado, o número reduzido de repetições GGC está associado à redução da atividade transcricional do gene *AR* (Rajender *et al.*, 2007).

O polimorfismo do número de glutaminas tem sido mais investigado do que o do número de glicinas. Como as regiões de DNA que codificam tanto a poliglutamina quanto a poliglicina são ricas em GC, a amplificação por reação em cadeia da polimerase (PCR) destes fragmentos torna-se difícil devido à conformação de fita simples que o DNA assume mediante a desnaturação e, também por este motivo, poucas mutações são descritas no *exon* 1 (Yong *et al.*, 2003; Rajender *et al.*, 2007; Rong *et al.*, 2010).

O NTD é o domínio menos conservado entre os receptores nucleares (Claessens *et al.*, 2008). Neste domínio, encontram-se regiões essenciais para a ação transativadora do AR. A região chamada *activation function 1* (AF-1) é importante, onde se encontram duas subregiões centrais com particular função na transativação. Essas subregiões são a Tau-1, localizada entre os resíduos 101 e 370 e, a Tau-5, que se estende do resíduo 360 ao 529, ambas desempenham funções através de interações intramoleculares e intermoleculares com co-reguladores. Além disso, os primeiros 30 aminoácidos do NTD são essenciais para a interação entre os terminais N e C do receptor, fundamental para a estabilização do ligante e ativação apropriada do AR, como observado na Figura 2 (McEwan *et al.*, 2007; Werner *et al.*, 2008).

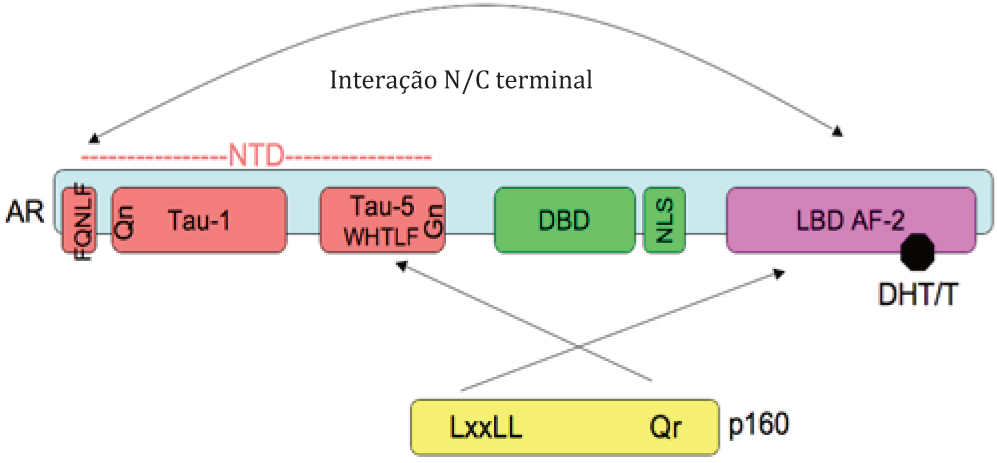


**Figura 2**: Representação esquemática do AR_NTD (vermelho), DBD (verde) e LBD (rosa). O *motif* FQNLF do NTD interage com o AF2 do LBD, levando a interação N/C-terminal (acima). A região Tau-1 do NTD contém a repetição do aminoácido glutamina (Qn). Já a região Tau-5 apresenta a repetição do aminoácido glicina (Gn). Ao ligar T/DHT, o AF2 é ativado e acontece a interação com cofatores da transcrição. O esquema representa a interação entre o coativador p160 com NTD e LBD. LxxLL e Qr são *motifs* do coativador p160, que se ligam diretamente com regiões específicas do AR. O sinal de localização nuclear (NLS) situado entre o DBD e LBD é responsável pela translocação do complexo hormônio/receptor do citoplasma para o núcleo da célula (Esquema adaptado de Claessens *et al.*, 2008).

### 1.2.2 Domínio de ligação ao DNA

O domínio de ligação ao DNA (DBD), o mais conservado da proteína AR, situa-se entre os aminoácidos 539 e 628, sendo seu tamanho de aproximadamente 80 aminoácidos. Este domínio é reconhecido pela presença de dois dedos de zinco (*zinc finger*). Os dedos de zinco apresentam estruturas e funções diferentes e são codificados pelos *exons* 2 e 3, respectivamente (Figura 3) (Melo *et al.*, 2005; Rajender *et al.*, 2007).

São ligados ao zinco por quatro cisteínas, produzindo uma estrutura de hélice-alça-hélice que interage com sequências especificas de DNA, os elementos de respostas hormonais (HRE) (Melo *et al.*, 2005; Rajender *et al.*, 2007).

O reconhecimento da sequência alvo no DNA é feita pelo primeiro dedo de zinco, que apresenta uma sequência específica de cinco aminoácidos chamada de P-box, como detalhado na figura 3. A região P-box também é encontrada em outros receptores capazes de reconhecer sequências específicas no DNA, como exemplo, podemos citar os receptores de progesterona, glicocorticóides e mineralocorticóides (Clinckemalie *et al.*, 2012).

O segundo dedo de zinco é responsável pela ligação do receptor ao DNA e por estabilizar a interação DNA-proteína. Essa ligação acontece pelo contato com os grupamentos fosfatos do DNA, tendo assim um papel fundamental em promover a ativação da transcrição. Esse sítio está diretamente relacionado com a estabilidade da dimerização do receptor (Giwercman *et al.*, 2000; Hughes *et al.*, 2006; Clinckemalie *et al.*, 2012).

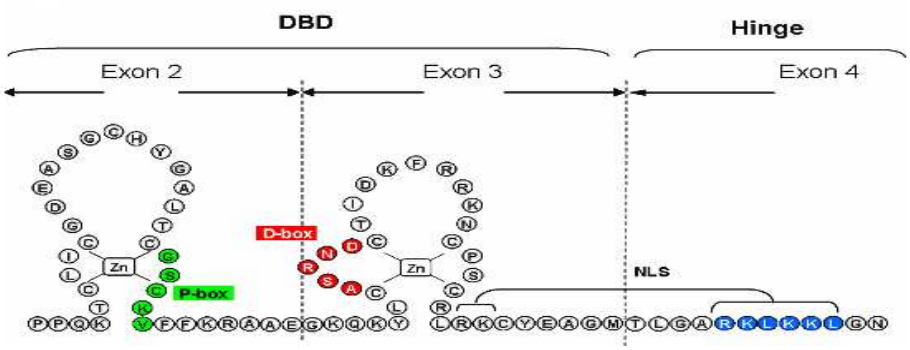


**Figura 3**: Estrutura do domínio de ligação ao DNA e região *Hinge*. O DBD apresenta dois dedos de zinco ligados. O primeiro dedo de zinco apresenta a região P-box (*proximal box*) que está associada ao reconhecimento ao elemento de resposta hormonal (HRE) no DNA e o segundo dedo de zinco, contém o D-box (*distal box*) que está envolvido na dimerização do complexo hormônio receptor. A região *hinge* é codificada pela porção 5' do *exon* 4 do gene *AR* e está associada a localização nuclear e a estabilidade do receptor (Clinckemalie *et al.*, 2012).

### 1.2.3 Região *hinge*

Entre os domínios de ligação ao DNA e o domínio de ligação ao andrógeno, encontra-se a região *hinge.* Esta região é codificada pela porção 5' do *exon* 4 do gene *AR* (Figura 3). Os aminoácidos localizados entre as posições 629 até 669 compõem esta região, também conhecida como região de dobradiça. Nesta região, especificamente os aminoácidos $^{629}$RKLKKL$^{634}$, encontra-se a região necessária para a translocação do complexo andrógeno/receptor do citoplasma para o núcleo da célula (NLS). Além desta função, pode-se atribuir à região *hinge* outras funções como regular o potencial transcricional e a estabilidade do receptor, promover a mobilização intra-nuclear, além de ser alvo de modificações pós-traducionais (Helsen *et al.*, 2012, Rajender *et al.*, 2007, Wong, *et al.*, 2008, Clinckemalie *et al*., 2012). Clinckemalie e colaboradores (2012) descrevem em seu trabalho a importância desta região também na interação com co-reguladores. Mutações ou deleções nesta região podem resultar em um prejuízo drástico na capacidade de ligação com o DNA, porém, curiosamente aumentam o potencial de transativação desse gene. Processos sub-nucleares evidenciam a importância dessa região no recrutamento de cofatores, porém este mecanismo ainda não foi esclarecido (Helsen *et al.*, 2012, Rajender *et al.*, 2007, Wong, *et al.* 2008, Wu *et al.*, 2010, Quigley *et al.*, 1995, Tanner *et al.*, 2010).

### 1.2.4 Domínio de ligação ao hormônio

O domínio LBD é o segundo domínio mais conservado, também conhecido como *carboxi-terminal domain* (CTD), localiza-se entre os aminoácidos 670 a 920 (Figura 1). Neste domínio, encontra-se o subdomínio *activation function* 2 (AF-2). Este subdomínio apresenta uma região altamente conservada na região *carboxi-terminal* deste receptor, fundamental para a transativação deste gene (Figura 2). Depois da ligação com o andrógeno, as proteínas de choque térmico que mantém o AR desativado são liberadas. O complexo hormônio/receptor dimeriza-se e é transportado para o núcleo celular onde é ativado por mudanças conformacionais na hélice 12 desta proteína, que favorece a interação entre os domínios N/C terminais (NTD e CTD) através das hélices 3, 4 e 12 (Jaaskelain *et al.* 2006). O AF-2 forma uma fenda hidrofóbica que se liga ao LXXLL *motif*

(L representa o aminoácido leucina e o X representa outro aminoácido), dos co-ativadores (SRC)/p160. O subdomínio AF-2 também se liga com alta afinidade ao FQNLF *motif,* localizado no domínio *NH2* do AR (Figura 2). Estas ligações intramoleculares são conhecidas como interações N/C terminais, como representado na figura 2 (Werner *et al.*, 2008). Atualmente, este tipo de interação tem sido usada para relacionar a capacidade de transativação do AR e o prejuízo associado ao fenótipo do paciente. Ensaios sobre interações N/C terminais têm-se comprovado úteis como ferramentas importantes em casos de síndrome da Insensibilidade Androgênica (AIS, do inglês *Androgen Insensibility Syndrome*) onde mutações no LBD não provocam prejuízos na ligação ao hormônio (Jaaskelain *et al.*, 2006; Werner *et al.*, 2008).

Ainda no domínio LBD, encontra-se e a região denominada *androgen receptor binding function-3* (AR BF-3) que compreende os aminoácidos Ile-672, Phe-673, Pro-723, Gly-724, Asn-727, Phe-826, Glu-829, Asn-833, Glu-837 e Arg-840. Assim como o subdomínio AF-2, a região BF-3 é altamente conservada entre os receptores de esteróides (Perpina *et al.,* 2007). Estudos revelam que a região BF-3 é um *hot spot* para mutações que estão associadas ao câncer de próstata e à AIS (Buzon *et al*.,2011, Perpina *et al.,* 2007). Buzón e colaboradores (2011), sugerem em suas análises estruturais que existe uma relação direta entre AF-2 e BF-3 no recrutamento de co-ativadores. Ainda nesse trabalho, os autores referem-se à região BF-3 como uma potencial candidata para o desenvolvimento de fármacos (Buzon *et al*.,2011, Perpina *et al.,* 2007).

### 1.3 Ação Androgênica

Outras proteínas importantes na ação androgênica são as chaperonas, como a HSP70, HSP90, HSP40 e FKBP52, essenciais para o estabelecimento da ligação do complexo hormônio/receptor, translocação do complexo do citoplasma para seu sítio de ação nuclear, acelerar a transcrição mediada pelo AR e manutenção da proteína AR (Figura 4) (Nitsche e Hiort, 2000; Heemers e Tindall, 2007).

A transativação do AR é dada pela interação entre diferentes subdomínios da proteína AR. Como já citado, os subdomínios mais conhecidos que atuam diretamente neste processo são os subdomínios AF1, localizado no domínio amino-terminal e o subdomínio

AF2, localizado no domínio carboxi-terminal da proteína AR. Interações entre AF1 e AF2 são comprovadamente necessárias para a transativação desse receptor (Werner *et al.*, 2008). Deleções no domínio LBD podem abolir completamente a ligação com o hormônio e consequentemente a função do AR. Deleções na região N-terminal que é o domínio NTD, não afetam a capacidade de ligação com o ligante, porém, podem afetar a capacidade de transativação do AR devido à presença dos subdomínio AF1 nesta região.

Entre as co-chaperonas que promovem a ação androgênica, podemos citar a FKBP52, que tem sido estudada em relação à sua atuação nos mecanismos de atividade androgênica e também quanto seu papel no fenótipo de indivíduos com hipospadias (Cheung-Flynn *et al.*, 2005; Cox *et al.*, 2007; Beleza-Meireles *et al.*, 2007; Chen *et al.*, 2010). Embora tenha comprovadamente um papel importante na ação transcricional do receptor de andrógenos, nenhuma mutação no gene *FKBP4*, que a codifica, foi identificada. Provavelmente, isto se deva ao fato de as triagens de mutações em indivíduos com os vários graus de insensibilidades androgênicas serem ainda incipientes.

Em um estudo feito por Sunnotel e colaboradores (2010) ficou demonstrado que o fator FKBPL é expresso em altas quantidades em testículo de rato, sendo que essa expressão aumenta na puberdade. A proteína FKBPL humana é expressa nos testículos em um padrão semelhante ao da FKBP52 e também é capaz de aumentar a atividade transcricional do gene *AR* em ensaios com *gene-reporter* (Sunnotel *et al.*, 2010). Em tal trabalho, que descreve mutações no gene *FKBPL*, ficou clara a importância dos genes que codificam co-chaperonas na regulação da atividade androgênica.

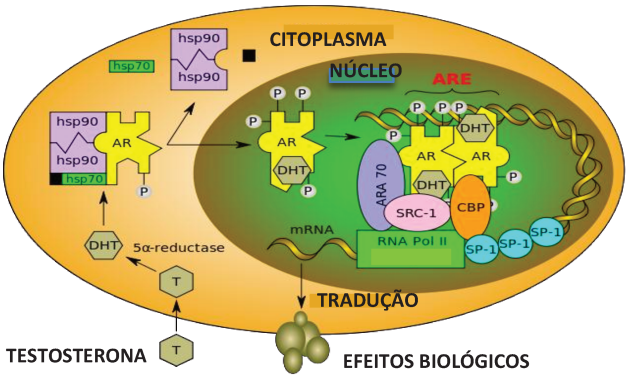


**Figura 4**: Esquema do mecanismo molecular do receptor de andrógenos. O receptor de andrógenos é ativado após a entrada de testosterona e/ou dihidrotestosterona no citoplasma da célula. A ativação do receptor envolve a dissociação de proteínas inibitórias associadas ao receptor, como as *heat-shock proteins* (HSPs). Depois da ligação com o hormônio, acontece a fosforilação e dimerização do receptor. No núcleo, os dímeros ligam-se aos elementos de resposta ao hormônio (ARE), recrutam cofatores para a iniciação da transcrição de genes alvo e assim acontecem os efeitos biológicos (Adaptado de Meehan e Sadar, 2003).

### 1.4 Mutações no Gene *AR*

A atuação dos hormônios testosterona e diidrotestosterona só acontecem quando o receptor para esses andrógenos e todos os mecanismos relacionados à ação androgênica estão e íntegros. Assim, a ação desses hormônios ocorrerá, permitindo sua atuação no desenvolvimento fetal e na diferenciação da genitália masculina normal (Heemers e Tindall, 2007).

Quando o gene *AR* apresenta alguma alteração ou outros genes de alguma proteína envolvida na ação androgênica não se encontram em suas formas íntegras, ocorrerá um prejuízo na ação do hormônio e o indivíduo apresentará a AIS (OMIM 300068) (Heemers e Tindall, 2007).

A AIS está associada principalmente a mutações no gene *AR*, porém pode ser associada a outros eventos envolvendo a atuação dos andrógenos, desde a ligação do andrógeno ao receptor, até sua ação nuclear (Werner *et al.*, 2006). Dependendo da gravidade da alteração,

podemos associá-la aos fenótipos completo, parcial ou brando da insensibilidade androgênica.

Mais de 800 mutações constam do banco de mutações específico do gene *AR* (http://androgendb.mcgill.ca/AR23.pdf, Gottlieb *et al.*, 2012), sendo que 15% foram encontradas no domínio ativador da transcrição, 20% no domínio de ligação ao DNA e 55% no domínio de ligação aos andrógenos.

A maioria das mutações encontradas no gene *AR* é do tipo *missense*. Este tipo de mutação, conhecido pela substituição de aminoácidos isolados pode estar associado a todos os fenótipos atribuídos a insensibilidade androgênica. A localização das mutações está relacionada diretamente à manifestação da doença. Mutações localizadas no subdomínio *AF-2* da proteína AR, geralmente estão associadas à forma CAIS (do inglês *complete androgen insensitivity syndrome*) ou parcial grave (Galani *et al.*, 2008; Wu *et al.*, 2010; Petroli *et al.*, 2011).

Não muito frequente, mas também relacionada a AIS, são as mutações que conduzem à alteração no quadro de leitura (mutações *frameshift*), inserções ou deleções de nucleotídeos, alterações no mecanismo de *splicing* ou até deleções de grandes segmentos deste gene. Todas essas mutações podem interromper ou alterar a sequência primária da proteína e estão associadas ao fenótipo CAIS (Maciel-Guerra *et al.*, 2002; Galani *et al.*, 2008; Brinkmann *et al.*, 2011).

## 1.5 A Insensibilidade Androgênica

A insensibilidade androgênica é a causa mais comum de distúrbios da diferenciação do sexo em indivíduos com cariótipo 46, XY. O espectro fenotípico dessa síndrome pode variar desde indivíduos com fenótipo tipicamente feminino, com testículos, com mamas e desenvolvimento reduzido de pêlos até indivíduos com fenótipo masculino, geralmente com ginecomastia, podendo ou não apresentar comprometimento na fertilidade (Figura 5) (Larrea *et al.,*1978; Quigley *et al.,* 1995; Yong *et al.,* 1998; Giwercman *et al.,* 2000; Boehmer *et al.*, 2001).

Os primeiros casos de AIS foram relatados por Morris em 1953, data que ficou conhecida como um marco histórico no estudo desta doença (Morris, 1953). Em seu

trabalho, Morris avaliou 82 casos, inicialmente conhecidos como "testículos feminilizantes" (Quigley *et al.*, 1995, Hughes *et al.*, 2006). Como sinais clínicos de AIS, foram estabelecidos os critérios: genitália externa feminina, com ausência de genitais internos femininos, exceto útero rudimentar, trompas uterinas ou ductos espermáticos, em alguns pacientes; desenvolvimento mamário feminino normal; ausência ou escassez de pêlos pubianos e axilares; gônadas com túbulos seminíferos, ausência de espermatogênese e aumento das células intersticiais; hábito feminino (Quigley *et al.*, 1995).

Dados de frequência na população ainda são imprecisos. Na população dinamarquesa, a incidência é estimada entre 1:20.400 a 1:99.000 recém-nascidos com sexo genético masculino (Quigley *et al.*, 1995; Hughes *et al.*, 2006).

Por ser uma anomalia recessiva ligada ao cromossomo X, somente indivíduos com sexo genético 46,XY são afetados por AIS, enquanto que indivíduos com sexo feminino 46,XX podem ser portadores e transmiti-la para a sua prole, porém sem manifestar o quadro clínico da doença (Hiort *et al.*, 2000). Em 70% dos casos, os indivíduos com características clínicas de AIS herdam a mutação da mãe, enquanto os 30% restantes são de mutações *de novo* (Kohler *et al.*, 2005).

Para a classificação da genitália de indivíduos com AIS, Quigley e colaboradores (1995) propõem 7 graus diferentes, ordenados de acordo com a gravidade da resistência androgênica (Figura 5). De acordo com esta classificação, as genitálias são diferenciadas de acordo com o grau de virilização, iniciando-se no grau 1, que inclui indivíduos com resposta normal aos andrógenos durante o desenvolvimento fetal e genitália externa normal, sendo caracterizado por homens com ginecomastia e inférteis ou, mais raro, férteis. Esses pacientes se enquadram no fenótipo leve desta síndrome, conhecida como MAIS (do inglês *mild androgen insensitivity syndrome*). A forma parcial, conhecida por PAIS (do inglês *partial androgen insensitivity syndrome*) é classificada entre os graus 2 ao 6, podendo variar desde ambiguidade genital até genitália típica feminina com pilificação. Já indivíduos com a resistência completa aos andrógenos, forma CAIS é classificada como grau 7 e compreende indivíduos com fenótipo feminino, porém sem desenvolvimento de pêlos pubianos e axilares na puberdade.

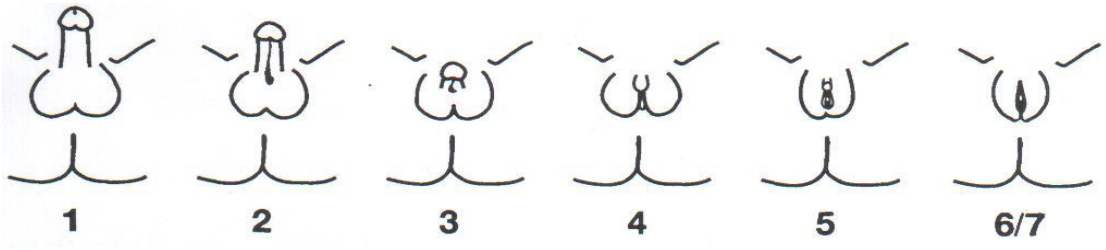


**Figura 5:** Classificação dos graus de virilização da genitália externa em indivíduos com AIS, de acordo com a gravidade da resistência androgênica (Quigley *et al.*, 1995). Em 1, genitália masculina típica, observado em casos de insensibilidade branda aos andrógenos. Em 2, compreende indivíduos com fenótipo masculino com manifestação de hipospádia. Em 3, engloba indivíduos com fenótipo predominantemente masculino, com anomalias graves na masculinização, hipospadias perineal, pênis diminuido com criptorquidia. Em 4, compreende indivíduos com limitações na masculinização, com falo intermediário entre clitóris e pênis. Em 5, indivíduos com fenótipo feminino, com mínima ação androgênica no período fetal, separação entre uretra e orifício vaginal, leve clitoromegalia e/ou pequeno grau de fusão labial posterior. Em 6, compreende indivíduos com genitália feminina normal, sem atuação de andrógenos no desenvolvimento fetal, com pêlos pubianos e axilares. De 2-6 compreende indivíduos com a forma parcial da insensibilidade androgênica. Em 7, compreende indivíduos com genitália feminina normal, sem atuação de andrógenos no desenvolvimento fetal, sem desenvolvimento de pêlos pubianos e axilares na puberdade, caracterizando a forma completa da AIS (Quigley *et al*.,1995).

### 1.5.1 A Insensibilidade Completa aos Andrógenos

A forma mais grave da resistência aos andrógenos, conhecida por insensibilidade completa a andrógenos, é caracterizada pela perda completa da função do receptor de andrógeno.

A CAIS acomete indivíduos 46,XY que produzem normalmente testosterona na vida fetal e na puberdade. Porém, tanto a genitália quanto os outros orgãos-alvo não respondem aos andrógenos nessas fases, causando diferenciação feminina da genitália externa e do seio urogenital, além de feminilização na puberdade.

O diagnóstico dessa síndrome pode ser realizado logo após o nascimento, devido à presença de testículos palpáveis e à discrepância entre o cariótipo 46,XY e o fenótipo dos pacientes, tipicamente feminino. No entanto, muitos casos podem não ter diagnóstico precoce e na infância. Nesses casos, o diagnóstico acontece na puberdade, pela amenorréia primária e ausência de pêlos (Quigley *et al.*, 1995). A manifestação clínica mais comum é a presença de hérnia inguinal bilateral. Segundo Galani e colaboradores (2008), a prevalência de pacientes com AIS e hérnia inguinal acontece em 1 a 2% desses casos.

O fenótipo clássico atribuído à forma CAIS caracteriza-se como ausência de ductos genitais internos, vagina em fundo cego e genitália externa feminina normal, exceto pela presença frequente de gônadas inguinais ou labioescrotais. Indivíduos com CAIS apresentam desenvolvimento feminino típico na puberdade, com distribuição de gordura ginecóide e pêlos sexuais escassos ou ausentes, mamas têm características femininas normais, contornos corporais femininos e ausência de acne, devido à produção de estrógeno pelos testículos e pela aromatização periférica da testosterona em estradiol (Guigley *et al.*, 1995; Rajender *et al.*, 2007).

### 1.5.2 Insensibilidade Parcial aos Andrógenos

Ao contrário do fenótipo relativamente uniforme dos casos da CAIS, a PAIS (OMIM 312300) é caracterizada por indivíduos com diferentes fenótipos devido ao defeito parcial na função do receptor de andrógeno, que pode variar. Os pacientes com PAIS apresentam diferentes graus de masculinização da genitália externa, interna e do seio urogenital. Devido ao grande número de fenótipos que podem estar associados a PAIS, a prevalência desta síndrome ainda é desconhecida (Ragender *et al.*, 2007).

### 1.5.3 Insensibilidade Leve aos Andrógenos

Indivíduos que apresentam ginecomastia com fertilidade comprometida ou não são classificados na forma MAIS, como descritos anteriormente. A fertilidade em pacientes com AIS é extremamente rara, com poucos casos descritos na literatura (Larrea *et al.,*1978, Yong *et al.,* 1994, Giwercman *et al.,* 2000). Segundo Ferlin e colaboradores (2006), 2 a 3%

de infertilidade masculina são causados por mutações no gene *AR*, em pacientes com fenótipo MAIS.

Embora mutações no gene *AR* estejam diretamente associadas à insensibilidade androgênica, em alguns casos essa síndrome se manifesta mesmo com o gene *AR* em sua forma íntegra. Como a ação androgênica é um evento complexo, não pode ser descartada a hipótese de co-reguladores da transcrição não estarem atuando em sua forma normal, o que poderia explicar a manifestação desta síndrome nestes casos.

Adachi e colaboradores (2000) descreveram o caso de uma mulher de 19 anos de idade, com amenorréia primária e resistência completa a andrógenos. Este diagnóstico foi baseado nos dados clínicos, citogenéticos e hormonais. No estudo do gene *AR* desta paciente, no entanto, nenhuma alteração que comprovasse o fenótipo foi observada. Segundo os autores, um possível prejuízo na interação do receptor de andrógenos com co-reguladores estaria impedindo a ação androgênica nesta paciente.

Neste trabalho, descrevemos a atividade funcional de sete mutações, sendo uma relacionada ao fenótipo MAIS, três ao fenótipo PAIS e três ao fenótipo CAIS. A forma mais grave da PAIS está descrita no artigo 1, no artigo 2 serão apresentados os estudos funcionais de mutações associadas às formas PAIS e CAIS e no artigo 3, descrevemos o estudo da mutação p.Pro695Ser encontrada em um paciente com fenótipo masculino cuja única queixa era ginecomestia, com fertilidade preservada.

## 2. Objetivos

### 2.1 Objetivos gerais:

- Investigar quais os prejuízos na atividade de transativação do AR, causados por novas mutações.

- Analisar como acontece a interação entre os domínios amino-terminal e carboxi-terminal do receptor de andrógeno mutante.

- Associar os prejuízos funcionais dos AR mutantes com os fenótipos associados às diversas formas de AIS.

## 2.2 Objetivos Específicos:

### 2.2.1 Artigo 1:

- Analisar o gene *AR* em uma família com quadro sugestivo de insensibilidade androgênica, através do sequenciamento automático.
- Realizar o estudo estrutural *in silico* das trocas aminoacídicas com base na estrutura da proteína AR previamente publicada (PDB-ID: 2AM9);
- Relacionar o genótipo com o fenótipo dos pacientes.

### 2.2.2 Artigo 2:

- Investigar as propriedades funcionais de sete mutações individuais e uma combinação de duas mutações, avaliando o impacto na atividade de transativação e na interação entre os domínios N-terminal e C-terminal do receptor de andrógenos.

- Relacionar os graus de comprometimento da função do receptor com os fenótipos dos pacientes.

### 2.2.3 Artigo 3:

- Investigar a atividade transcricional da proteína AR em um paciente com ginecomastia e fertilidade preservada.
- Analisar se a mutação p.Pro695Ser é responsável por alguma alteração entre as interações N/C terminal neste paciente.

**3. Capítulo 1**

**Artigo 1:**

# Severe forms of partial androgen insensitivity syndrome due to p.L830F novel mutation in androgen receptor gene in a Brazilian family

Reginaldo J Petroli, Andréa T Maciel-Guerra, Fernanda C Soardi, Flávia L de Calais, Gil Guerra-Junior and Maricilda Palandi de Mello.

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RESEARCH ARTICLE Open Access

# Severe forms of partial androgen insensitivity syndrome due to p.L830F novel mutation in androgen receptor gene in a Brazilian family

Reginaldo J Petroli[1], Andréa T Maciel-Guerra[2,3], Fernanda C Soardi[1], Flávia L de Calais[1], Gil Guerra-Junior[3,4] and Maricilda Palandi de Mello[1,3*]

**Abstract**

**Background:** The androgen insensitivity syndrome may cause developmental failure of normal male external genitalia in individuals with 46,XY karyotype. It results from the diminished or absent biological action of androgens, which is mediated by the androgen receptor in both embryo and secondary sex development. Mutations in the androgen receptor gene, located on the X chromosome, are responsible for the disease. Almost 70% of 46,XY affected individuals inherited mutations from their carrier mothers.

**Findings:** Molecular abnormalities in the androgen receptor gene in individuals of a Brazilian family with clinical features of severe forms of partial androgen insensitivity syndrome were evaluated. Seven members (five 46,XY females and two healthy mothers) of the family were included in the investigation. The coding exons and exon-intron junctions of androgen receptor gene were sequenced. Five 46,XY members of the family have been found to be hemizygous for the c.3015C>T nucleotide change in exon 7 of the androgen receptor gene, whereas the two 46, XX mothers were heterozygote carriers. This nucleotide substitution leads to the p.L830F mutation in the androgen receptor.

**Conclusions:** The novel p.L830F mutation is responsible for grades 5 and 6 of partial androgen insensitivity syndrome in two generations of a Brazilian family.

## Findings

The androgen insensitivity syndrome (AIS, OMIN #300068) is a recessive disorder linked to the X chromosome. It may result in the failure of external genitalia masculinization in individuals with 46,XY karyotype and normal androgen production and metabolism [1,2]. There is a wide range of clinical manifestation, therefore the syndrome can be divided in three subgroups according the degree of undermasculinization: 1) mild AIS (MAIS) that is characterized by gynecomastia and infertility in phenotypically male individuals; 2) partial AIS (PAIS) that may present with predominantly male development or ambiguous genitalia (AG) or even with predominantly female external genitalia with clitoromegaly and/or posterior labial fusion and breast and pubic hair development; 3) complete AIS (CAIS) resulting in female external genitalia, sparse to absent pubic and axilary hair and normal breast development [2-6]. Due to significant differences generally found among clinical manifestation features in PAIS, some authors assigned grades ranging from 1 to 6 to describe patients that presented with different phenotypes varying from male genitalia and infertility to female genitalia with pubic and underarm hair [2,5,6].

The androgen activity is mediated by the androgen receptor (AR), a member of nuclear receptor family, which is encoded by the androgen receptor gene (*AR*). The gene is located on the X-chromosome at Xq11 - 12 and is formed by eight exons and seven introns that spans ~ 90 kb of DNA [7,8]. The AR protein contains approximately 919 amino acid residues, but this number is variable due to the existence of both polyglutamine and polyglycine stretches in the amino terminal region

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that may vary in length conferring normal variability among individuals [9,10]. Like other members of the nuclear receptor superfamily, the AR contains four different functional domains: an amino-terminal domain encoded by exon 1, which is a non-conserved region involved in transcriptional activation of target genes [11]; a central DNA-binding domain (DBD) encoded by exons 2 and 3, which contains two zinc finger motifs [12,13]; a hinge region containing the nuclear targeting signal [14] and a C-terminal ligand binding domain (LBD) encoded by exons 4-8 that also encompasses subdomains involved in dimerization and transcriptional activation processes [15,16].

Mutations in the *AR* gene lead to AIS [5,17,18]. Such mutations are found differently distributed throughout the gene sequence [[19], website: http://www.mcgill.ca/androgendb]. Almost 70% of 46,XY affected individuals inherited mutations from their carrier mothers [20]. As discussed by Boehmer *et al.* [21] the identification of a specific AR mutation and its residual androgen action always provide more precise diagnosis and/or prognosis, which might contribute to the decision for sex assignment of 46,XY individuals with AIS and facilitate genetic counseling of carrier females. In addition, due to clinical and genetic heterogeneity of the condition, studies describing novel mutations in AIS provide important information for the function of a specific amino acid residue.

Therefore, the purpose of this study was to identify the *AR* gene mutation in a Brazilian family with five patients presenting PAIS corresponding to grades 5 and 6. The role of the novel p.L830F missense mutation in the AR within LBD is discussed by comparing structural characteristics of both normal and mutant proteins.

## Ethics and Consents

This study was approved by the Ethics Committee from Universidade Estadual de Campinas (São Paulo, Brasil) and informed consents were obtained from patients and relatives; informed consents from individuals III-10 and IV-1 were obtained separately for the publication of Figures 1B and 1C.

## Methods

Seven individuals from two generations of a family (Figure 1A) were included in this study. The index case (Figure 1A, III-10), an 18-year-old girl, was referred to us due to primary amenorrhea with spontaneous telarche and pubarche and palpable gonads in the inguinal region. She was born at term after an uneventful pregnancy by cesarian section with a birth weight of 3,330 g and height of 48 cm. Bilateral inguinal gonads were detected at birth. She was followed up over a period of eight months when assignment of

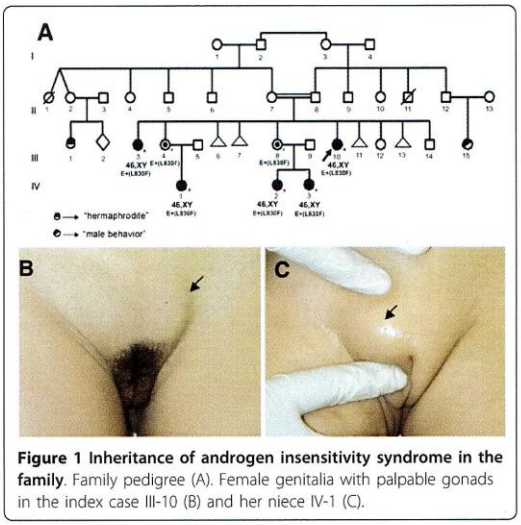


**Figure 1 Inheritance of androgen insensitivity syndrome in the family**. Family pedigree (A). Female genitalia with palpable gonads in the index case III-10 (B) and her niece IV-1 (C).

female gender was decided; corrective procedure for inguinal gonads was planned but did not occur. On physical examination, she had typical female external genitalia, with only a slight posterior fusion of labioscrotal folds. Palpable gonads were found bilaterally in the inguinal region with volumes of 15 $cm^3$, whereas pubertal development had reached Tanner stage B4P5 (Figure 1B). The uterus was absent under pelvic sonograms. Hormonal evaluation revealed normal FSH (5.4 mIU/mL; normal range (NR): 1.5 - 12.4 mIU/mL) and elevated levels of both LH (21.2 mIU/mL; NR: 1.7 - 8.6 mIU/mL) and total testosterone (>15 ng/mL; NR: 2.86 - 8.1 ng/mL); her karyotype was 46,XY. The bone mineral density test revealed femoral osteopenia and lumbar osteoporosis. Gonadectomy was performed a few months later and histological analysis revealed bilateral testes with no evidence of malignancy. She was subsequently referred to other services to perform vaginoplasty and to start hormone replacement therapy with estrogens and treatment of osteopenia/osteoporosis. Her parents were first cousins, and there was a positive family history with individuals presenting similar features: three nieces with palpable gonads and an older sister.

A 24-year-old sister (Figure 1A, III-3) that had inguinal gonads corrected in the first year of life, referred with primary amenorrhea, spontaneous breast development. On physical examination, pubertal development had reached Tanner stage B5P4. She was oriented to perform bilateral gonadectomy, vaginoplasty and hormone replacement.

A 3-year old niece (Figure 1, IV-1) was the only child of unrelated parents. She was born at term by normal

delivery after an uneventful pregnancy with birth weight of 2,800 g, and bilateral inguinal gonads were detected at birth. On physical examination, she had typical prepubertal female genitalia and bilaterally palpable gonads were found in the inguinal region (Figure 1C). Her karyotype was 46,XY. After receiving all relevant information about risks and benefits of early versus late gonadectomy, her parents decided to delay surgery in order to allow spontaneous puberty. The girl was then referred to the pediatric endocrinology service for follow up.

A 1.5-year-old niece (Figure 1A, IV-2) was also the first child of unrelated parents. She was born at term by normal delivery after an uneventful pregnancy with birth weight of 2,650 g, and palpable gonads in the inguinal region were detected at birth. On physical examination, she had typical female external genitalia, with only a slight posterior fusion of labioscrotal folds. Her karyotype was 46,XY. Her parents decided to delay surgery until after puberty and the girl was referred to the pediatric endocrinology service for follow up. Her sister (Figure 1A, IV-3) was brought to us when she was 3 month old due to palpable gonads in the inguinal region, which had been detected at birth. She was born at term after an uneventful pregnancy by normal delivery with a birth weight of 3,180 g and length 49.5 cm. On physical examination, she had typical female external genitalia and both gonads were palpable in the inguinal regions. Her karyotype was 46,XY.

Samples of genomic DNA were obtained from peripheral blood by proteinase K/phenol extraction method [22]. Molecular analysis was performed by amplifying the eight exons of *AR* gene using the polymerase chain reaction (PCR) followed by sequencing the fragments using Big Dye® Terminator Cycle Sequencing Kit V3.1 Ready Reaction (ABI PRISM/PE Biosystems). The sequences obtained in an ABI 3700 Sequencer (ABI PRISM/PE Biosystems) were compared with the normal sequence of the gene (ENSEMBL-ENSG00000169083) using Chromas (reduced version - free software) and GeneRunner v.3.05 (free software) or CLC Sequence Viewer v.6.2 (free software).

The model of human AR mutant protein was built using the resolved 3-D structure of human AR (PDB accession # 2AM9) as template. Molecular modeling was performed using MODELLER web-server program. The model images were examined and edited using PyMOL® program and Millennium STING (CNPTIA-Embrapa, Brasil). The human AR sequence was compared with the corresponding mammalian proteins sequences in the ClustalW http://www.genome.jp/tools/clustalw/.

## Results

Upon sequencing exons 2 to 8 of the *AR* gene, a novel c.3015C>T nucleotide change in exon 7 was identified in five 46,XY female hemizygote individuals and also in two heterozygote carrier mothers (Figure 2A). This nucleotide change cause the putative replacement of a leucine by a phenylalanine residue at codon 830 (p.L830F).

Exon 1 sequencing showed 21 and 20 repeats of each CAG (SNP # rs5902610) and GGC, respectively, for the affected individuals. In addition, three GGT codons preceded the GGC stretch instead the two normally found in the *AR* gene. Besides the mutation, the heterozygote carrier mothers (Figure 1A: III-4, III-8) were also heterozygous for the c.639G>A (SNP # rs6152) and c.2319-

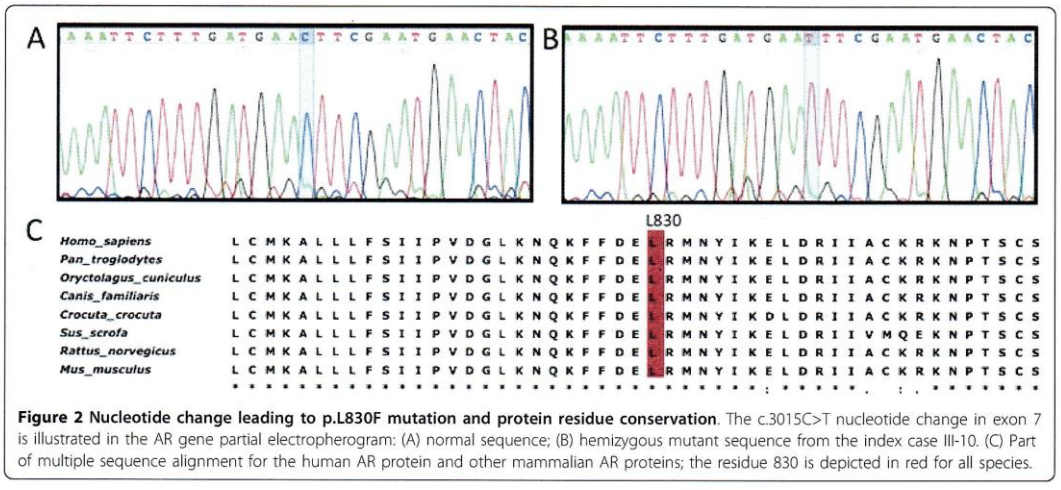


**Figure 2 Nucleotide change leading to p.L830F mutation and protein residue conservation**. The c.3015C>T nucleotide change in exon 7 is illustrated in the AR gene partial electropherogram: (A) normal sequence; (B) hemizygous mutant sequence from the index case III-10. (C) Part of multiple sequence alignment for the human AR protein and other mammalian AR proteins; the residue 830 is depicted in red for all species.

78T>G (SNP # rs1337076) polymorphisms in exon 1 and intron 5, respectively.

Multiple alignments comparing the human AR protein sequence to other mammalian AR proteins indicated the L830 as a highly conserved residue (Figure 2B). The structural analysis by modeling normal and mutant proteins (Figure 3A) revealed that the discrepancy of the mutant F830 compared to the normal L830 resides mainly in the abolishment of a hydrophobic interaction with F813 residue and in the creation of two different internal hydrophobic interactions with both G724 and N727 residues (Figure 3B and 3C). Since the interaction between L830 and F813 residues was suppressed by the mutation, several interactions involving F813 and other amino acids have also been disrupted (Figure 3D). It was also observed that the distance between F830 and either G724 or N727 residues has shortened to 2.94 Å (Figure 3B).

## Discussion

We report here the novel p.L830F mutation in the hormone binding region of the androgen receptor that is responsible for partial androgen insensitivity syndrome in a Brazilian family. Five patients in two generations carry the mutation. The index case (Figure 1A, III-10) and her niece (Figure 1A, IV-2) showed clinical and laboratorial data compatible with PAIS grade 5, since they had female external genitalia with a slight posterior fusion of labioscrotal folds and palpable gonads [2,6]. Whereas, her older sister (Figure 1, III-3) and two nieces (Figure 1, IV-1. and IV-3) presented with typical female external genitalia and palpable gonads [2,6], which classify them as PAIS grade 6. The presence of pubic hair was observed in the two sisters; however, it could not be verified in the nieces because they have not reached puberty yet.

In addition to the mutation, all patients presented 21 and 20 repeats for polyglutamine and polyglycine stretches, respectively, both within the range described as normal [23]. The 46,XX heterozygote mothers (Figure 1: III-4, III-8) were also heterozygous for both c.639G>A (SNP # rs6152) and c.2319-78G>T (SNP # rs1337076) polymorphisms. Since A and T nucleotides are, respectively, rare and very rare alleles (NCBI SNP database) in

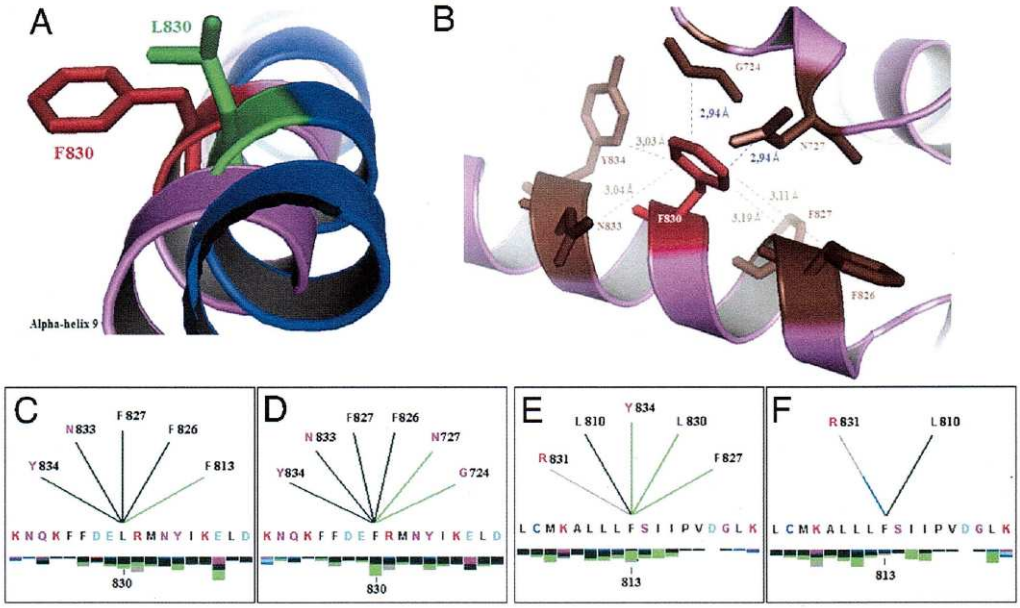


**Figure 3 Modeling for normal and mutant AR proteins**. (A) Comparison of normal and mutant human AR protein models at residue 830; normal leucine is denoted in green and phenylalanine in red. (B) Distances in Ångström (Å) for contacts for the F830 mutant residue estimated using PyMOL software: hydrogen bonds are shown in brown and hydrophobic interactions in purple. (C - F) Internal contacts provided by the analysis with BlueStar STING software. The native residue L830 (C) forms energetic hydrogen bonds with F826, F827, N833 and Y834 and a hydrophobic interaction with F813, whereas the mutant residue F830 (D) suppresses the interaction with F813 and introduces two additional hydrophobic interactions with G724 and N727. Effects upon internal contacts for F813 residue: in the normal protein, the F813 residue presents hydrophobic interaction with F827 and Y834 (E), whereas in the mutant protein those interactions are lost (F).

Caucasian populations it can be conclude that the paternal inherited allele also corresponds to a rare *AR* allele.

Missense mutations in AR protein may cause a spectrum of phenotypes that include complete androgen insensitivity in 46,XY individuals with female genitalia and partial androgen insensitivity in 46,XY individuals with male phenotype, except for perineoscrotal hypospadias, gynecomastia and/or infertility [19]. The phenotype variability appears to reflect the degree to which ligand-binding and receptor functions are disrupted by different substitutions [24]. In addition, genetic background also influences the resulting phenotype since a same mutation may cause different forms of AIS within a family [3,25]. The most frequent are missense mutations that are found within two important areas of the receptor protein: DBD and LDB domains [8]. The importance of the L830 residue for the AR activity may be inferred by p.L830V described before in a patient with CAIS [26]. In addition, the neighboring codon 831 was target for several missense mutations such as p.R831L and p.R831Q also causing CAIS [27,28]. Therefore, mutations in codon 830 may severely disturb the functional activity of LBD in which two hot spots for mutations have been identified [24].

X-ray crystallographic studies showed that the three-dimensional structure of the AR-LBD encompasses 12 α-helices [29]. In the normal molecule these α-helices undergo conformational changes in response to ligand-binding resulting in the assembly of the AF-2 domain. According to AR structure, the amino acid L830 is located within the α-helix 9 that comprises residues 825-847. This region has been proposed as part of an allosteric regulatory site termed binding function 3 (BF-3) where ligand interactions exert indirect effects on AF-2 to modulate co-regulator binding [30]. Mutations in BF-3 have demonstrated to diminish AR activity and they can be related with different phenotypes [30].

When the leucine is replaced by the phenylalanine in codon 830 the hydrophobic character of the residue is maintained, but the change of a high hydrophobic leucine to a less hydrophobic phenylalanine might affect the transcriptional activity as described for p.F826L mutation [31]. Considering the structural analysis for p.L830F, it can be proposed that the suppression of a hydrophobic interaction with residue F813 might destabilize the interaction between alpha-helices 8 and 9 which forms an important hydrophobic core to keep the AR binding capacity [32]. Conversely, the mutant residue established novel contacts with amino acids G724 and N727 that are located between alpha-helices 3 and 4, both residues are highly conserved and critical for ligand binding [17,31,33]. The creation of such hydrophobic contacts suggests that the p.L830F substitution would reduce the mobility of the region involving alpha-helix 9 and the region between alpha-helices 3 and 4. Also, those new interactions brought F830 and each G724 and N727 amino acids more close such as surface contacts of F830-G724 and F830-N727 enhanced leading to stronger interactions between the loop region located in the middle of alpha-helices 3 and 4 and alpha-helix 9. Those interactions give the region less flexibility than that necessary to allow properly co-factor assembly of AF-2 domain [34]. In conclusion, the decrease on the hydrophobicity of residue 830 and changes in the internal contacts caused by p.L830F mutation are probably responsible for a very low androgen receptor activity which might correlate with the severe PAIS phenotype observed for the patients. Similarly to other mutations described in this domain as causing different phenotypes within a family [3,25], p. L830F produced different PAIS grades in the family described here indicating an influence of genetic background on its effect.

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**Authors' contributions**

RJP and FLC carried out sequencing experiments and sequence alignment analysis; FCS contributed with the protein structural analysis; ATMG and GGJ were responsible for diagnosis and management of patients and participated in the design of the study; MPM conceived the study, and participated in its design and coordination and also drafted the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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**4. Capítulo 2**

**Artigo 2:**

# Functional analysis of six androgen receptor mutations identified in patients with complete and partial androgen insensitivity syndrome

Reginaldo J Petroli, Olaf Hiort, Dagmar Struve, Fernanda Caroline Soardi, Karla Melo, Ivo Jorge Prado Arnhold, Andréa Trevas Maciel-Guerra, Gil Guerra-Junior, Ralf Werner and Maricilda Palandi de Mello.

**Functional analysis of six androgen receptor mutations identified in patients with complete and partial androgen insensitivity syndrome**

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***Running title:*** Functional studies on androgen receptor mutations

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**Abstract**

The androgens are responsible for development and maintenance of male sex secondary characteristics. Dysfunctions in androgen action due mutations in androgen receptor (AR) gene can lead to androgen insensitivity syndrome (AIS). Depending on the impairment owing AR gene mutations, AIS manifestation can be mild, partial or complete. Here, we have analyzed the effects of p.Ser759Thr, p.Leu830Phe and p.Ile898Phe, p.Leu768Val, p.Cys806Phe+p.Gln798Glu, and p.Pro904Arg missense mutations that were clinically associated to partial AIS (PAIS) or to complete AIS (CAIS). All mutations have been identified by *AR* gene sequencing. To evaluate the transactivation ability of each AR mutant, the site-directed mutagenesis assay was performed on full-length AR cDNA, followed by transfection and expression in mammalian cells. The analysis of reporter gene transactivation with different dihydrotestosterone (DHT) concentrations was performed. N/C-terminal interactions for each AR mutant were analyzed using two-hybrid mammalian assay. PAIS mutations: p.Ser759Thr, p.Leu830Phe, p.Ile898Phe mutations showed transactivation activity higher than 20% in physiological hormone concentration. In the N/C interaction assays, the p.Ile898Phe revealed a complete disruption of N/C interaction at all hormone concentrations; the p.Ser759Thr and p.Leu830Phe showed positive response with increasing DHT. CAIS mutations: p.Leu768Val revealed a complete disruption of AR action; p.Gln798Glu and p.Cys806Phe mutations studied separately revealed responses to DHT induction similar to Mild and PAIS, respectively. However, when analyzed together, transactivation activities lower than 10% in physiologic hormone conditions were observed, consistent to CAIS phenotype. The p.Pro904Arg showed reduced transcriptional activity and abolished response in N/C terminal interaction, compatible with CAIS. We conclude that the functional analysis of the AR could elucidate the molecular mechanisms associated with each case of AIS, and may provide a basis for response to treatment in each particular case.

## Introduction

The androgen insensitivity syndrome (AIS) is clinically characterized by impaired virilization in 46,XY individuals. The phenotype associated with AIS can range from mild AIS (MAIS) to complete AIS (CAIS). MAIS is characterized by gynecomastia and in- or subfertility in phenotypically male individuals. Partial AIS (PAIS) may present with predominantly male development or ambiguous genitalia (AG) or even with predominantly female external genitalia with clitoromegaly and/or posterior labial fusion and development of breast and pubic hair. On the other hand, the most severe form known as complete AIS (CAIS) results in female external genitalia, sparse to absent pubic and axillary hair and normal female breast development (3, 14, 19, 23, 24, 28).

AIS can be caused by a de novo mutation or an inherited maternal mutation in the androgen receptor (AR) gene. The *AR* gene is localized on the X-chromosome at Xq11-12 region and mutations can be transmitted with an X-chromosomal recessive inheritance. The *AR* gene has eight exons and seven introns distributed in more than 186 kb. Within exon 1, encoding for the N-terminal region involved in transactivation of the receptor, there are two polymorphic regions. The first is a poly-Gln region which can range from 9 to 36 residues and the second is a poly-Gly region which ranges from 10 to 27 residues, in different populations (3, 15, 27, 28). Variations on the number of repeats can be associated with different conditions, for instance, a very long Gln-stretch causes spino-bulbar muscular atrophy (40).

The AR protein contains approximately 920 amino acid residues with molecular weight of about 110 kDa (1, 13, 28). The AR is a nuclear receptor (NR) that belongs to the superfamily of ligand-activated transcription factors. Important functions can be attributed to AR in male development such as muscular and male genitalia development, voice deepening and spermatogenesis. Therefore, impaired or increased AR activity can be associated to AIS or prostate cancer, respectively (1, 2, 27).

Similarly to other members of the NR superfamily, the AR exhibits three basic functional domains, which are a variable amino-terminal domain (NTD), a conserved DNA

binding domain (DBD) and a conserved ligand binding domain (LBD). Between DBD and LBD there is a flexible linker, the hinge region (1, 3, 16, 25).

The NTD (residues 1-538) is involved in the transactivation through interaction with transcriptional machinery (1, 25, 30). This domain also harbors a ligand independent activation function 1 (AF-1) which is the major activation domain for the AR. In the same region, there are FXXLF and WXXLF residues (where F is phenylalanine, L is leucine, W is tryptophan and x is any amino acid), which are important for N/C-terminal interaction and hormone-receptor complex stabilization (1, 5, 7, 27).

The DBD (residues 539-628) contains of two zinc fingers which are able to recognize and bind to specific DNA sequence (5, 11, 16, 25). The hinge region (residues 629-669) allows the nuclear translocation, receptor mobility, DNA binding and transactivation (3, 5, 31). The LDB (residues 670-920) contains 11 helices responsible for the organization of the ligand-binding pocket formed by helices 3, 4, 5, 7, 11 and 12 with also beta-sheet preceding helix 6 (16). The LBD harbors an important and conserved region, the ligand-depending activation function 2 (AF-2). The AF-2 is responsible for ligand-dependent transcriptional activation and is formed by helice 3, 4, 5 and 12. Mutation or deletion of the AF-2 domain dramatically reduces transcriptional activation in response to a ligand. In contrast to other nuclear receptors, the AR preferentially interacts with the FQNLF in the NTD causing the N/C interaction, an important step toward the activation of AR. Damage in N/C interaction is associated with AIS, even when the hormone binding is normal (5, 16, 27, 32, 34).

In the cytoplasm, the inactive AR form is complexed with heat shock proteins before binding to the hormone testosterone (T) or 5α-dihydrotestosterone (DHT). The heat shock proteins are dissociated from the AR after the hormone binding to LBD. The translocation to the nucleus leads to conformational changes, resulting in an AF-2 hydrophobic pocket formation. This hydrophobic pocket serves as a binding site for coactivators and for the AR NTD interactions. The coactivators interaction increases AR transcriptional activation (10, 32, 33).

Mutations in the *AR* gene usually result in a decrease of ligand affinity, co-activator interaction and also impaired DNA binding (33). However, in a substantial number of AIS cases, there is no clear correlation between AIS clinical phenotype and *AR* gene mutation

(4, 12, 21). Nevertheless, there are more than 800 different AR mutations (http://androgendb.mcgill.ca) which have been described in more than 850 patients with different AIS phenotypes (8). They are distributed in all AR domains, especially in LBD. There is a predominance of missense mutations in the LBD correlated with a significantly higher number of CAIS than PAIS cases (8, 9).

In order to clarify the functional consequences of missense mutations in AR gene, we investigated seven mutations, p.Gln798Glu+p.Cys806Phe, p.Leu768Val and p.Pro904Arg associated with CAIS, p.Ser759Thr, p.Leu830Phe and p.Ile898Phe associated with PAIS all located in the LBD. The combination of the new mutation p.Cys806Phe and the well-known p.Gln798Glu was particularly found in one patient with CAIS. Five of these variants have been previously described in Brazilian patients (22, 26, 29). In order to understand the functional properties of these mutations, the impact of each one on full length AR transactivation capacity and on AR N/C-terminal interactions were investigated.

## Material and Methods

### Patient descriptions

#### Patient with p.Gln798Glu+p.Cys806Phe mutations:

This girl was referred to us with 13 years of age. She was born at term by normal delivery after an uneventful pregnancy with a birth weight of 3,950 g. Inguinal hernias were detected in the neonatal period and were surgically repaired when she was 2 months old. Bilateral testes were found at inguinal herniorrhaphy, and both were removed when the child was 16 months old. Estrogens had been prescribed five months before her first visit to our service.

Her parents were unrelated, and family history was unremarkable. She had two normal sisters aged 19 and 17 years and a younger 2.5-year-old brother. On physical examination, her height was 166.2 cm and weight 80.3 kg. She had typical female external genitalia and pubertal stage was B2P2. Hormonal evaluation revealed high levels of FSH (92.6 UI/L; normal range (NR): 1.5-12.4 UI/L) and LH (20.6 UI/mL; NR: 1.7-8.6 UI/mL) and prepubertal levels for total testosterone (1.41 nmol/L; NR: 9.92-28.11 nmol/L); her karyotype was 46,XY.

Clinical data for patients with p.Ser759Thr, p.Leu768Val., p.Leu830Phe, p.Ile898Arg and p.Pro904Arg mutations have been previously described (22, 26, 29).

## Plasmids

### AR expression plasmids

All mutations were introduced into the full-length AR expression vector (pSVAR0), which was kindly provided by Dr. A. Brinkmann, from the University Hospital Rotterdam, Rotterdam, NL University. Each point mutation was inserted by PCR based site directed mutagenesis with specific *primers* (Table 1).

Table 1: Mutagenesis primers used in this study

| ***Primer*** | **Sequence 5'-3'** |
|---|---|
| AR_p.Ser759Thr_sense: | CTTCACCAATGTCAACACCAGGATGCTCTACTT |
| AR_p.Ser759Thr_antissense: | AAGTAGAGCATCCTGGTGTTGACATTGGTGAAG |
| AR_p.Le768Val_sense: | CTACTTCGCCCCTGATGTGGTTTTCAATGAGTA |
| AR_p.Leu768Val_antissense: | TACTCATTGAAAACCACATCAGGGGCGAAGTAG |
| AR_p.Gln798Glu_sense: | GAGTTTGGATGGCTCGAAATCACCCCCCAGG |
| AR_p.Gln798Glu_antissense: | CCTGGGGGGTGATTTCGAGCCATCCAAACTC |
| AR_p.Cys806Phe_sense: | CCCAGGAATTCCTGTTCATGAAAGCACTGCT |
| AR_p.Cys806Phe_antissense: | TAGCAGTGCTTTCATGAACAGGAATTCCAGT |
| AR_p.Leu830Phe_sense: | TTCTTTGATGAATTTCGAATGAACTAC |
| AR_p.Leu830Phe_antissense: | GTAGTTCATTCGAAATTCATCAAAGAA |
| AR_p.Ile898Phe_sense: | GAAATGATGGCAGAGTTCATCTCTGTGCAAGT |
| AR_p.Ile898Phe_antissense: | ACTTGCACAGAGATGAACTCTGCCATCATTTC |
| AR_p.Pro904Arg_sense: | ATCTCTGTGCAAGTGCGCAAGATCCTTTCTG |
| AR_p.Pro904Arg_antissense: | CAGAAAGGATCTTGCGCACTTGCACAGAGAT |

Mutagenesis primers were designed using GeneRunner v.3.05 (free software).

In brief, PCR mutagenesis was performed in two steps. Using pSVAR0 as template, in the first step AR_LBD644s (5´-GAGGCTTCCAGCACCACCA-3´) was used as sense primer together with each respective mutagenesis antisense primer. The second amplification was performed with the respective mutagenesis sense primer and pSV0seq_as (5'- TGATAGGCAGCCTGCACCTGA -3'). Both amplicons were gel purified, combined, annealed and amplified in a third PCR using the primer pair AR_LBD644s - pSV0seq_as. Mutant amplicons were double digested with *Tth*111 I/*Pst* I and subcloned into pSVAR. All inserts and their cloning borders were verified by sequencing.

**Two hybrid constructs**

AR N/C interaction was analyzed using the mammalian two-hybrid assay kit (Stratagene, La Jolla, CA). In order to generate mammalian two-hybrid clones containing the GAL4 DNA binding domain fused to $AR_{618-920}$, the AR $LBD_{618-920}$ was amplified using the primer pair AR1823s_*Bam*H I (5'- TGGATCCCGGAAATGTTATGAAGCAG -3') and pSV0seq_as using wt or mutant full length *AR* as template. Amplicons were double digested by *Bam*H I and *Pst* I and subcloned into pCMV_BD bait vector (Stratagene). The vector pAD_$NTD_{1-536}$ containing the GAL4 activation domain in fusion to the N-terminal domain of AR (aa 1-536) was previously described in (32).

**Luciferase genes**

The p$(ARE)_2$TATA-Luc vector which contains two androgen responsive elements 5' of the TATA-box preceding the firefly luciferase gene was a kind gift of Dr. G. Jenster from Josephine Nefkens Institute, Erasmus MC (Rotterdam, NL). The pFR-Luc vector (Stratagene, La Jolla, CA) contains a synthetic promoter with five tandem repeats of yeast GAL4 binding sites which control the expression of the firefly luciferase gene and served as a reporter gene in mammalian two hybrid assays. Plasmid phRGTK contains a

constitutive active *Renilla luciferase* reporter gene used to normalize transfection efficiency in Dual Luciferase Assays (Promega).

**Transient Transfections of Full length AR**

The Chinese hamster ovary (CHO) cells were used to express the wild type and mutant AR proteins. Transfection experiments were performed as previously described (31). In brief, 80,000 cells/well were cultured overnight in 24-well plates with Dulbecco's modified Eagles Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma) supplemented with 10% dextran charcoal-stripped fetal calf serum under 5% $CO_2$ at 37°C. After initial incubation, 30 ng wt-AR or mutant plasmid DNAs were co-transfected with 200 ng p$(ARE)_2$TATA-Luc and 5 ng phRGTK using 0.5 µl FuGENE HD (Promega Corp., Madison, WI). Five hours after transfection, 0–100 nmol/l DHT were added in each well. The luciferase activity was determined 18 h after hormone induction, using a Dual Luciferase assay (Promega Corp., Madison, WI) and normalized to transfection efficiency. For comparison of experiments performed at different days, we set the transactivity of wt-AR at 10 nmol/l DHT as 100%. Relative luciferase units (RLU) of other samples assayed at the same time were expressed relative to this value. For statistical analysis, all transfections were performed in triplicate and at least three independent experiments were carried out.

**Two hybrid assays**

N/C interaction assays have been described in detail previously (32). In brief, CHO cells were cultured and seeded in 24-well plates as described above. 100 ng pBD-$LBD_{618-920}$ (bait, wild type or mutant) and 100 ng of pAD-$NTD_{1-536}$ (prey) were co-transfected with 200 ng pFR-Luc (reporter gene) and 10 ng phRGTK per well using 1.0 µl FuGENE HD (Promega). Five hours post-transfection, LBDs were activated by adding the indicated amount of DHT (0–100 nM). The luciferase activities were measured 18 h after hormonal induction using the Dual Luciferase assay system (Promega). As for full-length assays, the luciferase activity of wild type NTD/LBD interaction at 10 nmol/l DHT was set to 100%. Transfection efficiency was normalized by *Renilla* luciferase. For statistical analysis, all

transfections were carried out in triplicate and at least three independent experiments were performed.

**Western Blots Analysis**

For protein expression analysis 300,000 CHO cells were grown in six well culture plates overnight. Cells were transfected using 1 μg $p(ARE)_2$ TATA-Luc plasmid, 150 ng wild type or mutant AR plasmid, 25 ng phRGTK and 3.35 μl Fugene HD reagent per well. After 16 h, cells were washed twice with cold PBS and lysed in 250 μl M-PER lysis buffer (Pierce, Rockford, IL) including complete proteinase inhibitor cocktail (Roche, Mannheim, Germany) on ice for 5 min. Cells were scraped and collected for Western blot analysis. Protein concentration was estimated by Bio-Rad protein assay (Bio-Rad, Richmont, CA). The remainder of protein was dissolved in SDS sample buffer and denatured by heating at 95°C for 10 minutes followed by SDS-PAGE.

Full-length AR and NTD were detected with primary antibody anti AR F39.4.1 (Innogenex, San Ramon, CA) diluted 1:500. GAL4-LBD fusion proteins were detected with primary antibody AR-C19 (sc-815, Santa Cruz Biotechnology Inc.) diluted 1:200. Actin was detected using anti-actin antibody A2066 (Sigma) diluted 1:1000. Signals were visualized using the corresponding conjugated anti-mouse or anti-rabbit secondary antibody applying the Western Lightning Chemiluminescent Reagent Plus (PerkinElmer, Boston, MA) and a Fusion SL chemiluminescent imager (Vilbert Lourmat).

**Results:**

In order to analyze the effect of AR mutant proteins on AR transactivation capacity associated with CAIS and PAIS phenotype, full-length AR plasmids were co-transfected with the firefly luciferase reporter plasmid $p(ARE)_2$TATA-Luc in CHO cells. Expression of comparable amounts of wild-type (wt) and mutants either for full-length AR proteins (Fig. 1A) or Gal4-LBD fusion protein constructions (Fig. 1B-D) were confirmed by Western blotting.

*Full Length AR Transactivation and N/C interactions in PAIS related mutations*

The mutations associated to PAIS phenotype that have been tested were p.Ser759Thr, p.Leu830Phe and p.Ile898Phe. In transactivation assays, all three mutations revealed low response activities at low hormone concentrations reaching approximately 27% (t-test, *P*<0,001), 30% (t-test, *P*=0,002) and 22% (t-test, *P*<0,001) of the wt at 1.0 nM DHT, respectively. At high and supraphysiological hormone concentration the responses reached 80% (t-test, P<0,0001) and 62% (t-test P<0,0001) for p.Ser759Tre and p.Ile898Phe, respectively, at 100 nM DHT; whereas it was totally recovered for p.Leu830Phe (t-test P<0,7263) (Figure 2A).

In N/C interaction analyses, the p.Ser759Thr mutation showed significantly reduced responses at all hormone concentrations. Transactivation of the reporter gene was almost undetectable at low DHT concentrations (0.1-1 nM) showing gradually higher responses with the increase in DHT concentration. It reached only 50% (t-test, P<0,0001) at a supraphysiological DHT concentration(100 nM) (Fig 2B).Similarly, the response of the reporter gene for p.Leu830Phe at low hormone concentrations (0.1-1.0 nM) was undetectable. However, it was totally recovered at 3.3 nM DHT and it showed a transcriptional activity 2.5 times higher than the wt in the presence of 100 nM DHT (Figure 2B).

Conversely, p.Ile898Phe did not show any detectable activity of reporter gene in the N/C interaction assay, indicating that in this case N/C interation was completely abolished (Figure 2B).

*Full Length AR Transactivation and N/C interactions in CAIS related mutations*

The mutations associated to CAIS phenotype that have been tested were p.Leu768Val and p.Pro904Arg.

In the experiment using full-lengh *AR* the reporter gene activation by p.Leu768Val mutant was undetectable from 0.1 to 3.3 nM DHT and reached only 36% (t-test, P<0,0001) at 100 nM (Figure 3A). Conversely, p.Pro904Arg showed a better response to DHT stimulus presenting 20% (t-test, P<0,0001) of the reporter gene activation at 1.0 nM (Fig.

3A). At high and supraphysiological hormone concentrations the response was approximately that of the wt. However, a drastic effect upon the reporter gene activation is observed for both mutations in N/C interaction assays. Whereas p.Leu768Val did not show any response even in supraphysiological hormone concentration, p.Pro904Arg showed approximately 30% (t-test, P=6934) of the reporter gene activation only at 100 nM DHT. Those results indicate that N/C interaction was severely disrupted by both mutations, (Figure 3B).

*Full Length AR Transactivation and N/C interactions for p.Gln798Glu+p.Cys806Phe*

The novel p.Cys806Phe AR mutation was identified in association with p.Gln798Glu, a well known AR mutation. Therefore, full-length and N/C interaction experiments were performed for individual and combined mutations . Individually, p.Gln798Glu retained 80-90% (t test, P=0,0356) the capacity of transactivating reporter gene expression in both full-legth and N/C interaction experiments (Figure 4A, B); whereas, p.Cys806Phe showed less than 30% activity (t-test, P<0,0001) at 1.0 nM DHT and recovered to almost 95% (t-test, P<0,0001) at 100 nM in the full-length experiment (Figura 4C). Similarly to the effect observed for p.Leu830Phe in the N/C interaction assay, p.Cys806Phe also showed increasing activity of the reporter gene as hormone concentration increased being almost 3 times higher than the wt at 100 nM (Figura 4D) with a drastic effect upon the reporter expression at 1.0 nM. When those two mutations were combined in one construct the capacity of activating the reporter gene expression at 1 nM DHT remained below 10% in both assays (Figure 4E, F). For higher DHT concentrations, the combination p.Gln798Glu+p.Cys806Phe showed increased response although remained below 80% (t-test, P>0,0031) at 100 nM DHT in the full-lengh assay. Those results indicate a synergistic effect since a less severe effect was individually observed for those mutations either in low or in high DHT concentrations. However, the results under high DHT conditions (10-100 nM) in the N/C interaction assays followed that of p.Cys806Phe reaching 2.5 times the wt transactivation capacity (Figure 4F).

## Discussion

The androgen insensitivity syndrome is described as a dysfunction in the AR, which can be associated with a mutation in *AR* gene or due to unknown mechanisms (12, 14, 15, 21). Thus, patients with AIS are initially screened for AR mutations by AR gene sequencing. In this study we describe functional activities of six missense mutations and one combination of two missense mutations, all of them located in the LBD region, three point mutations were associated with PAIS, other mutations were associated with CAIS phenotype.

PAIS mutations p.Ser759Thr, p.Leu830Phe and p.Ile898Phe showed increasing transactivation capacities on the reporter gene with increasing hormone concentration. At physiological hormone concentration (1.0 nM DHT), the response was lower than 40%, which indicated PAIS classification. However, they differed in the N/C interaction assays. AR p.Ser759Thr was previously described in a patient with PAIS by Takahashi *et al.* (47). In our study, the patient with p.Ser759Thr presented micropenis and hormone dosages suggesting PAIS phenotype, as described by Tobo *et al.* (29). Transactivation assay with full-length AR cDNA with p.Ser759Thr showed a reduced activity at low hormone concentrations (0.1-1 nM) that reached 80% at high and supraphysiological levels of DHT. The residue Ser759 is in the loop between helix 5 and helix 6 of AR protein. As this region is part of the ligand-binding pocket, alterations might be associated with an impairment of N/C interaction (8, 32, 33, 36, 39). This is supported by N/C interaction data presented here where loss of transactivation ability at low to physiological hormone concentrations (0.1-1 nM) was demonstrated, whereas a moderate rescue at high to supraphysiological levels (3.3-100 nM) reaching only 50% of the wt activity. Differently to p.Ser759Tre, the p.Ile898Phe revealed a complete disruption in N/C interaction at all hormone concentrations indicating a CAIS phenotype. However, this mutation was described in a family with severe PAIS (20) suggesting that other interactions could be involved in AR activity in this case. The residue Ile898 is located at the end of helix 11, near helix 12. This region is conserved in steroid receptors due to the important role in the protein-protein interactions performed with AF-2 (21, 25, 32, 37).

A quite different profile was observed for p.Leu830Phe and p.Cys806Phe mutants. Despite low activity in the N/C interaction experiment at low to normal hormone values (0.1-1 nM), they equal the wt with high hormone values (around 3.3 nM) and surprisingly at high to supra physiological DHT concentrations transactivation activities were almost two times that of the wild type. The mechanism of this unusual hormone response is not known yet. Similar result was obtained with the CAIS related double mutant p.Gln798Glu+p.Cys806Phe. Likewise, our group had observed similar patterns in N/C interaction experiments with p.Arg840His mutation (41) as well as with p.Arg840Cys (unpublished results). Szafran *et al*. (42) using HeLa cells also report absence of N/C interaction at low (0.2 nM) testosterone concentrations and increased N/C interaction at 20-200 nM T for p.Arg840Cys AR mutant. This effect was first reported by Wong *et al.* (33) who described an increased N/C interaction and an increased TIF2 coactivation at high hormone concentrations for PAIS mutation p.Phe826Leu. Independent assays using two different promoters and three different ligands were performed to test p.Phe826Leu and similar results were obtained (33). Those results differed from ours in that p.Cys806Phe, p.Leu830Phe and p.Arg840His/Cys mutations led to a loss of N/C interaction at low hormone concentrations and switch to an increased N/C interaction at high hormone concentrations, whereas p.Phe826Leu always led to an increased N/C interaction, at both low and high hormone concentrations. Nevertheless, these results indicated that some residues could impact AF2 function despite the location outside AF2 or ligand binding pocket. Residues Arg826, Leu830 and Arg840 are located on helix 9 and are part of binding function 3 (BF-3), a concave surface topographically adjacent but distinct from AF2, that allosterically regulates coactivator binding (43). Residue Cys806 is located on helix 8, just below Arg840 on helix 9 (Figure 5). Substitution of the cysteine by a more bulky phenylalanine at residue 806 may disturb the BF-3 conformation; consequently, it may destabilize coactivator binding. Crystal structures of AR-LBDs bound to co-activator peptides differ from those without bound peptides indicating that structural rearrangements outside the AF2 pocket in different residues located at BF-3, for instance, Phe826 and Arg840, possibly transmit their effect though residues located at the boundary between them (43). Leu830 divides BF-3 and the conformation of the side chain is responsible for further dissecting of BF-3 into 2 sockets (43). The more bulky phenylalanine residue may

impact on this BF-3 dissection thereby may influence AF2 peptide binding in the BF-3/AF2 communication. Hormone insensitivity at low steroid concentrations caused by p.Cys806Phe, p.Leu830Phe and p.Arg840His/Cys points to an interference with chaperone/cochaperone/immunophilin interaction. Steroid hormone receptor amino acid sequence alignments identified six residues, including Cys806 and Arg840 that are conserved among the FKBP52-regulated AR receptors, glucocorticoid receptor (GR) and progesterone receptor (PGR), but differ in the FKBP52-insensitive mineralocorticoid receptor (MR) (44). The alteration p.Cys806Tyr was described previously, associated with PAIS (38). Interestingly, functional assays in yeast demonstrated an increased dependence on FKBP52 for function of AR mutation p.Cys806Tyr (44). Grosdidier *et al.* (37) described the same effect on N/C interaction with the CAIS associated alterations p.Asn833Arg and p.Arg840Ala. These results can be associated with many events in AR action, such as transcriptional activity and alteration in co-activator binding. Therefore, such interesting effects suggest this region as a special place for future investigations (37).

Two mutations associated to CAIS phenotype have been investigated. The AR p.Leu768Val resulted in a severe effect upon transcriptional activity in both assays showing a weak transcriptional activity only at supraphysiological hormone concentrations in the full-length cDNA experiment. The residue Leu768 is located in the loop between helixes 5 and 6. Alterations in this region can present an important role in conformation of N/C interaction or a disruption in AF2, resulting in the impairment in the hormone binding (16, 17, 23). Helsen *et al.* (11) described the residue Pro766 involved in DBD-LBD interaction. This alteration was found in a patient with amenorrhea and testes in *labia majora* (23). Other mutations have been described in the same residue, such as the CAIS associated mutation p.Leu768Pro and PAIS associated mutation p.Leu768Met (8, 17). The amino acid Leu768 is conserved in other hormone receptors (36). Due to the localization and conservation of Leu768, alteration in this residue can result in damage of AR ligand-binding pocket stabilization and also in the binding of co-regulators (23, 38). The other CAIS related mutation studied here was p.Pro904Arg. Residue Pro904 is highly conserved among steroid receptors (45, 46). CAIS mutations p.Pro904His and p.Pro904Ser have been previously described in this residue (21, 32). AR p.Pro904Arg is located at the end of helix 12 which together with helix 3, 4 and 5 forms AF2. A response as low as 20% at

physiologic hormone concentration (1.0 nM DHT) was observed in the full-length experiment whereas it equaled the wt at high and supraphysiological hormone concentrations. However, the transcriptional activity in the N/C interaction assay did not exceed 40% even in supraphysiological hormone concentrations. The residue Pro904 has not a significant contact with ligand in LBD; however alterations in Pro904 might affect the reconstitution of AF2 and the sealing of the ligand-binding pocket (21, 30).

Finally, the combination p.Gln798Glu+p.Cys806Phe were identified in a girl with CAIS. To understand the effect of this mutation, we tested each separately and the combination. At physiologic hormone concentration (1.0 nM DHT) p.Gln798Glu, p.Cys806Phe and AR p.Gln798Glu+p.Cys806Phe showed a transcriptional activation of the reporter gene showed a residual activity of 77%, 22% and >10%, respectively, in both full-length cDNA and N/C interaction experiments. At high hormone concentration (>10 nM of DHT), these mutations recovered the activity reaching ≥ 80% in the full-length cDNA assays. In N/C assays, those mutations presented increasing transactivation activity reaching almost 3 times the wt, except p.Gln798Glu that did not exceed the wt activity. Mutation AR p.Gln798Glu was previously investigated *in vitro*, although using different reporter genes and cell lines (12, 16). Those results together with ours indicate that mutations in residue Gln798 seem not to be very harmful since they were described associated with MAIS, PAIS and prostate cancer (13, 14, 15). However, it seems to exert a synergistic negative effect on the AR transcriptional ability when combined in the p.Gln798Glu+p.Cys806Phe double mutant since this function was slightly more compromised than in each mutation separately. The N/C-terminal interaction of AR p.Gln798Glu+p.Cys806Phe and AR p.Cys806Phe at low to normal hormone concentrations was abolished whereas, at supra physiologic hormone concentration they showed enhanced N/C-terminal interaction, similar to AR p.Leu830Phe. Mutation p.Gln798Glu had no significant impact on N/C interaction, indicating that mutation p.Cys806Phe is the more severe mutation mostly responsible for the phenotype.

In conclusion, we described six mutations associated with PAIS and CAIS phenotypes. Dysfunctions of the mutations were confirmed by *in vitro* assays. Functional analysis can clarify the mechanism of AR action and can be very helpful for choosing the appropriate therapy for the patient as well. Although many studies on the AR protein have

been described in the literature, new results can support new strategies aiming the identification of the real AR mutation effect in the phenotype of each patient.

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**Figures**:

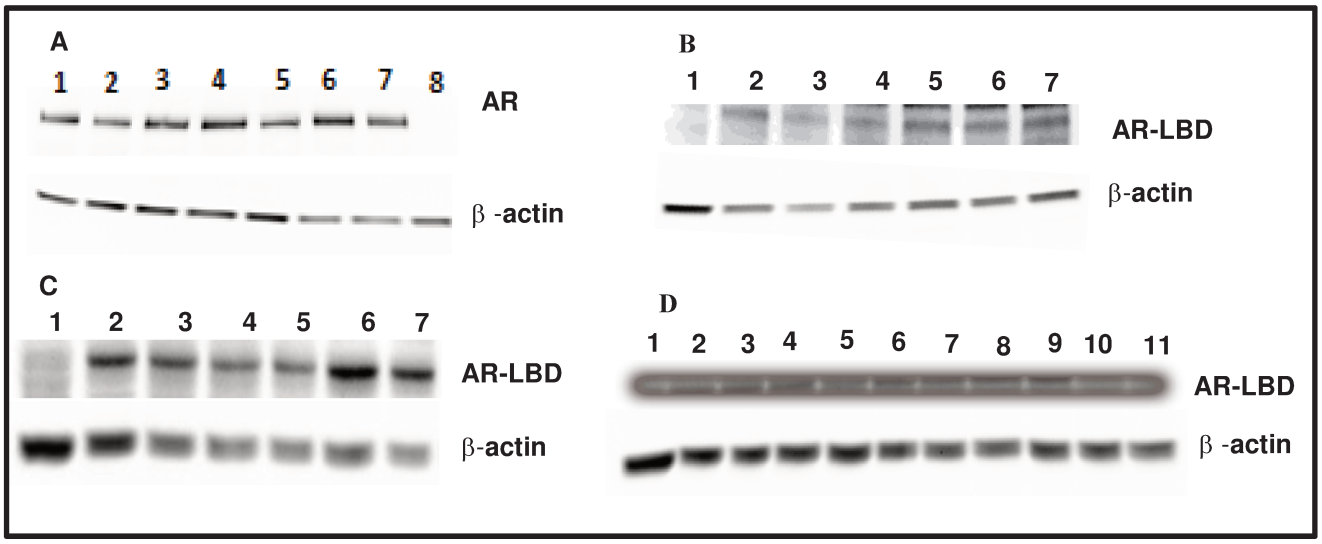


**Figure 1.** Western blot analysis of wt and mutant AR. (A) Full-length AR wt and mutants were incubated with the anti-AR antibody F39.4.1. Detection of actin protein by actin antibody was performed on the same membrane. Lanes 1-7: p.Pro904Arg, p.IleI898Phe, p.Leu830Phe, p.Cys806Phe, p.Leu768Val, p.Ser759Thr and wt; lane 8: non-transfected CHO cells. (B-D) GAL4-DBD/AR-LBD fusion constructs detected with AR-C19 antibody; (B) lane 1: non-transfected CHO cells, lanes 2-4: wt AR fusion protein incubated with 0 nM, 1 nM and 10 nM DHT and lanes 5-7: p.Cys806Phe fusion protein incubated with 0 nM, 1 nM and 10 nM DHT. (C) lane 1: non-transfected CHO cells, lanes 2-4: wt AR-LDB fusion protein incubated with 0 nM, 1 nM and 10 nM DHT and lanes 5-7: p.Leu830Phe fusion protein incubated with 0 nM, 1 nM and 10 nM DHT. (D) lane 1: non-transfected CHO cells, lanes 2-3: wt AR-LDB fusion protein incubated with 0 nM and 10 nM DHT and lanes 4-5: p.Ser597Thr fusion protein incubated with 0 nM and 10 nM DHT, lanes 6-7: p.Ile898Phe fusion protein incubated with 0 nM and 10 nM DHT, lanes 8-9: p. Leu768Val fusion protein incubated with 0 nM and 10 nM DHT and lanes 10-11: p.Pro904Arg fusion protein incubated with 0 nM and 10 nM DHT. Detection of β-actin was performed on the same membrane as a loading control for the experiment.

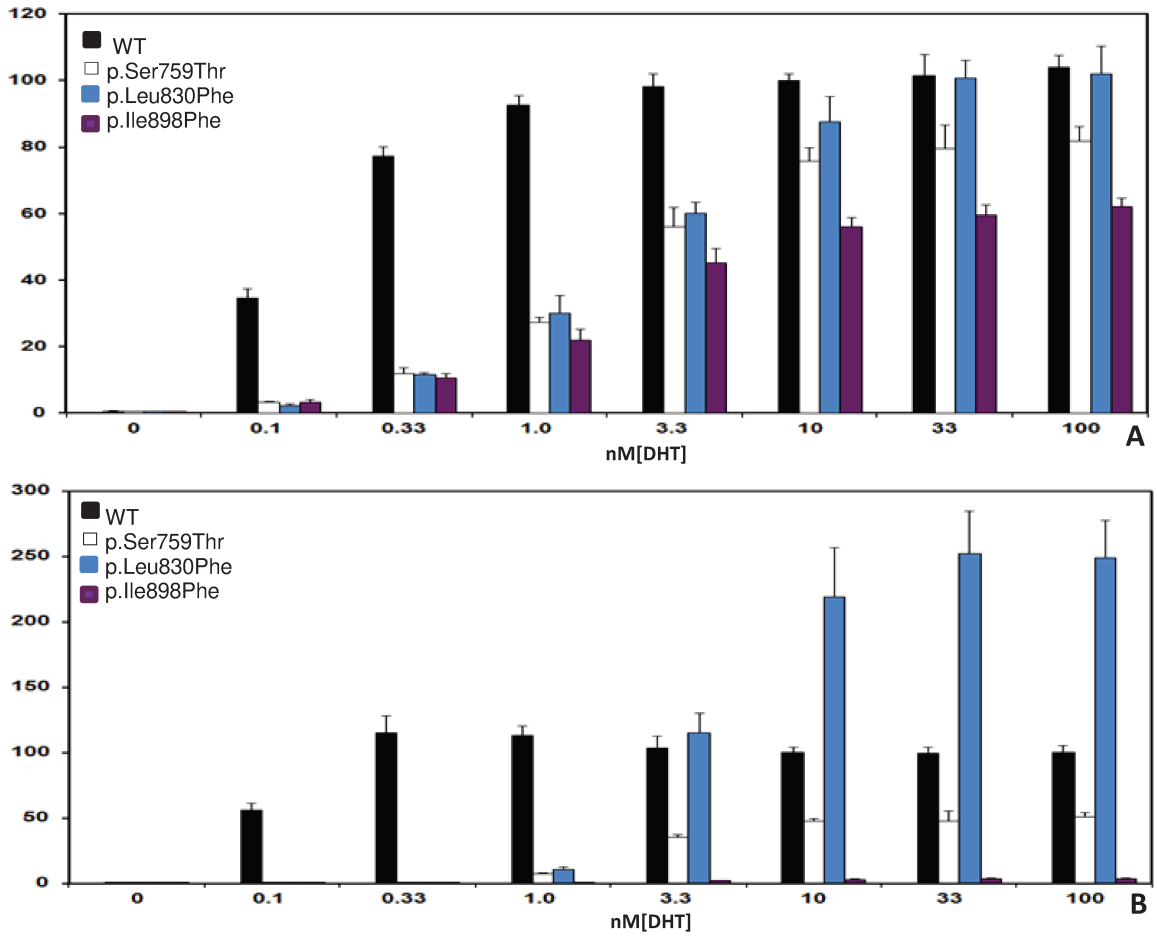


**Figure 2.** Transcriptional activity of the full-length (A) and N/C interaction (B) of PAIS related mutations. In A, CHO cells were transfected with the androgen-responsive firefly-luciferase reporter p(ARE)$_2$ TATA-Luc, the *Renilla* luciferase plasmid phRGTK and full-length AR constructs (pSVAR_wt and mutants: pSVAR_p.Ser759Thr, pSVAR_p.Leu830Phe and pSVAR_p.Ile898Phe. In B, CHO cells were transfected with the bait vector: pBD_LBD_wt or mutants: p.BD_LBD_p.Ser759Thr, pBD_LBD_p.Leu830Phe and p.BD_LBD_p.Ile898Phe, the target fusion construct pAD_NTD, the firefly reporter gene pFR_Luc and the constitutively expressed *Renilla* luciferase phRGTK. The cells were treated with indicated amount of hormone five hours after transfection. Transfection efficiencies were normalized using the Dual Luciferase Assay kit. The activity of the wt construct at 10 nM DHT was set to 100%. The statistical analysis was performed with data of 3 independent experiments in triplicates with + 1 SD error bars. RLU: relative luciferase units.

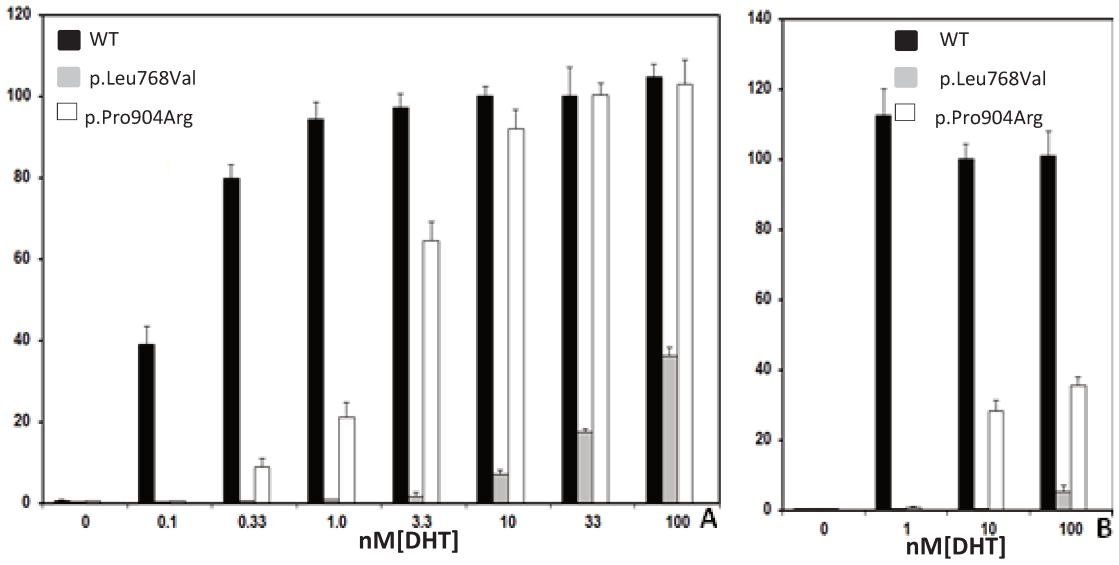


**Figure 3.** Transcriptional activity of full-length (A) and N/C interaction (B) of CAIS related mutations. In A, CHO cells were transfected with the androgen-responsive firefly-luciferase reporter p(ARE)$_2$ TATA-Luc, the *Renilla* luciferase plasmid phRGTK and full-length AR constructs: pSVAR_wt and mutants: pSVAR_p.Leu768Val, pSVAR_p.Pro904Arg. In B, CHO cells were transfected with the bait vector: pBD_LBD_wt or mutants: pBD_p.Leu768Val, pBD_p.Pro904Arg, the target fusion construct pAD_NTD, the firefly reporter gene pFR_Luc and the constitutively expressed *Renilla* luciferase phRGTK The cells were treated with indicated amount of hormone five hours after transfection. Transfection efficiencies were normalized using the Dual Luciferase Assay kit. The activity of the wt construct at 10 nM DHT was set to 100%. The statistical analysis was performed with data of 3 independent experiments in triplicates with + 1 SD error bars. RLU: relative luciferase unit.

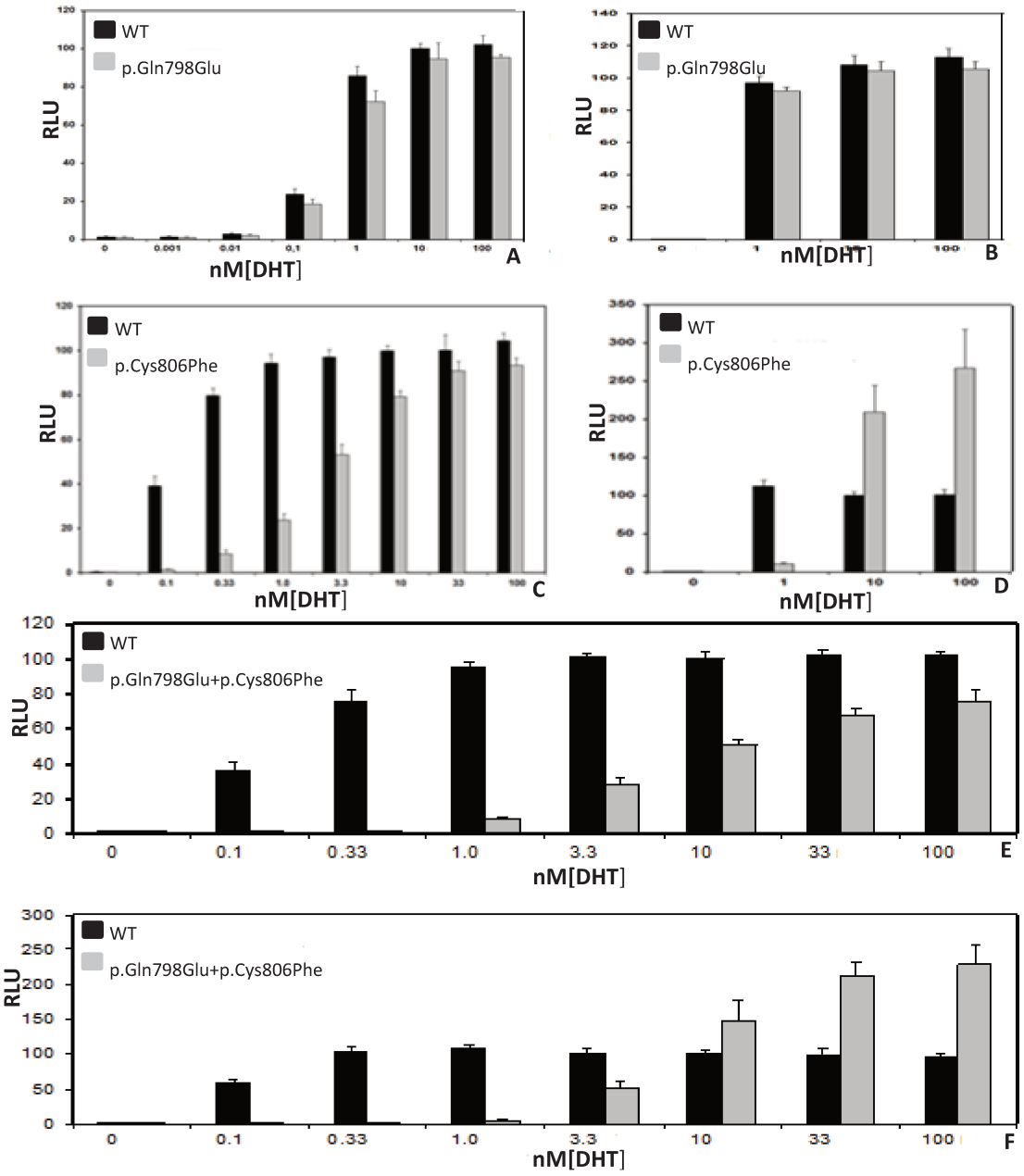


**Figure 4.** Transactivation of full-length and N/C interaction and of p.Gln798Glu, p.Cys806Phe and double mutant p.Gln798Glu+p.Cys806Phe**.** In A, C and E CHO cells were transfected with androgen-responsive firefly-luciferase $p(ARE)_2TATA$-Luc, the *Renilla* luciferase plasmid phRGTK and full-length wt AR or AR_p.Gln798Glu, AR_p.CysC806Phe and AR_p.Gln798Glu+p.Cys806Phe. In B, D and F, CHO cells were transfected with pBD_LBD_wt or pBD_LBD_p.Gln798Glu, pBD_LBD_p.CysC806Phe, pBD_LBD_p.Gln798Glu+p.Cys806Phe, pAD_NTD fusion constructs, pFR_Luc and the

phRGTK (*Renilla* luciferase). Five hours after transfections, cells were treated with indicated concentration of DHT. Transfection efficiencies were normalized using the Dual Luciferase Assay kit. The activity of the wt or mutant constructs at 10 nM DHT was set to 100%. At least three independent experiments were performed in triplicates, error bars + 1 SD. RLU: relative luciferase units.

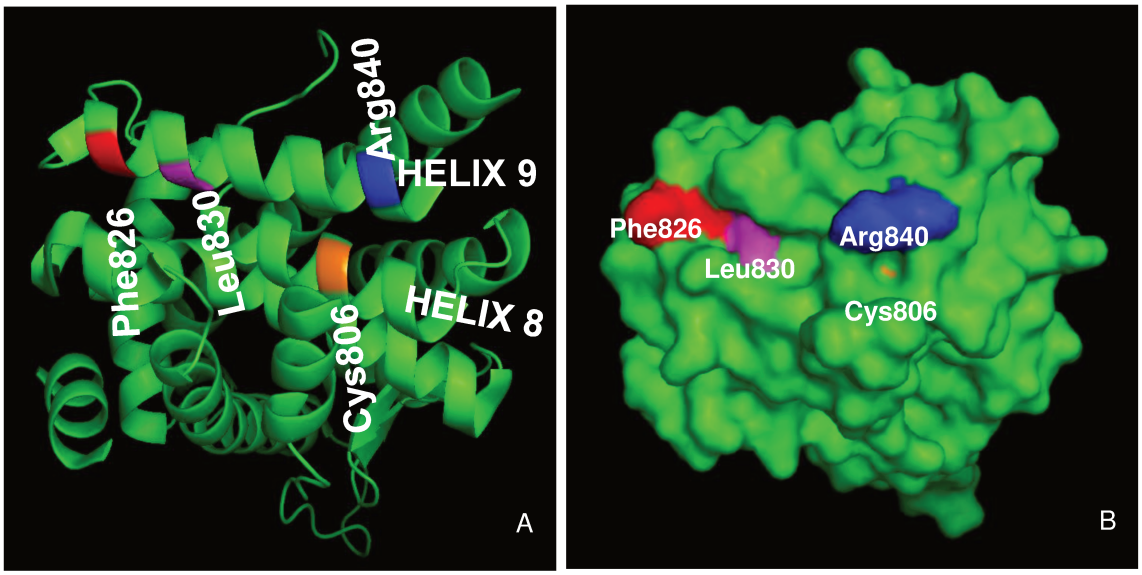


**Figure 5**: Simplified model representation of AR LBD structure. A, helix 8 showing the residue Cysteine 806 in orange; and, the helix 9 showing residues Phenylalanine 826 in red, Leucine 830 in purple and Arginine 840 in blue. B, Solid-surface representation of AR LBD in green showing the residues Cys 806, Phe 826, Leu 830 and Arg 840.

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**5. Capítulo 3**

**Artigo 3:**

# Functional studies for p.Pro695Ser androgen receptor mutation: association with gynecomastia and fertility

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• ***Running title.*** Male Fertility associated with *AR* mutation

• ***Title.*** Functional studies for p.Pro695Ser androgen receptor mutation: association with gynecomastia and fertility

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*Capsule*

Gynecomastia and fertility in androgen insensitivity syndrome correlate to p.Pro695Ser mutation in the androgen receptor gene. The expression profile suggests a high dose DHT therapy to overcome functional deficits.

**Structured Abstract**

**Objective:** To search for mutation in the androgen receptor gene and to evaluate its influence on the androgen receptor transactivation ability.

**Design:** Androgen receptor gene sequencing and *in vitro* assays for androgen receptor transactivation activity comparing normal and mutant proteins.

**Setting:** University research.

**Patient(s):** A fertile man with gynecomastia.

**Intervention(s):** Peripheral whole blood.

**Main Outcome Measure(s):** A mutation in the androgen receptor gene presenting transactivation activities that correlate to mild form of androgen insensitivity syndrome.

**Result(s):** Androgen receptor gene sequencing revealed the p.Pro695Ser mutation. It is located within the androgen receptor ligand-binding domain. Bioinformatics analysis indicated a deleterious role, which was verified upon testing transctivation activity and N/C interaction by *in vitro* expression of a reporter gene and two hybrid assays. p.Pro695Ser showed low levels of both transactivation activity and N/C interaction at low dihydrotestosterone conditions. As the ligand concentration increases both transactivation activity and N/C interaction also increase and reach normal levels.

**Conclusion(s):** This study provides functional insights for the p.Pro695Ser mutation described here for the first time in a patient with mild androgen insensitivity syndrome. The expression profile p.Pro695Ser not only correlates to the patient's phenotype but also suggests that a high dose DHT therapy may overcome the functional deficit of the androgen receptor.

**Key Words:** androgen receptor gene, gynecomastia, male fertility, p.Pro695Ser mutation.

## Introduction

The androgen insensitivity syndrome (AIS) is known as a disorder caused by mutations in the androgen receptor gene (*AR*) but in the absence of *AR* mutations it can result from abnormalities in some unknown mechanism (1-3). Individuals 46,XY with AIS present undermasculinization with external genitalia that varies from normal male to female. Based on the degree of masculinization the disorder is divided in three main subgroups: mild AIS (MAIS) characterized by infertility or gynecomastia; partial AIS (PAIS) that is associated with different degrees of masculinization of external genitalia, that manifests as micropenis, severe hypospadia, bifid scrotum or clitoris enlargement with labial fusion; and, complete AIS (CAIS) that results in a female phenotype with sparse to absent pubic and axillary hair and normal breast development (1, 3, 4-6).

The *AR* gene maps to the X-chromosome at Xq11 – 12 region. Mutations on this gene can be either inherited with a X-chromosomal recessive inheritance or sporadic. The *AR* gene has eight exons and seven introns distributed on more than 186 kb (7-9).

The *AR* gene codes for a protein with approximately 920 amino acids and molecular weight of about 110 kD. The AR protein exhibits three functional domains: the variable amino-terminal domain (NTD) that extends from residue 1 to 538, and is involved in the AR transactivation activity through interaction with the transcriptional machinery (10); the conserved DNA-binding domain (DBD) formed by residues 539 to 628 (11, 12); and, the conserved ligand-binding domain (LBD) from residue 670 to 920. The LBD contains 11 helices that are responsible for the organization of ligand-binding pocket which is formed by helices 3, 4, 5, 7, 11 and 12 and also the beta-sheet preceding helix 6 (13). The LBD also harbors an important and conserved region, the ligand-dependent activation function 2 (AF-2) (14, 15). Interaction between N-terminal domain and C-terminal domain of AR protein is an important step toward the activation of AR. Any damage in the N/C interaction can be associated with AIS, even if the hormone binding is normal (17-20).

Mutations in the AR may result in decrease of ligand affinity, cause a disturbance in co-activator interactions or also cause damages in the DNA-binding activity depending on which residue is involved (15, 21). There are more than 800 different AR mutations (22) reported in the literature that are associated to phenotypes that range from MAIS to CAIS.

In general, mutations associated to MAIS cause male infertility. Here, we describe a rare case of a fertile patient with MAIS due to the p.Pro695Ser AR mutation. To understand the functional properties of this mutation, we studied its impact mutation on both full length AR transactivation ability and AR N/C-terminal interaction.

**Patient and Methods**

*Patient*

The patient, a 27-year-old man, was referred to us due to persistent gynecomastia. Breast development began spontaneously at the age of 11.5 years and progressed during a period of 4 years; afterwards, the gynecomastia stabilized. There was no history of use of drugs such as androgenic or estrogenic hormones and antiandrogens that could have a causative association with gynecomastia. His parents were not consanguineous, and there was no family history of gynecomastia, genital ambiguity or infertility. On physical examination, his weight was 87.6 kg, height 169.5 cm and arm span 170 cm; breast development was equivalent to Tanner stage III, and both glandular and fat tissues were palpable. He had male external genitalia with a small phallus (7.7 cm stretched length) and testes were located in the scrotum (right testis = 25 $cm^3$, left testis = 20 $cm^3$). Facial and axillary hair was sparse. He had a normal 46,XY karyotype. Levels of FSH (2.03 IU/L; normal range (NR)=1.5-12.4) and estradiol (36.04 pg/mL; NR=7.6-42.6 pg/mL) were within the normal male reference range; whereas LH (8.77 IU/L; NR=1.7-8.6) and testosterone (30.99 nmol/L; NR=9.92-28.11 nmol/L) levels were slightly elevated. The Androgen Sensitivity Index (LH x Testosterone in U x $nmol/L^2$) was elevated (271 U x $nmol/L^2$; NR=< 138 U x $nmol/L^2$) suggesting AIS (23). He had two sperm analyses that showed concentrations within the normal range (29 and 25 millions/mL; normal = >20 millions/mL) and indicated normal sperm morphology and motility. Each of two ex-wives had miscarriages, and his current wife gave birth to a healthy boy. This study had been approved by the Institutional Review Board at Universidade Estadual de Campinas (# 434/2006).

*Molecular Analysis of AR Gene*

Genomic DNA was isolated from whole blood samples with standard procedures using Proteinase K lysis and phenol/chloroform extraction. The eight *AR* exons and exon-intron junctions had been amplified by PCR using primers and PCR conditions described

elsewhere (6). PCR products were purified using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, WI, USA). Further direct sequencing using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI PRISM/PE Biosystems, Foster City, CA, USA) was carried out using sense and antisense primers. Sequences were obtained in an ABI PRISM 3130 DNA Analyzer (ABI PRISM/PE Biosystems). Free softwares such as Chromas Pro v.1.5 and CLC Sequence Viewer v.6.6.2 were used to analyze and compare sequences with the *AR* reference sequence at Ensembl database (ENSG00000169083, 24).

*Preparation of AR expression vectors*

The mutation was introduced into the full-length AR expression vector pSVAR0, which was kindly provided by Dr. A. Brinkmann from the University Hospital Rotterdam, Rotterdam, NL. PCR based site-directed mutagenesis was carried out in two steps using the following primers: AR_Pro695Ser_sense: 5'-CACGACAACAACCAGTCCGACTCCTTTGCAG-3'; AR_Pro695Ser_antissense: 5'-CTGCAAAGGAGTCGGACTGGTTGTTGTCGTG-3'. In the first step, primer AR_LBD644s (5´-GAGGCTTCCAGCACCACCA-3´) and the respective mutagenesis antisense primer were used. The second amplification was performed with the respective mutagenesis sense primer and pSV0seq_as (5'- TGATAGGCAGCCTGCACCTGA -3'). Both amplicons were gel purified, combined, annealed and amplified in a third PCR using the primer pair AR_LBD644s - pSV0seq_as. Mutant amplicons were double digested with Tth111I/PstI and subcloned into pSVAR0. The insert and its cloning borders were verified by sequencing.

*Two Hybrid Constructs*

AR N/C interaction was analyzed using the mammalian two-hybrid assay kit (Stratagene, La Jolla, CA). In order to generate mammalian two-hybrid clones containing the GAL4 DNA binding domain fused to $AR_{618-920}$, the AR $LBD_{618-920}$ was amplified using specific primers, previously described in Werner *et al.* (19).

*Luciferase Gene Assays*

The $p(ARE)_2$TATA-Luc vector, which contains two androgen response elements 5' of the TATA-box preceding the firefly luciferase gene, was a kind gift of Dr. G. Jenster from Josephine Nefkens Institute, Erasmus MC (Rotterdam, NL). The pFR-Luc vector (Stratagene, La Jolla, CA) contains a synthetic promoter with five tandem repeats of yeast

GAL4 binding sites which control expression of the firefly luciferase gene and served as a reporter gene in mammalian two hybrid assays. Plasmid phRGTK contains a constitutive active *Renilla luciferase* reporter gene used to normalize transfection efficiency in Dual Luciferase Assays (Promega).

*Transient transfections of full length AR and two hybrid assays*

The Chinese hamster ovary (CHO) cells were used to express the wild type and mutant AR proteins. Transfection experiments were performed as previously described (25). 80.000 cells/well were cultured overnight in 24 well plates with Dulbecco's modified Eagles Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma-Aldrich Co., St. Louis, MO) supplemented with 10% dextran charcoal-stripped fetal calf serum in 5% $CO_2$ at 37°C. Then 30 ng wt-AR or mutant plasmid DNA were co-transfected with 200 ng p$(ARE)_2$TATA-Luc and 5 ng phRGTK using 0.5 µl FuGENE HD (Promega Corp., Madison, WI).

For N/C interaction assays CHO cells were cultured and seeded in 24 well plates as described above. 100 ng pBD-$LBD_{618-920}$ (bait, wild type or mutant) and 100 ng of pAD-$NTD_{1-536}$ (prey) were co-transfected with 200 ng pFR-Luc (reporter gene) and 10 ng phRGTK per well using 1.0 µl FuGENE HD (Promega). For both experiments, five hours after transfection, 0–100 nmol/l DHT were added to each well. The luciferase activity was determined 18 h after hormone induction, using a Dual Luciferase assay (Promega) and normalized to transfection efficiency. For comparison of experiments performed at different days we set the transactivity of wt-AR at 10 nmol/l DHT as 100%. Relative luciferase units (RLU) of other samples assayed at the same time were expressed relative to this value. For statistical analysis, all transfections were performed in triplicate and at least three independent experiments were performed.

*Western blot analysis*

For protein expression analysis of AR full length constructs 300.000 CHO cells were grown in six well culture plates overnight. Cells were transfected using 1 µg $(ARE)_2$ TATA-Luc plasmid, 150 ng wild type or mutant AR plasmid, 25 ng phRGTK and 3.35 µl Fugene HD reagent per well. For analysis of two-hybrid constructs 80.000 CHO cells were grown in twenty four well culture plates overnight and were transfected as described above. After 18 h, cells were washed twice with cold PBS and lysed in 250 µl M-PER lysis buffer

(Pierce, Rockford, IL) including complete proteinase inhibitor (Roche, Mannheim, Germany) on ice for 5 min. Cells were scraped and collected for Western blot analysis. Protein concentration was estimated by Bio-Rad protein assay (Bio-Rad, Richmont, CA). The remainder of protein was dissolved in SDS sample buffer and denatured by heating at 95°C for 10 minutes followed by SDS-PAGE.

Full-length AR was detected with primary antibody anti AR F39.4.1 (Innogenex, San Ramon, CA) diluted 1:500. GAL4-LBD fusion proteins were detected with primary antibody AR-C19 (sc-815, Santa Cruz Biotechnology Inc.) diluted 1:200. Signals were visualized using the corresponding conjugated anti-mouse or anti-rabbit secondary antibody applying the Western Lightning Chemiluminescent Reagent Plus (PerkinElmer, Boston, MA) and a Fusion SL chemiluminescent imager (Vilbert Lourmat).

**Results**

*AR* gene sequencing identified the c.2083C>T nucleotide change within exon 4. This change leads to p.Pro695Ser mutation in the LBD region.

To investigate the effect of the AR p.Pro695Ser mutant protein on AR transactivation capability, the full-length AR plasmids were co-transfected with the firefly luciferase reporter plasmid $p(ARE)_2TATA$-Luc in CHO cells (Fig. 1A). Expression of comparable amounts of wild-type (wt) and mutant proteins was confirmed by Western blotting (Fig. 2A).

At low DHT concentrations (0.1-0.33 nM) the AR p.Pro695Ser mutation showed a reduced transactivation of the reporter gene reaching approximately 60% of the wild type level (Fig. 1A). Physiological DHT concentrations of 1.0-3.3 nM still showed a small but significant reduced transactivation (t-test: $P<0.001$), while high and supraphysiological DHT concentrations (10-100 nM) rescued full AR transactivation activity.

The effect of AR p.Pro695Ser on N/C terminal interaction was tested using mammalian two hybrid assays (Fig. 1B). Similar results were obtained, where DHT concentrations of 1 nM still lead to a significantly reduced transactivation of the reporter gene (t-test, $P<0.001$), indicating a reduced binding of the FxxLF motif to AF-2. On the other hand, higher DHT concentrations (3.3-100 nM) fully restored N/C-interaction (Fig. 1B). Expression of comparable amounts of wild-type (wt) and mutant Gal4-LBD fusion proteins was confirmed by Western blotting (Fig. 2B).

**Discussion**

The clinical manifestation of p.Pro695Ser mutation in the patient described here was gynecomastia with elevated LH and testosterone serum levels. These levels contributed to a high androgen sensitivity index suggesting MAIS. Besides clinical features of the patient and the molecular findings, we describe in this study the functional activity of p.Pro695Ser mutation that is located in the LBD of AR protein.

The alignment of human AR protein sequence to other mammalian AR proteins indicated that P695 residue is conserved among all mammalian species (data not shown). Mutations located in this residue are expected to drastically damage the receptor structure and function as mutations surround it were mainly found in 46,XY female patients with normal or ambiguous genitalia (22, 26-28).

The c.2083C>T nucleotide change responsible for p.Pro695Ser mutation have not been annotated yet in the Androgen Receptor Gene Mutations Database (22), whereas it was reported as a single nucleotide variation (SNV) in the NHLBI GO Exome Sequencing Project (ESP) with the reference # rs372903533 (29). As the individual carrying the mutation was a heterozygous female, no clinical significance has been associated to it. Conversely, SIFT and PolyPhen predictive algorithms theoretically considered the p.Pro695Ser as a deleterious mutation.

The functional significance of the mutant p.Pro695Ser AR protein was tested *in vitro* and the results indicated impairments in both transcriptional activity and in N/C interaction under low hormone concentration conditions. Both functionalities are gradually recovered as DHT concentration increases. Although they still remained significantly lower at physiological hormone concentrations, they were very close to the wild-type; whereas, at high DHT concentrations they were fully recovered.

The mild impairment of AR transactivation capacity observed *in vitro* for p.Pro695Ser mutant under physiological conditions associated it to MAIS phenotype. N/C-terminal interaction is critical for AR transactivation and is predominantly mediated by binding of the N-terminal FxxLF motif (residues 23-27) to the hydrophobic binding groove of AF-2 in LDB (30). According to the crystallographic structure of the ligand-binding domain of the androgen receptor, the residue P695 is located in the loop between helix 1 and helix 3 of AR protein (13). Based on results for the p.Asp696Asn mutation, this region

was considered to be important for N/C interaction (15). This mutation did not affect ligand-binding but drastically reduced transactivation activity through impairment of N/C interaction. Therefore, we tested N/C interaction for p.Pro695Ser to verify the possible influence upon transactivation activity. A significant reduction in the N/C interaction was only observed at low DHT concentrations (≤ 1.0 nM) confirming the mild effect of the mutation.

In general, fertility in MAIS patients is a very rare feature. Larrea *et al.* (31) described four individuals in a family with gynecomastia and normal fertility due to MAIS, but molecular evaluation was not available at that time. Besides p.Pro695Ser here discussed, two other mutations, p.Arg825Lys and p.Arg841Cys, have been associated to MAIS without affecting fertility (32, 33). Similarly to the mutation described here, those mutations present an increase in AR activity as DHT concentration increases. In the family described by Chu *et al.* (33) the p.Arg841Cys mutation was associated with fertile and infertile 46,XY individuals. Based on the improvement in the AR activity in higher androgen concentrations, the authors discuss that the variation in phenotype among the different patients may be explained by fluctuation in androgen secretion during certain periods of sexual development. Among morphological features in fertile individuals with MAIS described by Chu *et al.* (33) and Giwercman *et al.* (32) hypospadias, gynecomastia and diminished penis size can be found. Gynecomastia was the only morphological feature in the patient we describe. As this condition is a result of excessive estrogen actions due to an increased ratio of estrogen to androgen associated with low activity of androgen receptor, it is reasonable to think that the level of testosterone in the breast was not enough to rescue the activity of the mutated androgen receptor in the patient; whereas, a higher testosterone concentration in the testis might have preserved fertility as the mutant receptor recovers its transactivation activity under ligand high level conditions.

In conclusion, we describe for the first time the association of p.Pro695Ser with a mild form of androgen insensitivity in a fertile individual whose complain was gynecomastia. *In vitro* analysis confirmed the role of the mutation on MAIS ethiology and gave clues for choosing an appropriate therapy for the patient as well. The results suggest that a high dose DHT therapy may overcome the functional deficit of the receptor. Although many studies on the AR protein have already been described in the literature,

novel mutations and their effects can give insights for understanding how they are reflected in the phenotype of each patient.

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**Figures:**

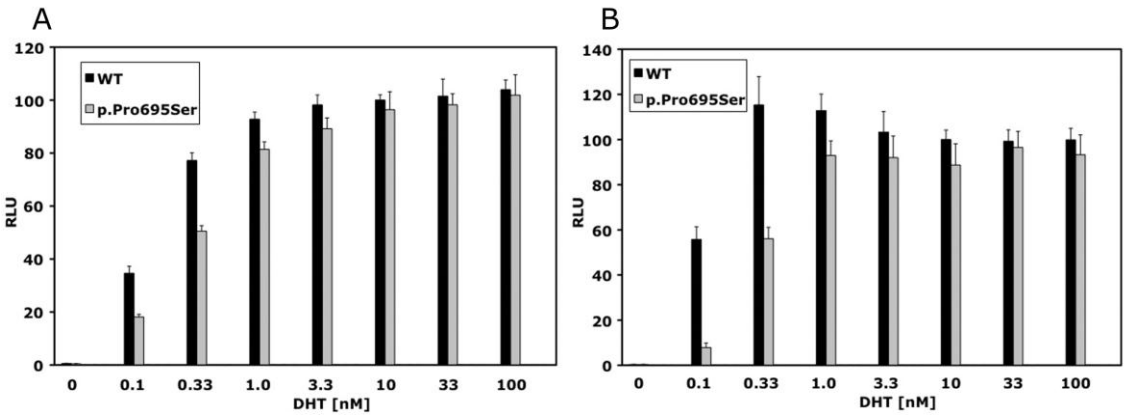


**Figure 1.** Transactivation and N/C interaction for wt and mutant AR. **A**) Transactivation assay: CHO cells were transfected with the androgen-responsive firefly-luciferase reporter gene p(ARE)$_2$ TATA-Luc, the *Renilla* luciferase plasmid phRGTK and full-length wt AR or AR_ p.Pro695Ser. Five hours after transfections, cells were treated with indicated concentrations of DHT. **B**) N/C-Interaction assay: CHO cells were transfected with pAD_NTD fusion construct, pFR_Luc, phRGTK (Renilla luciferase) and pBD_LBD_wt or pBD_LBD_p.Pro695Ser. Five hours after transfections, cells were treated with indicated concentrations of DHT. In both experiments, transfection efficiencies were normalized using the Dual Luciferase Assay kit. The activity of the wt constructs at 10 nM DHT was set to 100%. At least three independent experiments were performed in triplicates, error bars + 1 SD. RLU: relative luciferase units.

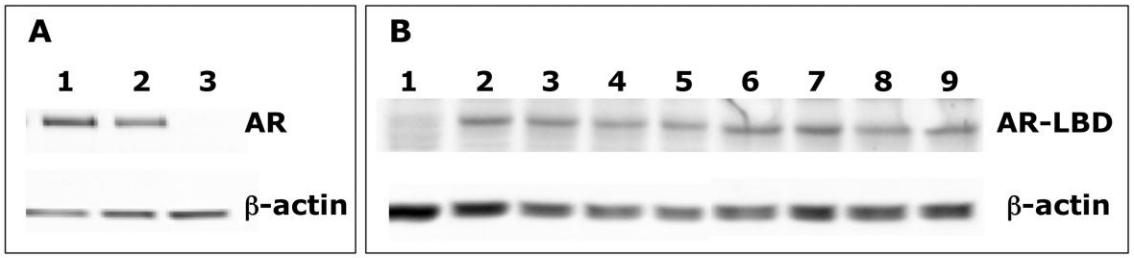


**Figure 2.** Western blot analysis of wt and mutant AR. (A) mutant p.Pro695Ser (lane 1), wt full length AR (lane 2) and non-transfected CHO cells (lane 3) incubated with anti-AR antibody F39.4.1. (B) GAL4-DBD/AR-LBD fusion constructs detected with AR-C19 antibody; lane 1: non-transfected CHO cells, lanes 2-5: wt AR fusion protein incubated with 0 nM, 0.1 nM, 1 nM, and 10 nM DHT and lanes 6-9: p.Pro695Ser fusion protein incubated with 0 nM, 0.1 nM, 1 nM and 10 nM DHT. Detection of β-actin was performed on the same membrane as a loading control for the experiment.

## 6. Discussão:

Através do sequenciamento automático do gene *AR*, pode-se observar as mutações descritas nos artigos 1 a 3 e a partir delas, as análises funcionais foram realizadas. No artigo 1 descrevemos uma família com quadro clínico sugestivo de AIS, o que foi confirmado com a análise por sequenciamento, onde foi observada a troca nucleotídica c.3015C>T no *exon* 7 do gene *AR*, levando à mutação p.Leu830Phe, associada ao fenótipo PAIS. Esta alteração, descrita pela primeira vez neste trabalho, foi avaliada quanto à conservação do resíduo e a suas alterações estruturais. As análises indicaram que o resíduo é bastante conservado e que mudanças no microambiente onde se deu a mutação ocorreram, sendo possivelmente relacionadas com o comprometimento funcional do AR.

O artigo 2 mostra a análise funcional de seis mutações e uma combinação entre duas mutações associadas aos fenótipos PAIS e CAIS, incluindo a p.Leu830Phe descrita no artigo 1. As alterações relacionadas ao fenótipo PAIS: p.Ser759Tre, p.Leu830Phe e p.Ile898Phe apresentaram resultados semelhantes em concentrações sub-fisiológicas (0,1-0,33 nM DHT) e fisiológica (1 nM DHT). Na concentração fisiológica, a resposta foi inferior a 40% para todas as alterações acima. Uma baixa capacidade de transativação do gene repórter, inferior a 50% quando comparado com o wt, é observada em ensaios realizados com AR mutante em pacientes com diferentes graus da forma PAIS, assim os dados obtidos estabelecem uma boa correlação com o diagnóstico clínico (Werner *et al.*, 2008, Jaaskelainen *et al.*, 2006). No entanto, estas mutações influenciam a interação N/C de formas diferentes e respondem às concentrações crescentes de DHT também de formas distintas. Essa diferença pode ser relacionada às posições que ocupam na estrutura. Enquanto o resíduo Ser759 encontra-se no sulco de ligação ao ligante, o resíduo Ile898 ocupa uma região responsável por interações proteína-proteína (anexo 1). Essas diferenças podem se refletir na resposta ao tratamento com andrógenos a que os pacientes geralmente são submetidos.

Para as mutações associadas ao fenótipo CAIS, as alterações p.Leu768Val e p.Pro904Arg apresentaram uma resposta baixa à luciferase em concentração fisiológica (1 nM DHT) no ensaio com o cDNA completo do AR. A alteração p.Leu768Val anulou a função do AR, entretanto, a alteração p.Pro904Arg apresentou 30% de ativação do receptor,

resultado que considerado isoladamente poderia associá-la ao fenótipo PAIS. Entretanto, a resposta da interação N/C terminal da Arg904, assim como a Val768 foi praticamente abolida, prejudicando desta forma a capacidade de ativação transcricional do AR, embora para a Arg904 esta interação se recupere parcialmente atingindo 30-40% da capacidade transcricional em concentrações altas de DHT. A alteração p.Pro904Arg está localizada na extremidade da hélice 12 que em conjunto com hélice 3 e hélice 4, compõe o subdomínio AF2 (anexo 1). Esta alteração mostrou uma baixa resposta à luciferase em todas as concentrações hormonais. Várias mutações relacionadas ao fenótipo CAIS têm sido descritas no resíduo 904, como exemplo, as alterações p.Pro904His e p.Pro904Ser (Melo *et al.*, 2003; Werner *et al.,* 2006) indicando que este resíduo seja realmente importante para a manutenção da função do AR. Para a mutação p.Leu768Val encontrada em um paciente com CAIS mostra apenas atividade transcricional em concentrações hormonais supra-fisiológicas. Em concentrações baixas ou fisiológicas, a atividade foi completamente abolida. O resíduo Leu768 apresenta-se conservado entre outros receptores nucleares, desta forma, alterações nessa região resulta em prejuízo na ligação com o ligante, como observado nas análises funcionais, além de comprometer as interações com cofatores da transcrição (Sack *et al.*, 2001). Outras alterações no resíduo Leu768 foram descritos associados ao fenótipo PAIS e CAIS (Boehmer, 2001; Ahmed *et al.*, 2000).

Ainda relacionadas ao fenótipo CAIS, descrevemos a combinação de alterações p.Gln798Glu+p.Cys806Phe. Estas mutações foram encontradas no *exon* 6 em um paciente com CAIS. Para o completo entendimento da contribuição de cada mutação para o fenótipo da paciente, estudamos as mutações primeiro separadamente e depois em conjunto. A alteração p.Gln798Glu, apresentou padrão de transativação compatível com MAIS em todas as concentrações hormonais, tanto no ensaio de transativação do AR quanto nas interações N/C terminais. A mutação p.Gln798Glu foi anteriormente investigada *in vitro*, e foi associada com PAIS (Hughes, 2008). Além disso, mutações no resíduo Gln798 foram descritas associados com MAIS, PAIS e câncer da próstata (Hughes *et al.*, 2012). Sua localização periférica contribui para que mudanças nesse resíduo não causem grande impacto na função proteica (anexo 1). Quando os receptores com as alterações p.Cys806Phe e p.Gln798Glu+p.Cys806Phe foram expostos a concentrações fisiológicas de DHT, as respostas foram aproximadamente 30% e <10%, respectivamente, no experimento

com o cDNA completo, sendo que para o experimento de interação N/C as atividades para a mesma concentração de DHT foram menores ainda para cada mutação. No entanto, a partir de 10 nM de DHT, houve uma resposta aumentada à luciferase, principalmente no experimento de interação N/C terminal onde a resposta superou a do controle. A combinação entre as mutações p.Gln798Glu+p.Cys806Phe foi encontrada em um paciente com CAIS. Os resultados de transativação para a combinação p.Gln798Glu+p.Cys806Phe apresentou um prejuízo maior na transativação, quando comparada com as alterações estudadas separadamente. Esse resultado mostra que a combinação das duas alterações promove um efeito aditivo ou sinérgico diminuindo ainda mais a atividade funcional do AR mutante em concentrações fisiológicas de DHT, embora o aumento de atividade em concentrações maiores tenha sido também observado. Este padrão é conhecido, mas ainda pouco explicado na literatura (Werner *et al.,* 2008; Wong *et al.*, 2008; Perpina *et al.*, 2007). Alguns autores especulam a possibilidade desta alta resposta estar associada a uma disfunção no recrutamento de co-reguladores da transcrição, podendo haver um aumento na transativação. Perpina e colaboradores em 2007 descrevem em seu trabalho uma alteração que apresentou o mesmo padrão de resposta à luciferase, como o aqui mostrado. Segundo os autores, a alteração p.Arg840Cys apresentou uma alta resposta à luciferase quando exposto a altas concentrações de diferentes andrógenos. Este efeito anômalo pode estar associado a alterações no mecanismo de recrutamento de cofatores, levando em consideração a importante região que esta alteração se encontra, a região AF-2 da proteína AR. O mesmo resultado foi observado por Wong e colaboradores (2008), para a alteração p.Phe826Leu (Wong *et al.,* 2008). O subdomínio AF-2 é conhecido entre os receptores nucleares pela sua conservação (Berrevoets *et al.*, 1998) e por apresentar fundamental importância no recrutamento e ligação de cofatores. Alterações encontradas neste subdomínio podem aumentar a interação entre AF-2 e o domínio N-terminal da proteína AR, levando a uma capacidade de transativação aumentada (Szafran *et al.*, 2009).

A mutação p.Leu830Phe descrita acima também apresentou efeito semelhante e, acima de 10 nM de DHT, a resposta à luciferase foi bastante elevada. O resíduo Leu830 mostra-se importante para a função do AR visto que, além da mutação aqui descrita, foi alvo de outra mutação associada ao fenótipo CAIS (Chaves *et al.*, 2001). A gravidade dos fenótipos dos pacientes com alterações neste aminoácido explica-se pela importante região

que elas se encontram, sendo esta a hélice 9 formada pelos aminoácidos 825 ao 847. O domínio LBD da proteína AR apresenta duas regiões fundamentais: a primeira é o subdomínio AF2 que compreende os aminoácidos 892 ao 896, como descrito acima, e a segunda região é conhecido como subdomínio BF3. Alterações nestas regiões estão diretamente associadas ao câncer de próstata e AIS (Perpina *et al.,* 2007).

Já no artigo 3, realizamos a análise funcional da proteína AR com a mutação p.Pro695Ser. O paciente que apresenta esta mutação foi encaminhado por ginecomastia. Na análise por sequenciamento automático do gene AR, foi verificado a alteração p.Pro695Ser, primeiramente descrita neste trabalho. A fertilidade deste paciente foi confirmada através da análise da viabilidade dos espermatozóides no espermograma. Os resultados obtidos na análise funcional da alteração p.Pro695Ser está de acordo com o fenótipo MAIS. Sua localização periférica na estrutura proteica, à semelhança do resíduo Gln798 citado acima, indica um baixo impacto na atividade do AR (anexo 1). Tanto a transativação da proteína AR completa mutante com p.Pro695Ser quanto a análise das interações N/C terminais não revelaram reduções significativas em concentrações fisiológicas de hormônio, quando comparadas com a proteína selvagem na mesma concentração hormonal. Uma drástica redução na transativação foi observada em baixas concentrações hormonais, o que pode explicar a manifestação da ginecomastia. A ação da testosterona pode estar prejudicada devido à baixa concentração periférica, não sendo suficiente para ativar o receptor, em contrapartida, a ação gonadal preserva a fertilidade.

Na literatura, existem poucos casos de pacientes com AIS e fertilidade preservada. As análises funcionais destes casos são de extrema importância, uma vez que a atividade do AR pode ser recuperada em concentrações altas de hormônio. Esses dados colaboram para o desenvolvimento de estratégia medicamentosa para pacientes portadores de MAIS.

A síndrome de insensibilidade androgênica é conhecida como uma disfunção na proteína AR, que pode ser associado com mutações no gene de AR ou por mecanismos ainda desconhecidos (Hiort *et al.*, 2000; Hughes, 2008). O sequenciamento completo do gene *AR* tem sido uma peça fundamental no grande número de mutações descritas neste gene. Isso favorece primeiramente o esclarecimento do fenótipo do indivíduo, direciona o estudo funcional, de modo a analisar o efeito de mutações nos diferentes domínios da proteína AR além de elucidar o mecanismo molecular do receptor de andrógenos.

## 7. Conclusões:

- A mutação AR_Leu830Phe
    - Está localizada próxima a AF-2 e BF-3;
    - Foi associada aos graus 5 e 6 do fenótipo PAIS em duas gerações de uma família Brasileira;
    - Foi descrita pela primeira vez na literatura;
    - A análise *in silico* da proteína AR mostrou alterações entrem o resíduo normal e mutante.
- As mutações p.Ser759Tre, p.Leu830Phe e p.Ile898Phe apresentaram resultados funcionais compatíveis com o fenótipo PAIS;
- As mutações p.Leu768Val, p.Cys806Phe e p.Pro904Arg:
    - Apresentaram baixo padrão de expressão do gene repórter;
    - Resposta baixa ou alterada nas interações N/C terminais;
    - Dados compatíveis com o fenótipo CAIS.
- As mutação AR_p.Gln798Glu+p.Cys806Phe
    - Apresentou efeito sinérgico na transativação da proteína AR mutante.
- As alterações p.Leu830Phe e p.Cys806Phe apresentaram efeitos ativadores de função transcricional em concentrações supra-fisiológicas de hormônio.
- A mutação AR_Pro695Ser foi descrita pela primeira vez na literatura;
    - Altas doses hormonais podem recuperar a atividade do receptor;
    - Atividade transcricional compatível com o fenótipo MAIS.
- A análise funcional de mutações pode ajudar a esclarecer os mecanismos moleculares associados à proteína AR.
- O estudo do efeito de AR mutantes pode auxiliar na escolha da terapia apropriada em casos de MAIS e PAIS.

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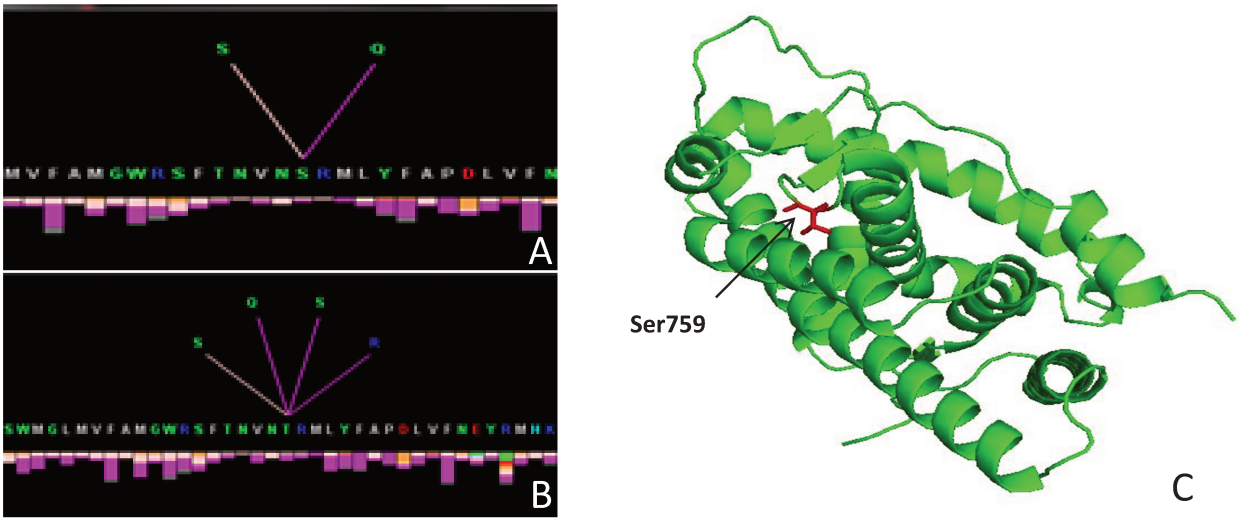
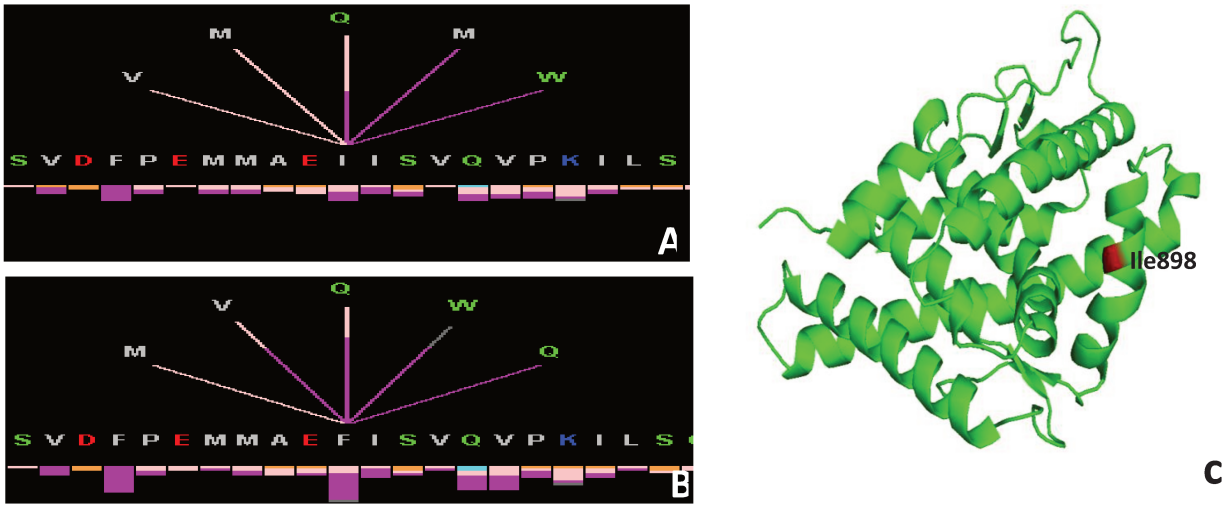
9. Anexos

9.1 Anexo 1: Análise estrutural das proteínas AR mutantes

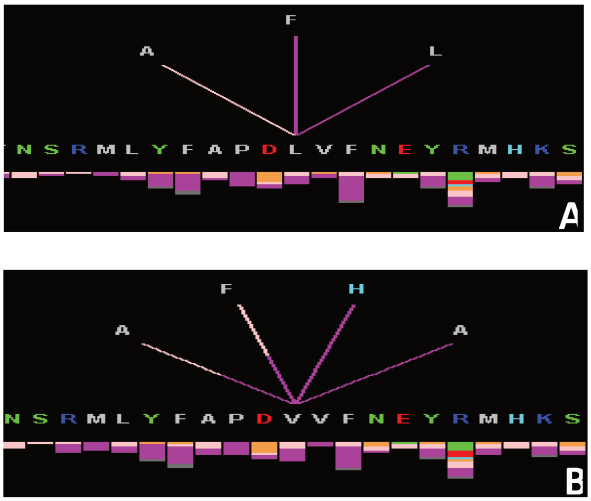


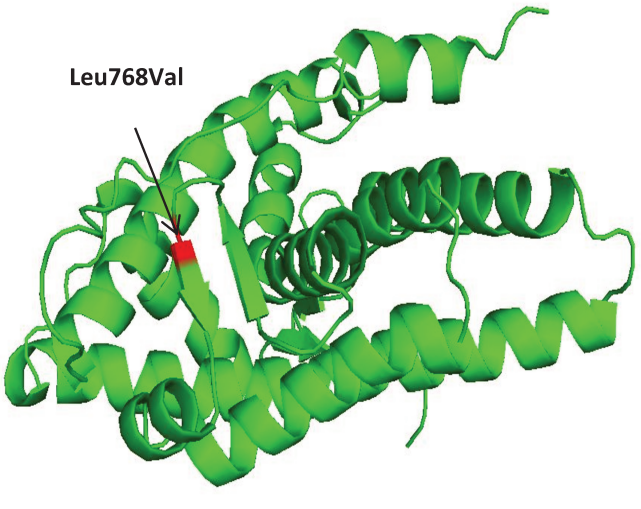

Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido 759. Em A, o resíduo selvagem Ser759 realiza uma ponte de hidrogênio com o resíduo Ser725 e uma interação hidrofóbica com o resíduo Phe764. Em B, resíduo mutante Tre759 mantém as ligações feitas pelo resíduo normal e cria mais três interações hidrofóbicas com os resíduos Arg760 e Phe764. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o aminoácido Serina 759, localizado entre as hélices 5 e 6.



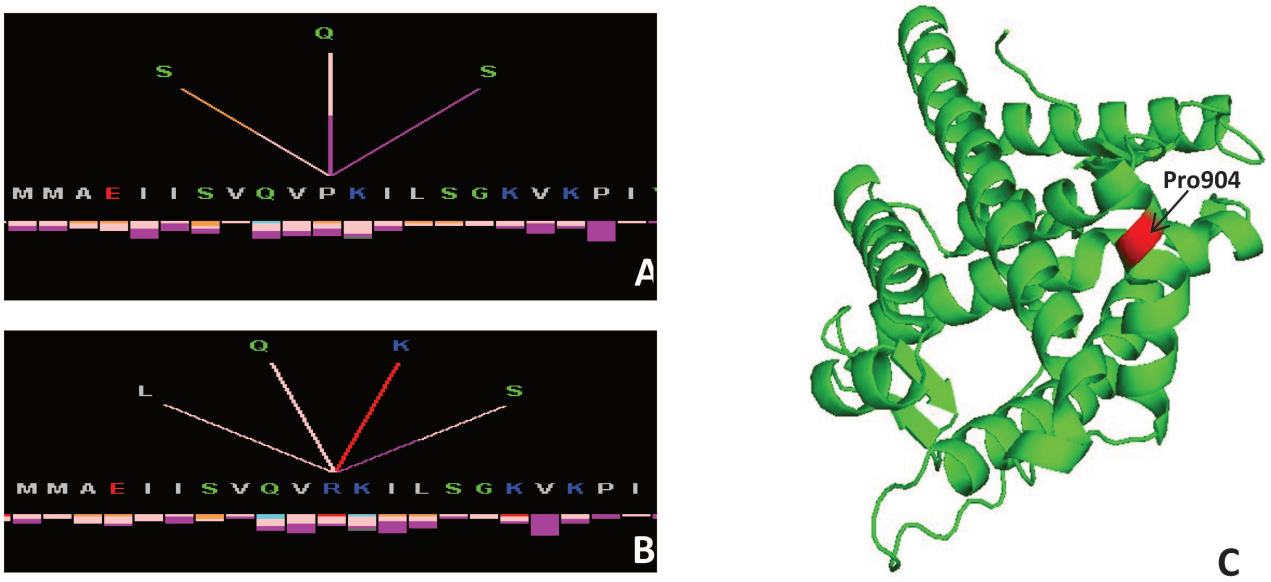

Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido 898. Em A, o resíduo selvagem Ile898. A isoleucina na posição 898 apresenta 3 pontes de

hidrogênio com os resíduos: Val902, Met893 e Gln900 e 4 interações hidrofóbicas com os resíduos: Gln900, Met712 e Trp740. Em B, resíduo mutante Phe898. A fenilalanina na posição 898 mantém as pontes de hidrogênio com Val902 e Met893 e cria interações com os resíduos Val902, Gln900, Trp740 e Gln737. O número de interações hidrofóbicas no resíduo mutante aumenta para 12 quando comparado com resíduo normal. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o aminoácido Isoleucina 898, localizado na hélice 11.

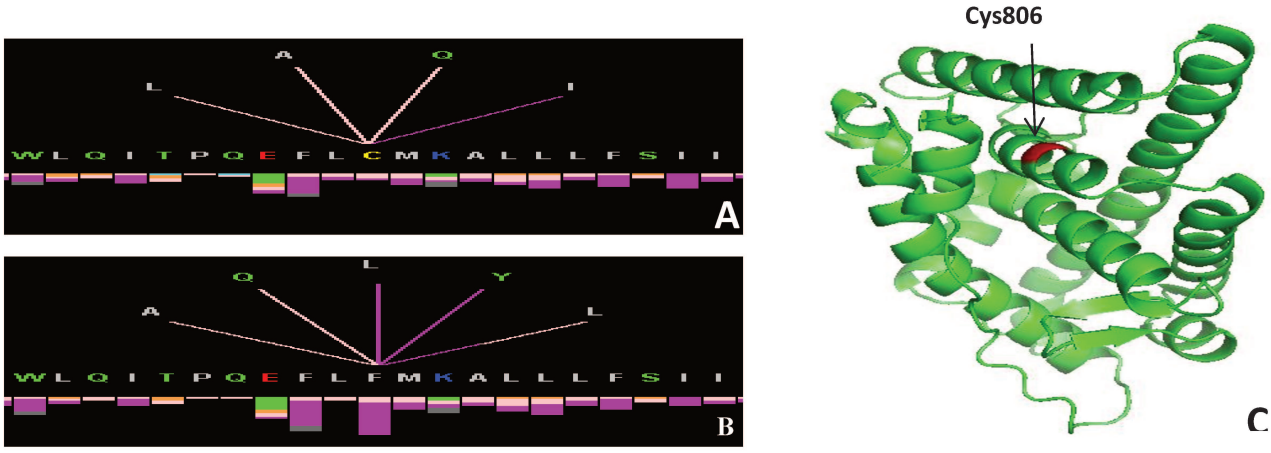


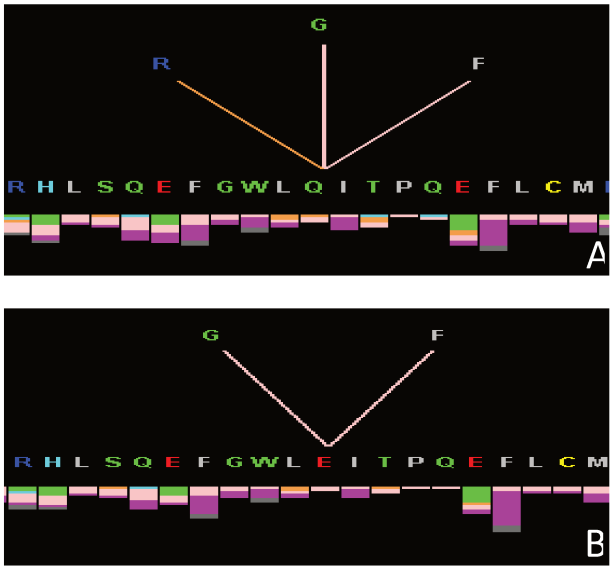

Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido 768. Em A, o resíduo selvagem Leu768 faz duas pontes de hidrogênio com a Ala765 e quatro interações hidrofóbicas com Phe770 e Leu702. Em B, o resíduo mutante Val768 mantém somente o contato com o aminoácido Ala765 e cria mais três novos contatos com os aminoácidos Phe764, His689 e Ala687. As interações hidrofóbicas também são alteradas, o resíduo selvagem Leu768 faz quatro interações hidrofóbicas com Phe770 e Leu702 e o resíduo Val768 fazendo seis interações hidrofóbicas com Phe764, His689 e Ala687. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o aminoácido Leucina 768, localizado entre as hélices 5 e 6.

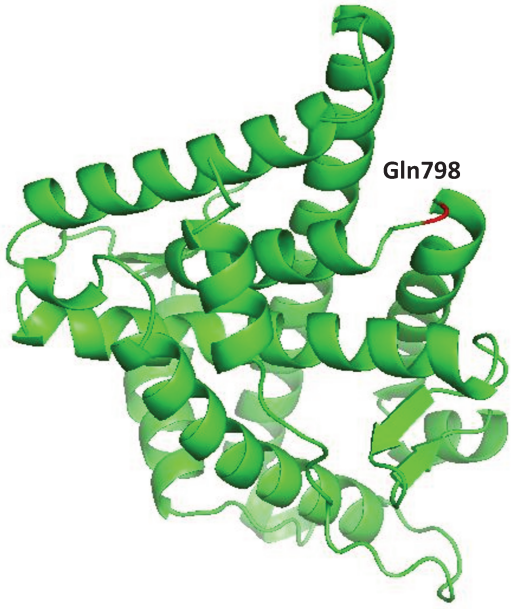



Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido 904. Em A, o resíduo selvagem Pro904 faz duas pontes de hidrogênio com os aminoácidos Ser908 e Gln902 e três interações hidrofóbicas com o aminoácido Ser 900. Em B, o resíduo mutante Arg904, mantém a interação com os aminoácidos Ser908 e Gln902 e cria novos contatos com os aminoácidos Arg910 e Leu907 e perde a ligação Ser900 . Duas interações hidrofóbicas são perdidas no resíduo mutante Arg904. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o aminoácido Prolina, localizado na hélice 12.

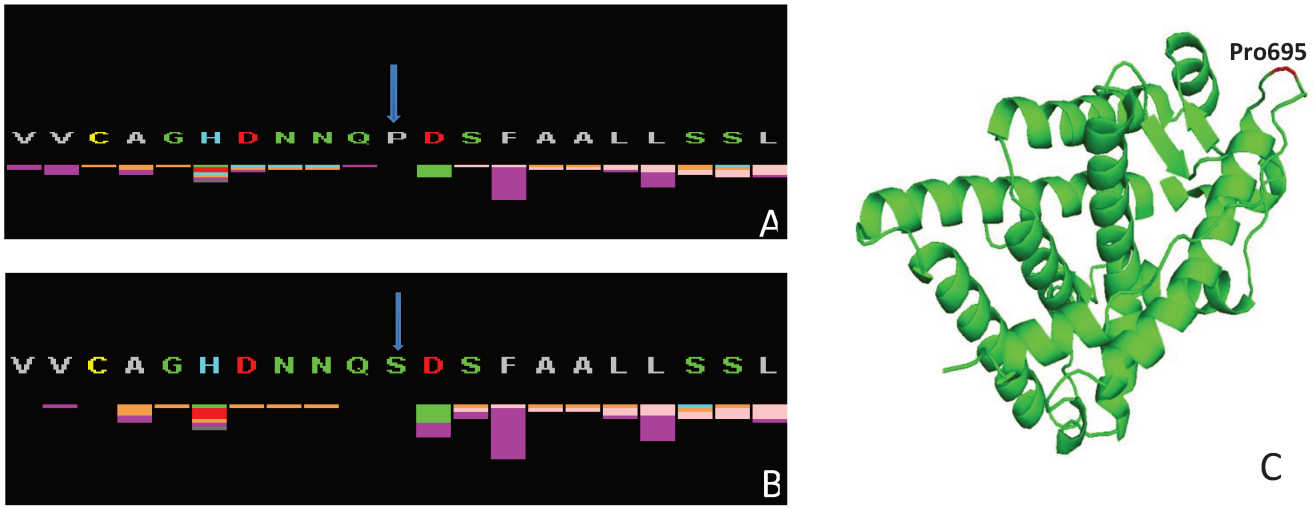


Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido 806. Em A, o resíduo selvagem Cys806 O resíduo normal apresenta 3 pontes de hidrogênio com os aminoácidos Leu810, Arg808, Gln802 e Ile841. O resíduo mutante mantém as pontes de hidrogênio com os resíduos Leu810, Arg808 e Gln802 e cria interações hidrofóbicas com Leu838, Tyr834, Leu810 e Leu839. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o aminoácido Cisteína, localizado na hélice 8.




Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido da posição 798. Em A, o resíduo selvagem Gln798 faz conto com os aminoácidos Arg855, Gln795 e Phe794. Em B, O resíduo mutante Glu798 perde uma ponte de hidrogênio feita com aminoácido Arg855 e mantém duas pontes de hidrogênio com o aminoácido Phe766. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o local da alteração o aminoácido Gln798, no loop entre as hélices 7 e 8 da proteína AR.




Análise estrutural do domínio de ligação da proteína AR, evidenciando o aminoácido da posição 695. Em A, o resíduo selvagem Pro695 e em B, o resíduo mutante Ser695. Ambos resíduos não estabelecem contatos internos. Em C, estrutura simplificada do domínio de ligação ao hormônio da proteína AR, evidenciando o local da alteração p.Pro695Ser no loop entre as hélices 1 e 3 da proteína AR, uma posição superficial externa.

9.2 Anexo II – Declaração Bioética e Biossegurança



Cidade Universitária "Zeferino Vaz",
18 de junho de 2014.

CIBio – Declaração 05/2014.

**Identificação:**
**Doutorado**: Reginaldo José Petroli, CPG-GBM UNICAMP
Projeto: EFEITOS DE NOVAS MUTAÇÕES NO RECEPTOR DE ANDRÓGENOS SOBRE OS MECANISMOS MOLECULARES.

**Parecer:**
Projeto aprovado pela CIBio/CBMEG sob número 03/2003.
Coordenador: Profa. Dra. Maricilda Palandi de Mello

**Profa. Dra. Edi Lúcia Sartorato**
Presidente da CIBio/CBMEG - UNICAMP