Universidade Federal do Rio de Janeiro

Paulo Alonso Garcia Alves Junior

AVALIAÇÃO MOLECULAR DO CARCINOMA DIFERENCIADO DE TIREOIDE EM CRIANÇAS E ADOLESCENTES E ASSOCIAÇÃO COM A EXPRESSÃO DO CO-TRANSPORTADOR SÓDIO-IODO (NIS / *SLC5A5*) E PUBERDADE.

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Medicina (Endocrinologia) da Universidade Federal do Rio de Janeiro, como requisito parcial para a obtenção do título de Doutor em Medicina (Endocrinologia)

Orientadora: Profª. Fernanda Vaisman Balieiro

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Aprovado em:

_____

Profª. Izabel Calland Ricarte Beserra
Professora Associada da Faculdade de Medicina da Universidade Federal do Rio de Janeiro

_____

Profª. Denise Pires de Carvalho
Professora Titular do Instituto de Biofísica Carlos Chagas Filho da Universidade Federal do Rio de Janeiro

_____

Prof. Sonir Roberto Rauber Antonini
Professor Titular da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo

_____

Prof. Rui Monteiro de Barros Maciel
Professor Emérito da Escola Paulista de Medicina da Universidade Federal de São Paulo

_____

Prof. Marcus Vinicius Leitão de Souza
Professor Adjunto da Faculdade de Medicina da Universidade Federal do Rio de Janeiro

À Princesa-Guerreira Helena. Obrigado por me inspirar a tentar ser o melhor pai do mundo.

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“Se você escutar uma voz dentro de você dizendo ‘você não é capaz de pintar’, então pinte de todas as maneiras e essa voz será silenciada”

(Vincent Van Gogh)

Resumo

O Co-transportador Sódio-Iodo (NIS) é a porta de entrada para a avaliação de metástases e tratamento adjuvante do carcinoma diferenciado de tireoide (CDT). Apesar de uma apresentação mais agressiva comparada a adultos, por sua raridade, poucos estudos avaliaram as alterações moleculares do CDT pediátrico e sua associação com a expressão do NIS e puberdade. A presente tese tem o objetivo de avaliar o perfil genético tumoral e analisar sua associação com o padrão de puberdade completa, com a expressão do gene *SLC5A5* e a detecção da proteína NIS, além de demonstrar a performance da pesquisa de corpo inteiro (PCI) e da tireoglobulina estimulada (Tgs) na detecção de metástase à distância em pacientes pediátricos com diagnóstico de CDT. A partir de amostras de tecido tumoral de pacientes menores de 19 anos diagnosticados entre os anos de 1976 e 2022 com CDT no Instituto Nacional de Câncer - Brasil, foram avaliados a expressão do gene *SLC5A5* por reação de cadeia de polimerase, a detecção da proteína NIS por imunohistoquímica, o perfil genético tumoral por sequenciamento de nova geração, a PCI com Iodo-131 e a Tgs por eletroquimioluminescência. Foram revistas as características clínicas, histológicas, categorização de puberdade completa, resposta ao final do tratamento e a PCI na modalidade diagnóstica e pós-radioiodoterapia dos pacientes selecionados. Foram selecionados 142 pacientes avaliados clinicamente com 97 amostras tumorais avaliadas para a detecção do NIS, 72 para a expressão do gene *SLC5A5* e 41 para o perfil genético tumoral. Esta tese resultou em três artigos que descreveram que somente em 25% das amostras foi detectada a proteína NIS, apesar de 54% apresentarem seu gene codificante expresso. Pacientes com maior expressão do gene *SLC5A5* necessitaram de menos tratamento com Iodo-131. O perfil molecular tumoral mais encontrado foi o de rearranjos/fusões, sendo os genes mais frequentes *RET*, *NTRK, ALK e BRAF*. Estes perfis moleculares não se relacionaram à detecção ou expressão do NIS, mas se associaram de maneira significativa com a puberdade e com a apresentação inicial da doença. A puberdade completa se associou com uma apresentação mais branda e um maior diagnóstico entre as mulheres. A PCI diagnóstica de 66 pacientes na modalidade prognóstica apresentou uma baixa performance comparada à Tgs, além de se associar ao encontro de imagens falso-negativas. Assim, a detecção da proteína NIS foi inferior à expressão do gene *SLC5A5*, não se associando à

parâmetros clínicos, moleculares, puberdade ou resposta ao tratamento. Possivelmente, não o NIS, mas fatores genéticos e pós-transcricionais, como a puberdade, se associam e determinam apresentações clínicas e respostas ao tratamento diferenciadas no CDT pediátrico. A PCI deve ser interpretada com cautela no CDT pediátrico.

Palavras-chave: 1. Tumor de tireoide; 2. Carcinoma diferenciado de tireoide pediátrico; 3. NIS; 4. Molecular; 5. Puberdade.

Abstract

The Sodium-Iodine Cotransporter (NIS) is the gateway to the evaluation of metastases and adjuvant treatment of differentiated thyroid carcinoma (DTC). Despite a more aggressive presentation compared to adults, due to its rarity, few studies have evaluated the molecular changes of pediatric DTC and its association with NIS expression and puberty. The present thesis aims to evaluate the tumor genetic profile and analyze its association with the pattern of complete puberty, with the expression of the *SLC5A5* gene and the detection of the NIS protein, in addition to demonstrating the performance of whole-body scan (WBS) and of stimulated thyroglobulin (Tgs) in the detection of distant metastasis in pediatric patients diagnosed with DTC. Using samples of tumor tissue from patients under 19 years old diagnosed between 1976 and 2022 with DTC at the Instituto Nacional de Câncer - Brazil, the expression of the *SLC5A5* gene was evaluated by polymerase chain reaction, the detection of the NIS protein by immunohistochemistry, tumor genetic profile by next generation sequencing, WBS with Iodine-131 and Tgs by electrochemiluminescence. The clinical and histological characteristics, categorization of complete puberty, response at the end of treatment and WBS in the diagnostic and post-radioiodine therapy modality of the selected patients were reviewed. 142 patients were clinically evaluated with 97 tumor samples evaluated for the detection of NIS, 72 for the expression of the *SLC5A5* gene and 41 for the tumor genetic profile. This thesis resulted in three articles that described that the NIS protein was detected in only 25% of the samples, despite 54% having its coding gene expressed. Patients with higher expression of the *SLC5A5* gene required less treatment with Iodine-131. The most common tumor molecular profile was that of rearrangements/fusions, with the most frequent genes being *RET*, *NTRK*, *ALK* and *BRAF*. These molecular profiles were not related to the detection or expression of NIS but were significantly associated with puberty and the initial presentation of the disease. Completed puberty was associated with a milder presentation and greater diagnosis among women. The diagnostic WBS of 66 patients in the prognostic modality presented a low performance compared to Tgs, in addition to being associated with the finding of false-negative images. Thus, the detection of the NIS protein was lower than the expression of the *SLC5A5* gene, not being associated with clinical, molecular parameters, puberty or response to treatment. Possibly, not

NIS, but genetic and post-transcriptional factors, such as puberty, are associated and determine differentiated clinical presentations and treatment responses in pediatric DTC. WBSI should be interpreted with caution in pediatric DTC.

Keywords: 1. Thyroid tumor; 2. Pediatric differentiated thyroid carcinoma; 3. NIS; 4. Molecular; 5. Puberty.

Lista de abreviaturas

CDT – Carcinoma diferenciado de tireoide

ATA – Do inglês, *American Thyroid Association* (Associação Americana de Tireoide)

Tg – Tireoglobulina

Tgs – Tireoglobulina estimulada

TSH – Do inglês, Thyroid-Stimulating Hormone (Tireotropina)

mCi - miliCurie

RIT – Radioiodoterapia

NIS – Do inglês, *Sodium-Iodide Symporter* (Simportador Sódio-Iodeto, Co-Transportador Sódio-Iodo)

IHC – Imuno-histoquímica

FFPE – Do inglês, Formalin-fixed paraffin-embedded (Fixado em formalina e armazenado em blocos de parafina)

PCI – Pesquisa de corpo inteiro

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Sumário

1 - Introdução

1.1 – Carcinoma diferenciado de tireoide pediátrico

O carcinoma diferenciado de tireoide (CDT) na população pediátrica é considerado uma doença rara, representado por um tipo de tumor maligno bem diferenciado da glândula tireoide em pessoas menores de 18 – 20 anos de idade e que vem apresentando aumento em sua prevalência nos últimos anos.[1-4] Esta malignidade corresponde a 3 % de todos os casos de cânceres pediátricos e é responsável por apenas 1,9 a 2,3 % do total de diagnósticos de câncer de tireoide, sendo muito mais comum entre os adolescentes.[1-2] Para fins comparativos, o número estimado de casos novos de câncer de tireoide no Brasil para os anos de 2023 a 2025 entre todas as idades é de 16.600 casos por ano, correspondendo a um risco estimado de 7,68 casos novos para cada 100.000 pessoas por ano.[5] No Brasil, a taxa de incidência do CDT pediátrico é de 0,51 casos por milhão para crianças entre 0 – 9 anos de idade, 4,4 casos por milhão entre as idades de 10 – 14 anos e de 17,5 casos por milhão para adolescentes entre 15 – 19 anos de idade. Ainda assim, é considerado o tumor endócrino mais comum na faixa etária pediátrica, com o Brasil sendo o país de maior incidência entre aqueles da América Central e do Sul.[6]

Devido a escassez de dados científicos robustos, durante muitos anos foram usadas as diretrizes estabelecidas em adultos para o tratamento e acompanhamento dessas crianças, sem levar em conta as características distintas e peculiares do CDT pediátrico.

Em crianças e adolescentes são vistas apresentações iniciais mais agressivas da doença, com maiores taxas de metástases cervicais (40-80 % em comparação a 20–50 % em adultos) e à distância (25 % em comparação à 4 % em adultos), de forma que a maior parte dos pacientes pediátricos necessitará de tratamento adjuvante após a cirurgia de tireoidectomia.[1-2] Apesar destes dados desfavoráveis, crianças e adolescentes diagnosticados com esta malignidade possuem respostas dramáticas ao tratamento, com uma sobrevida maior que 98 % (versus 95 % em adultos) em 5 anos.[7-9] Além destes aspectos, as células tumorais apresentam uma assinatura genética diversa da apresentada por adultos, como será melhor descrito à frente.

Outras características instigantes do CDT pediátrico são as mudanças na proporção de acometimento entre o sexo masculino e feminino a partir da idade de 10 anos[10] e o processo de puberdade associado à melhores apresentações e desfechos da doença.[11]

Pela sua raridade, comparativamente, existem poucos estudos sobre o tumor de tireoide na infância. Como norte, há somente duas diretrizes (*Guideline*) de recomendação para diagnóstico e acompanhamento do CDT em menores de 18 – 20 anos de idade, sendo uma primeira divulgada pela *American Thyroid Association (ATA)* em 2015 e uma última recentemente publicada em 2022 pela *European Thyroid Association* (*ETA*). Apesar destas Sociedades reconhecerem as diferenças entre o CDT no adulto e na população pediátrica, a maioria das recomendações destes *Guidelines* manteve-se baseada em estudos de adultos. Mesmo mais atual, não houve mudanças significativas entre o *Guideline* europeu e o americano, sendo este último o mais amplamente utilizado.

Norteado pela diretriz da *ATA* Pediátrica[1], um sistema de estadiamento foi padronizado e recomendado para separar os pacientes em risco de recidiva / recorrência para doença estrutural. Este sistema de estadiamento divide os pacientes pediátricos em 3 categorias, sendo elas: Baixo, Intermediário e Alto Risco de recidiva de acordo principalmente com o encontro de metástases linfonodais ou à distância. A tabela 1 resume as categorias e os achados relacionados ao sistema de estadiamento propostos. Diferentemente, para a classificação de risco em adultos, a *ATA* incorporou novas variáveis prognósticas para refinar este processo presuntivo, sendo adicionados mudança na idade do paciente, o perfil molecular do tumor, a avaliação da extensão extranodal e o número e tamanho de linfonodos acometidos.[7] Estas variáveis ainda não puderam ser incorporadas à população pediátrica.

Desde 2015, com a *ATA* Pediátrica, pacientes estadiados com Risco Intermediário ou Alto de recorrência passaram formalmente a ser tratados com terapia adjuvante após a cirurgia de tireoidectomia, diferentemente dos de Baixo Risco em que apenas cirurgia é apresentada como proposta de tratamento. Para esta finalidade, a avaliação de tecido remanescente, doença residual, metástase cervical ou à distância é fundamental e realizada através da dosagem da tireoglobulina sérica e da utilização de métodos por imagem, como a ultrassonografia cervical e a pesquisa de corpo inteiro (PCI) com iodo radioativo (Iodo-131).

A tireoglobulina (Tg) é uma proteína sintetizada pelas células foliculares da tireoide sob a influência da tireotropina (TSH). Esta proteína pode servir como marcador tumoral em pacientes submetidos à tireoidectomia, pois também é produzida por células tumorais bem diferenciadas.[1-2,7] A Tg não está incluída no sistema de estratificação de risco para recorrência da doença, apesar de ser uma das ferramentas utilizadas no estadiamento pós-operatório, indicando tratamento adjuvante quando aumentada.[1] A tireoglobulina estimulada (Tgs) é representada pelos níveis séricos de Tg obtidos após TSH estimulado para mais de 30 mUI/L, sendo estes valores alcançados a partir da suspensão do uso da reposição de Levotiroxina ou pelo emprego do TSH recombinante, e possui um valor preditivo maior que a Tg basal na detecção de doença metastática após a tireoidectomia em adultos.[7] Não se sabe sobre valores séricos de Tgs para a detecção de doença metastática em crianças e adolescentes.

Como dito anteriormente, outra ferramenta para detectar doenças tumorais é a utilização de exames de imagem. A PCI com Iodo-131 é um dos principais métodos de imagem utilizados para detectar células tireoidianas ou seus tumores. Além disso, a presença de tecido com captação de iodo na PCI sugere a possibilidade de uma doença capaz de ser tratada com radioiodoterapia (RIT). A diretriz pediátrica[1] recomenda a realização da PCI após a tireoidectomia e antes da RIT para identificar a presença de doença metastática (PCI diagnóstica), estadiar o paciente e definir assim a necessidade e a atividade do RIT. Uma nova PCI é realizada durante ou logo após o RIT (PCI pós-tratamento) para revelar os locais em que o tratamento está sendo efetivo e avaliar programação de possíveis novas doses naqueles que mantém doença estrutural após o primeiro tratamento.

O encontro de doença metastática (estrutural) pelos exames de imagem (Risco Intermediário e Alto de recorrência) ou valores de Tgs > 10 mUi/L após a tireoidectomia levam à indicação de terapia adjuvante com Iodo-131 com a finalidade da destruir as células tireoidianas remanescentes e tumorais a partir de sua propriedade ionizante. Portanto, o RIT representa uma importante estratégia terapêutica adjuvante adotada para facilitar o seguimento dos pacientes e reduzir a chance de recorrência ou mesmo mortalidade associada à doença.[12]

1.2 – Co-Transportador Sódio-Iodo ou Simportador Sódio-Iodeto (NIS)

O Co-Transportador Sódio-Iodo, melhor descrito como Simportador Sódio-Iodeto, vastamente conhecido como NIS, é uma glicoproteína encontrada principalmente na membrana basolateral das células foliculares tireoidianas, sendo codificada pelo gene *SLC5A5 (solute carrier family 5 member 5)* localizado no cromossoma 19 (19p13.11). Sua principal função é mediar a captação ativa de iodo da corrente sanguínea para dentro da célula através de uma bomba sódio-iodeto (2 Na^+ / I^-) permitindo assim a síntese dos hormônios tireoidianos na glândula tireoidiana.[13] Nos tumores tireoidianos que mantém sua detecção, o NIS representa a porta de entrada para a captação do Iodo-131, levando à destruição da célula tumoral através de sua ação ionizante mediada por partículas β.[13-14]

Alguns estudos demostraram falhas na expressão e detecção do NIS em células tumorais de tireoide, determinando resistência ao tratamento adjuvante com Iodo-131 e se associando com maiores taxas de recorrência e mortalidade.[15-17] Terapias de desdiferenciação que poderiam reincorporar o NIS às células tumorais vêm sendo atualmente estudadas com o objetivo de permitir retratamento com o Iodo-131 em pacientes antes considerados iodorefratários.[18-20]

Poucos estudos avaliaram a detecção do NIS ou a expressão de seu gene codificante *SLC5A5* em células de CDT na faixa etária pediátrica.[21-23] Estes estudos são importantes pois, como relatado anteriormente, o CDT pediátrico se apresenta de forma mais agressiva, com maiores taxas de metástases, requerendo tratamento adjuvante em mais de 70 % dos casos. Ainda mais, o perfil genético diferenciado do tumor de crianças e adolescentes poderia modificar a expressão do NIS e determinar diferentes atividades de Iodo-131 ou resposta ao tratamento e ser um possível alvo de tratamento naqueles com doença metastática iodorefratária em progressão.

1.3 – Perfil genético dos CDT

Desde a criação do Atlas do Genoma do Câncer, o panorama do perfil molecular dos tumores papilíferos de tireoide vem sendo mais bem compreendido. Lesões genéticas em células tireoidianas levam a alterações na funcionalidade celular e conferem a ela um crescimento seletivo, assim como outros marcadores de transformação neoplásica. De forma geral as mutações associadas ao CDT codificam proteínas envolvidas na cascata da MAPK (*mitogen-activated protein*

kinase), incluindo os genes *RET* (encoding proto-oncogen tyrosine-protein kinase receptor), *RAS* (*Rat Sarcoma Virus*) e o *BRAF* (*B-Raf proto-oncogene*). Pelo menos 1 lesão somática está presente em mais de 96 % dos CDT, sendo encontrada em mais de 60 % a mutação pontual *BRAF_V600E*; em 13 % a mutação em genes da família *RAS* (*NRAS* e *HRAS*); em 10 % as alterações moleculares de rearranjo com gene *RET* e menos frequentemente nos genes *ALK, NTRK1, NTRK3, MET, FGFR2, LTK, PPARG, THADA*. Para um melhor entendimento de comportamento e prognóstico em relação ao perfil molecular, os CDT foram divididos em 2 classes maiores: aqueles que carreiam a mutação *BRAF_V600E* (chamados de tipo-BRAF) ou aqueles tipo-RAS (determinados pelas mutações *RAS*, *BRAF* não-V600E e fusões *PPARG*). Esta diferença está relacionada à uma maior atuação na via da MAPK nos tipo-*BRAF* comparada aos tipo-RAS. Isto faz com que a classe tipo-RAS seja mais bem diferenciada, mantendo uma histologia folicular predominante e possua menores riscos de recorrência e melhor expressão do NIS, diferentemente do tipo-BRAF.[24-25]

Dentro deste contexto e mantendo o raciocínio de que crianças não são “adultos pequenos”, a assinatura molecular do CDT pediátrico segue um padrão diferente do anteriormente descrito. Nestes pacientes, alterações genéticas do tipo fusões são muito mais prevalentes, representada principalmente pelo rearranjo *RET/PTC*, e fusões com gene *NTRK* e *ALK*. A mutação no gene *BRAF* pode também ser encontrada, porém em uma frequência muito menor na forma pontual *BRAF_V600E* e aparecendo em alguns pacientes na forma de fusão como com outros genes, como exemplo o *AGK*.[26-28] Além disso, estas alterações genéticas parecem estar associadas com a idade do paciente e podem influenciar a expressão do NIS nas células tumorais.[29] Esta poderia ser a chave para responder a perguntas de o porquê de o CDT pediátrico se apresentar diferentemente de adultos e possuir uma resposta mais sensível ao tratamento.

2 – Objetivos

Entendendo que na faixa etária pediátrica os carcinomas diferenciados de tireoide possuem suas peculiaridades em relação à predileção por sexo e idade, possuem uma classificação de estadiamento ainda com poucos critérios prognósticos e baseados em diretrizes de adultos, que possuem um perfil molecular diferenciado e uma resposta excelente ao tratamento, foram objetivos desta tese em relação ao CDT pediátrico:

2.1 - Principal:

Descrever as alterações moleculares, a expressão do gene *SLC5A5* e a detecção da proteína NIS em células tumorais de pacientes diagnosticados na faixa etária pediátrica.

2.2 - Secundários:

a) avaliar a pesquisa de corpo inteiro com Iodo-131 como preditora de metástase à distância e de resposta ao tratamento;

b) avaliar o perfil de puberdade completa, e não a idade, como associada à predileção pelo sexo feminino, apresentação da doença e resposta ao tratamento;

c) avaliar o perfil molecular tumoral e sua associação com características clínicas, puberdade e resposta ao tratamento;

d) avaliar a detecção da proteína NIS e sua associação com as características clínicas, puberdade, perfil molecular tumoral e resposta ao tratamento; e

e) avaliar a expressão do NIS a partir do gene *SLC5A5* e sua associação com a detecção da proteína NIS, características clínicas, puberdade, perfil molecular tumoral e resposta ao tratamento.

3 – Materiais e métodos

Foram selecionados para participar do estudo os pacientes com diagnóstico de CDT, acompanhados de 1976 a 2022, no principal centro de referência de câncer do Brasil (Instituto Nacional de Câncer – INCA – Ministério da Saúde - Brasil).

Após aprovação pelo Comitê de Ética -*CEP/CONEP 2.146.715 e CAAE 66569517.8.0000.5257* (Figura 1), foram feitas buscas por pacientes pediátricos com diagnóstico de CDT contidos em registros físicos e informatizado da Instituição.

Foram inclusos os pacientes menores de 19 anos com diagnóstico histológico revisto e confirmado de CDT, com dados clínicos passíveis de interpretação e com material tumoral registrado como armazenado na Instituição. Foram excluídos aqueles com seguimento menor que 6 meses, que não desejavam participar ou com material tumoral em quantidade ou qualidade insuficiente para análise.

A puberdade foi diferenciada em 2 grupos: Grupo 1 – pacientes pré ou peri-púberes, denominados “puberdade não-completa” e Grupo 2 – pacientes pós-púberes, denominados “puberdade completa”. Para estes critérios, consideramos a categoria “puberdade completa” quando a idade ao diagnóstico de CDT fosse maior que a data de menarca em meninas ou maior/igual a 16 anos de idade em meninos.

O sistema de estadiamento utilizado foi o da ATA pediátrico,[1] padronizando os pacientes em 3 categorias de risco: Baixo, Intermediário e Alto (Tabela 1). A resposta ao tratamento foi classificada como sugerido para a ATA adulto[7], visto não haver sistema de referência para a população pediátrica. Foram apenas utilizados os padrões de Resposta Excelente e Resposta Estrutural Incompleta para fins analíticos (Tabela 2).

Em relação a avaliação da Tg, nossa instituição utilizou diferentes ensaios durante o período do estudo. A sensibilidade funcional do ensaio foi de ~1 µg/L entre 1986 e 1997 e 0,5 µg/L entre 1998 e 2001. A partir de 2001, os níveis de Tg foram medidos usando um ensaio imunométrico (*Immulite 1000 Immunoassay System; Siemens Healthcare Diagnostics Inc., Tarrytown, NY, EUA*) com sensibilidade funcional de 0,2 µg/L entre 2001 e 2014 e 0,1 µg/L após 2014.

Da mesma forma, vários ensaios anti-Tg foram utilizados durante o período do estudo. A sensibilidade funcional do ensaio foi de ~60 UI/L entre 1986 e 2009 e 20 UI/L de 2009 a 2013. A partir de 2014, os níveis séricos de anti-Tg foram medidos

usando um ensaio de eletroquimioluminescência com sensibilidade funcional de 10 UI/L.

A PCI foi realizada no Serviço de Medicina Nuclear da Instituição após administração de Iodo-131 e interpretada por profissionais treinados e com experiência no diagnóstico de câncer de tireoide. Doses de 2 mCi foram utilizadas para PCI-diagnóstica. Doses entre 30 e 200 mCi foram então administradas quando a RIT foi necessária, com a atividade individual definida por um conselho multidisciplinar institucional com base no estadiamento pós-operatório inicial do câncer. PCI pós-tratamento foi realizado entre 3 e 10 dias após Iodo-131 e usado como padrão ouro de imagem para a detecção de doença residual e metastática. Não foi possível determinar o número de pacientes com metástases cervicais por esse método porque o tecido de captação na imagem poderia representar o remanescente da tireoide, neste caso a ultrassonografia do pescoço foi usada como padrão ouro.

A amostra das células tumorais foi obtida a partir do tecido tumoral primário originário da cirurgia de tireoidectomia, fixada em formalina e armazenada em blocos de parafina (FFPE) que estavam registrados no Serviço de Patologia da Instituição, tomando-se o cuidado para que não houvesse o esgotamento do material no momento do processamento das análises propostas.

Os estudos de avaliação molecular do CDT, de detecção da proteína NIS e de expressão do gene *SLC5A5* foram realizados em momentos diferentes, de maneira independente e “às cegas”, a partir da transformação da identificação das amostras em números, para que os resultados encontrados não pudessem influenciar os examinadores dos diversos processos. Somente um membro da equipe poderia decodificar os resultados atribuídos à avaliação de um determinado paciente.

A avaliação molecular dos CDT pediátrico se deu a partir da extração e fragmentação de RNA de amostras tumorais FFPE, sendo anteriormente avaliadas por um patologista para a seleção da seção tumoral mais apropriada para extração. Em seguida foi realizada a identificação molecular específica, captura e enriquecimento da região-alvo com kit customizado. O sequenciamento de nova geração (NGS) foi realizado por meio da plataforma NextSeq500 (Illumina). Em seguida, foi realizado o alinhamento das sequências e detecção de variantes com base na versão do genoma *GRCh37*, utilizando o *pipeline* de bioinformática desenvolvido e validado internamente versão 3.6. O critério mínimo de qualidade do

sequenciamento exigiu média de cobertura maior ou igual a 200X. Foram reportadas fusões gênicas detectadas com um score maior ou igual a 0,90. A classificação de patogenicidade das fusões foi realizada pelo software de inteligência artificial *Franklin by Genoox*. Os seguintes genes foram analisados: *ABL1, AKT3, ALK, AXL, ARID1A, BRAF, EGFR, ERG, ESR1, ETV1, ETV4, ETV5, ETV6, EWSR!, FGFR1, FGFR2, FGFR3, FUS, JAK2, KIF5B, KMT2A, MAST1, MET, MSH2, MYH11, NOTCH1, NOTCH2, NR4A3, NGR1, NTRK1, NTRK2, NTRK3, PAX3, PBX1, PDGFRA, PDGFRB, PIK3CA, PPARG, RAF1, RARA, RET, ROS1, RSPO2, RSPO3, RUNX1, TAF15, TERT, TFE3, TMPRSS2*.

Para a avaliação da expressão do gene *SLC5A5*, o RNA foi extraído de amostras FFPE, utilizando uma seção de 10 µm por amostra, com kit *Purelink FFPE RNA* (*Ivitrogen*) seguindo as instruções fornecidas pelo fabricante. As amostras de RNA foram quantificadas no *Nanodrop* (*Thermo*), avaliando as razões de pureza 260 nm/280 nm e 260 nm/230 nm. O cDNA foi sintetizado a partir de 1,0 µg de RNA de cada amostra pela *Transcriptase Reversa SuperScript IV* (*Invitrogen*). O PCR quantitativo (qPCR) foi realizado em um volume de reação de 10,0 µL com o kit *Quantinova SYBR Green PCR* (*Qiagen*) em um termociclador *Rotor-Gene* (*Qiagen*) em 45 ciclos. As amostras foram analisadas em triplicata, utilizando 1,0 mcL de cDNA por reação. A expressão gênica de NIS foi pelo método ΔCt usando *GAPDH* como um gene constitutivo para quantificação relativa. Amostras que apresentaram amplificação de NIS com Ct ≥ 40 ou que não apresentaram curva de amplificação foram consideradas negativas. Amostras sem amplificação de *GAPDH* foram excluídas. As sequências de primers utilizadas neste projeto foram: NIS-F 5’ ACCTCATCAAACCTCGGCTG-3’; NIS-R 5'-GATCCGTAGATGAGTGAGAGC-3'; GAPDH-F 5'-CAACAGCCTCAAGATCATCAGCAA-3'; GAPDH-R 5' AGTGATGGCATGGACTGTGGTCAT-3'.

Para a avaliação da detecção da proteína NIS, a técnica de imuno-histoquímica (IHQ) foi realizada em dois dias consecutivos. Lâminas comerciais, previamente tratadas com preenchedores (*immunoSlide-Easy Path*) contendo cortes de 3 mícrons, foram imersas em 3 banhos de 5 minutos em xilol, seguidos de banhos rápidos em álcool 100%, 90%, 80% e 70%. O excesso de álcool foi retirado em água corrente por 3 minutos. A recuperação antigênica foi realizada em *Tampão Trilogy* (*Cell Marque*), à temperatura de 98°C, utilizando o processo a vapor, por 30 minutos. O bloqueio da peroxidase e o bloqueio proteico foram realizados utilizando

o kit *NovoLink Max Polymer Detection*, (*Leica Microsystems*), durante 5 minutos cada. A incubação com anticorpo policlonal de coelho NIS numa diluição de 1:2800 foi realizada durante a noite, no frigorífico. No segundo dia da técnica, as lâminas foram incubadas com o anticorpo pós-primário e com o polímero (*Novolink*), ambos por 30 minutos. Para revelar a reação, utilizou-se o cromogênio DAB por 3 minutos. A contrastação foi realizada com hematoxilina por 30 segundos. Foram utilizados o DAB do kit *Novolink* e a Hematoxilina *Haris*. Após a retirada do excesso de hematoxilina em água corrente, as lâminas foram imersas em banhos de álcool 70%, 80%, 90%, 100% e xilol. O bálsamo foi utilizado para montagem das lâminas, que foram analisadas em microscópio óptico, observando coloração citoplasmática/membrana (NIS).

Todos os dados coletados e resultados foram plotados no programa *Excel v.365* (*Micorsoft, Redmond, Washington, USA*). A análise estatística foi avaliada a partir do programa *GraphPad Prism 8.0 (Graphpad Software Inc., San Diego, CA, USA*). Variáveis categóricas foram analisadas por frequência absoluta e relativa. Variáveis contínuas foram avaliadas quanto à normalidade (paramétricas) e descritas como média/mediana, desvio-padrão, valores mínimo e máximo e intervalos. Teste de significância estatística das variáveis contínuas foi obtido pelo *Teste t de Student* em caso de dados não paramétricos ou pelo *One-Way ANOVA* quando distribuição normal; *Qui-quadrado* (x^2) ou *Teste Exato de Fischer* foi realizado para as variáveis categóricas. Um p valor $\leq 0,05$ foi considerado estatisticamente significativo.

4 - Resultados

Nesta tese são apresentados os resultados do estudo na forma de 3 artigos que se propuseram avaliar o CDT pediátrico levando em consideração a PCI como preditora do encontro de metástase à distância e a associação com a resposta ao tratamento e a avaliação da detecção e expressão do NIS associada a fatores clínicos e moleculares peculiares à população infanto-juvenil.

4.1 - Artigo 1

Endocrine

Stimulated thyroglobulin and diagnostic 131-iodine whole-body scan as a predictor of distant metastasis and association with response to treatment in pediatric thyroid cancer patients

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Corresponding Author:	Fernanda Vaisman, MD,PhD Instituto Nacional de Cancer BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Instituto Nacional de Cancer
Corresponding Author's Secondary Institution:	
First Author:	Paulo Alonso Garcia Alves-Junior
First Author Secondary Information:	
Order of Authors:	Paulo Alonso Garcia Alves-Junior
	Marise Codeço de Andrade Barreto
	Fernanda Aciolly de Andrade
	Daniel Alves Bulzico
	Rossana Corbo
	Fernanda Vaisman, MD,PhD
Order of Authors Secondary Information:	
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Abstract:	Introduction: Differentiated thyroid carcinoma (DTC) is a rare oncological disease in the pediatric population, presenting with a more aggressive form. Stimulated thyroglobulin (sTg) and the 131-iodine whole-body scans (WBSs) are known adult markers related to the presence of distant metastasis. Little is known about their roles in the pediatric population. Purpose: To evaluate sTg levels and diagnostic WBS (DxWBS) as predictors of distant metastasis after thyroidectomy and to correlate with the response to treatment at the end of follow-up in pediatric DTC. Materials and methods: Patients under 19 years old diagnosed with DTC from 1980 to 2022 were retrospectively evaluated. sTg values and WBS were assessed after thyroidectomy and prior radioiodine treatment (RIT) and correlated with the possibility of finding distant metastasis and response to treatment at the end of follow-up. Results: In a total of 142 patients with a median age of 14.6 (4-18) years who were followed for 9.5 ± 7.2 years and classified according to the ATA risk of recurrence as low (28%), intermediate (16%), and high risk (56%), 127 patients had their sTg evaluated. A sTg value of 21.7 ng/dl yielded a sensitivity of 88% compared to 30% for DxWBS in predicting distant metastasis. Specificity was 100% and 60% respectively. 42% of patients obtained discordant results between DxWBS and RxWBS. In high-risk patients, sTg levels were particularly able to differentiate those who would have distant metastasis with better diagnostic accuracy than the WBSs.

	Conclusions: The sTg level had better performance in detecting distant metastases in pediatric DTC than the DxWBS. DxWBS's low performance suggests that caution should be taken in interpreting their findings in terms of the underdiagnosis for metastatic disease, especially when the sTg level already suggests distant disease.
Suggested Reviewers:	Rafael Scheffel Universidade Federal do Rio Grande do Sul rscheffe@gmail.com expert in the field
	Maria Isabel Cordiolli isabelcordioli@gmail.com expert in the field

Stimulated thyroglobulin and diagnostic 131-iodine whole-body scan as a predictor of distant metastasis and association with response to treatment in pediatric thyroid cancer patients

Paulo Alonso Garcia Alves-Junior[1,2],M.Sc., Marise Codeço de Andrade Barreto[1,2], M.D, Fernanda Aciolly de Andrade[1],M.Sc., Daniel Alves Bulzico[1],Ph.D., Rossana Corbo[1],Ph.D., Fernanda Vaisman[1,2],Ph.D

[1]Endocrinology Service, Instituto Nacional do Cancer, INCA, Rio de Janeiro, RJ Brazil

[2]Facudade de Medicina, Federal University of Rio de Janeiro – UFRJ, Rio de Janeiro, RJ, Brazil

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Corresponding author

Fernanda Vaisman, vaismanfe@gmail.com, phone: +552125126474

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Stimulated thyroglobulin and diagnostic 131-iodine whole-body scan as a predictor of distant metastasis and association with response to treatment in pediatric thyroid cancer patients

Abstract

Introduction: Differentiated thyroid carcinoma (DTC) is a rare oncological disease in the pediatric population, presenting with a more aggressive form. Stimulated thyroglobulin (sTg) and the 131-iodine whole-body scans (WBSs) are known adult markers related to the presence of distant metastasis. Little is known about their roles in the pediatric population.

Purpose: To evaluate sTg levels and diagnostic WBS (DxWBS) as predictors of distant metastasis after thyroidectomy and to correlate with the response to treatment at the end of follow-up in pediatric DTC.

Materials and methods: Patients under 19 years old diagnosed with DTC from 1980 to 2022 were retrospectively evaluated. sTg values and WBS were assessed after thyroidectomy and prior radioiodine treatment (RIT) and correlated with the possibility of finding distant metastasis and response to treatment at the end of follow-up.

Results: In a total of 142 patients with a median age of 14.6 (4-18) years who were followed for 9.5 ± 7.2 years and classified according to the ATA risk of recurrence as low (28%), intermediate (16%), and high risk (56%), 127 patients had their sTg evaluated. A sTg value of 21.7 ng/dl yielded a sensitivity of 88% compared to 30% for DxWBS in predicting distant metastasis. Specificity was 100% and 60% respectively. 42% of patients obtained discordant results between DxWBS and RxWBS. In high-risk patients, sTg levels were particularly able to differentiate those who would have distant metastasis with better diagnostic accuracy than the WBSs.

Conclusions: The sTg level had better performance in detecting distant metastases in pediatric DTC than the DxWBS. DxWBS's low performance suggests that caution should be taken in interpreting their findings in terms of the underdiagnosis for metastatic disease, especially when the sTg level already suggests distant disease.

Keywords: differentiated thyroid cancer, pediatric thyroid carcinoma, whole-body scan, radioiodine therapy, thyroglobulin

Introduction

Pediatric differentiated thyroid carcinoma (DTC) is a type of malign tumor that is found in about 3% of all pediatric cancer cases and accounts for 2.3% of thyroid cancer diagnoses with an increase in its prevalence in recent years.[1-4] It is considered the most common endocrine tumor in children, with Brazil having the highest incidence among South and Central American countries. In Brazil, the incidence rate is 0.51 per million for children aged 0-9 years, 4.4 per million for those aged 10-14 years, and 17.5 per million for adolescents aged 15-19 years.[5]

Compared to adult DTC, pediatric cases have distinct clinical features. Despite an excellent better prognosis, children and adolescents present with more aggressive behavior with a higher likelihood of nodal and distant metastasis, therefore, most of them will require adjuvant therapy with radioiodine therapy (^{131}Iodine) to control the disease.[6-11]

Accurately cancer staging is crucial in determining the most effective treatment and predicting the patient's prognosis,[12] with The *American Joint Committee on Cancer* (AJCC) - 8th edition being the standard system to be used in DTC. As there is currently no validated staging system for pediatric DTC, we rely on the *American Thyroid Academy* (ATA) guidance published in 2015[8] that considers tumor size, lymph node involvement, and distant metastases to categorize patients as low, intermediate, or high-risk for disease recurrence. This classification based on recurrence risk helps in developing personalized treatment plans, recommends adjuvant therapy when necessary, and determines appropriate follow-up care.

Thyroglobulin (Tg) is a protein that is produced by thyroid follicular cells under the influence of thyrotropin (TSH). It can serve as a tumor marker in patients who have undergone thyroidectomy, as it is still produced by well-differentiated tumor

cells. Despite this, Tg is not currently included in the postoperative staging system. Stimulated thyroglobulin (sTg) is represented by serum levels obtained after TSH increased to more than 30 mIU/L and has a higher predictive value than basal Tg for the presence of disease after initial treatment in adults,[7,14-16] but few studies have demonstrated its predictive ability in children and adolescents.[17,18]

Another tool for detecting tumor disease is the use of imaging tests. Whole-body scanning (WBS) with radioactive iodine is one of the primary imaging methods utilized for detecting thyroid tumors. Furthermore, the presence of iodine-uptake tissue on WBS suggests the possibility of a disease that could be treated with adjuvant radioiodine therapy (RIT). The pediatric ATA guideline[8] recommends a WBS after thyroidectomy and before RIT (diagnostic WBS – DxWBS) to identify distant or residual disease and define RAI treatment and activity.

The current pediatric guidelines for evaluating distant metastasis using sTg and WBS with radioactive iodine are based on adult populations findings and it is not clear if this approach is applicable to children and adolescents' patients with DTC. Therefore, this study aims to investigate the effectiveness of sTg and DxWBS in detecting distant metastasis in pediatric patients with DTC.

Materials and methods

Patients diagnosed with DTC and followed up from 1980 to 2022 at the main cancer referral center in Brazil (Instituto Nacional de Cancer - INCA) were selected to participate in the study.

After approval by the local Ethics Committee (CEP/CONEP 2.146.715 and CAAE 66569517.8.0000.5257), the clinical, histopathological, laboratory, and

imaging data stored in the physical and computerized records of the institution were retrospectively searched.

Patients younger than 19 years of age who were submitted to thyroidectomy with a histological diagnosis of DTC and available clinical, laboratory, and imaging data amenable to interpretation were included in the study. Those with a follow-up of less than 6 months, those who did not wish to participate or those who did not sign the informed consent form were excluded.

The levels of TSH (mIU/L) and Tg (ng/ml) were evaluated by electrochemiluminescence. Antithyroglobulin antibodies were measured by chemiluminescence and categorized as present or absent based on the reference values of the kit used on the processing date. sTg was considered when TSH levels were > 30 mIU/L in the same sample. For this strategy, patients were asked to stop the use of levothyroxine for at least 21 days or were administered recombinant human TSH (*Thyrogen* 1.1 mg/ampoule) intramuscularly, 24 and 48 hours prior to measurement, with a dose of 0.55 mg in children younger than 10 years and 1.1 mg in children aged 10 years and older (local protocol), to achieve TSH values > 30 mIU/L. An iodine-free diet was instituted for 30 days before the procedure. To this study, we consider the sTg measurements performed after thyroidectomy and immediately before or on the same day of RIT.

WBS was performed in the Nuclear Medicine Department of the institution following administration of ^{131}I and interpreted by trained professionals experienced in the diagnosis of thyroid cancer. Doses of 2 mCi were used for DxWBS. Doses between 30 and 200 mCi were then administered when RIT was necessary with the individual activity defined by an institutional multidisciplinary tumor board based on the initial postoperative cancer staging (pediatric ATA guidance). RIT posttreatment

WBS (RxWBS) was performed between 3 and 10 days after ^{131}I and used as an image gold standard for the detection of residual and metastatic disease. It was not possible to determine the number of patients with cervical metastasis using this method because the uptake tissue on the image could represent the thyroid remnant instead, for this case neck ultrasound was used as the gold standard.

The risk of recurrence was evaluated as published by ATA Pediatric Guideline with Low Risk for those with N0/Nx or incidental N1a disease, Intermediate Risk with extensive N1a or minimal N1b disease, and High Risk for those with regionally extensive disease or locally invasive disease, with or without metastasis. Response to therapy was also based on ATA-published Guideline, with excellent response for those with negative imaging and Tg under 0.2 ng/ml at the end of follow-up.

All data were plotted in Microsoft Excel v.16.77.1 (Microsoft, Redmond, Washington, USA). Statistical analysis was performed using Statistical Package for Social Science (SPSS) v.23.0 for Windows (IBM, Chicago, Illinois, USA). Categorical variables were described using relative frequency while continuous variables were described using the median, standard deviation, minimum and maximum values, and intervals. Statistical significance was assessed for continuous variables using Student's T test or the Mann–Whitney U test; the chi-square (χ^2) test was performed for categorical variables. A p value 0.05 was considered to indicate statistical significance. The receiver operating characteristic (ROC) curve was used to select the best sTg cutoff point for predicting distant metastasis. Sensitivity, specificity, and positive and negative predictive values were calculated for each detection method and compared to advocate the best predictor.

Results

From a total of 165 patients registered at the Institution with a diagnostic of pediatric DTC, 142 met the inclusion eligibility criteria. Table 1 shows the characteristics of the studied population.

Ninety-six percent had papillary DTC, with a median tumor size of 2.7 cm. Seventy-three percent of the patients presented with metastasis to lymph nodes and 25% with distant metastasis, all in the lung region. No metastases to sites other than the cervical or thoracic sites were detected. According to the pediatric *ATA* criteria, more than half were classified as having a high risk of persistence or recurrence of the disease. Nevertheless, approximately half of the patients had no evidence of tumor at the end of follow-up (median 9.5 years).

Of the 142 patients, 127 underwent ablative/adjuvant treatment (RIT) with an initial mean ^{131}I dose of 150 mCi and a maximum cumulative dose of 950 mCi. 15 patients classified as low risk for recurrence did not undergo RIT and were therefore excluded from sTg and WBS analysis.

sTg level evaluated after thyroidectomy during preparation for RIT showed a median value of 30 ng/dl for the entire cohort. When evaluating the sTg values differentiated by *ATA* pediatric DTC risk, a significant difference was observed between those at high risk and those at the other risk levels (Table 2).

When comparing the sTg values with the response to treatment at the end of follow-up, those with excellent response had a median sTg level approximately 3 times lower than that of patients with the other response levels (13 vs 48.9 ng/dl, respectively), and this difference was statistically significant (Table 3). Table 3 also analyzes the performance of sTg and DxWBS stratified by ATA risk group for disease persistence/recurrence.

114 patients underwent imaging for evaluation after RIT - RxWBS (13 patients did not return for RxWBS), of which 66 also were submitted to DxWBS previously. Among the 114 patients who underwent RxWBS, 25.4% had lung metastases (Table 4).

When comparing those who underwent DxWBS with RxWBS, there was agreement in only 38 patients (57% of the patients), with RxWBS revealing a greater number of tumor-affected sites not detected on DxWBS. Fourteen (50%) of the 28 patients with Dx and RxWBS disagreement required new treatments with RIT.

Table 5 compares the ability of diagnostic sTg and DxWBS to detect distant metastasis. DxWBS achieved a specificity of 100% but the sensitivity was only 30%. Figure 1 shows the sTg ROC curve and the ability to detect distant metastasis. A sTg value of 21.7 ng/dl proved to be the best cutoff point balancing the test performance metrics, conferring a sensitivity of 88% and specificity of 22%.

Discussion

As DTC is the most prevalent endocrine tumor and has demonstrated an increasing incidence in recent years,[1] many studies have addressed this disease, but few have included children and adolescents. Until 2022, there is only 2015 *ATA* pediatric guideline[8] with the goal of standardizing diagnostic and treatment behaviors worldwide but using, most of the time as support, studies with adult populations. A new pediatric guideline was raised in 2022, this time from Europe [9], bringing new questions but maintaining basically the most known recommendations for the management of these patients due to the rarity of pediatric DTC studies. With this new study, we were able to expand knowledge about pediatric DTC, this time

evaluating that, although sTg and WBS are good methods to detect thyroid metastases as in adults, they must be individualized for children and adolescents.

Here we suggest a post-thyroidectomy sTg cutoff point for discriminating this population and correlating with the ATA pediatric recurrence risk[8] and with the response to treatment at the end of follow-up. We also brought an alert about the indication of DxWBS in managing metastatic cases.

Pediatric DTC has been well demonstrated as presenting with a unique genetic signature leading to a different presentation, behavior, and response to treatment from adult DTC. Rearrangement mutations are a notable difference, which could be the factor related to the more aggressive presentation and higher rates of regional and distant metastases but an extremely low mortality.[20-24] Our previous research showed that the infant and juvenile populations should be evaluated individually and suggested molecular, immunohistological, and clinical markers that could contribute to the optimization of the pediatric DTC approach.[25-28]

One of the main findings of this study was the Tg as a marker of distant metastasis where a sTg cutoff of 21.7 ng/ml could predict distant disease with a sensitivity of 94%, and therefore, being an important factor that should be considered as a recommendation for adjuvant therapy (RIT). *ATA* pediatric Guidelines suggest a sTg cutoff of 10 ng/ml for RIT, despite a potentially negative WBS. We believe that higher values are more suitable for the presence of metastases in the pediatric population since cutoff values of 10 ng/ml were based on studies that included the adult population.

Cistaro et al.[29] demonstrated in their study that sTg values correlated with the treatment response one year after RIT. In their study, sTg values of 4.4 ng/dl were related to an excellent response, while values of 52.5 ng/dl were related to a no

excellent response pattern, and an sTg value of 27.2 ng/dl was found to best predict the treatment response. In our study, we also observed an association between the sTg values and the response to treatment, but we have a longer follow-up (approximately 9.5 years). Nevertheless, a very similar sTg value (48.9 ng/dl) was related to a no-excellent response at the end of the follow-up. Thus, we understand that sTg is an important prognostic factor in pediatric DTC and must be used as a predictor tool with the appropriate cutoff.

Radioactive iodine-based WBS is an important and well-established imaging method used for both the detection and treatment of DTC-related metastatic disease.[19] Because most tumor cells maintain the surface expression of the sodium-iodide symporter, the use of I^{131} is an important theragnostic tool.[30-32]

In our institution, DxWBS was used as an imaging method to stage DTC pediatric patients until mid-2014, after which it was reserved only for individual cases. At that time, subjectively, we were already noticing discrepancies between Dx- and Rx-WBS. Through this study, we observed that although the DxWBS correctly predicted distant metastasis (100% positive predictive value), we were able to statistically confirm our suspicion of its low sensitivity, with a negative predictive value (NPV) of 74%. We emphasize this finding since the 2015 *ATA* pediatric Guideline recommends the use of DxWBS for adjuvant therapy.

When analyzing our data more carefully, we observed that in 66 patients who underwent DxWBS, disagreement with RxWBS was found in 42%; that is, DxWBS was unable to detect metastases that RxWBS detected. In 14 of 28 patients with disagreement Dx vs. RxWBS, a new therapeutic dose was required during follow-up due to the detection of structural iodine-sensitive disease. Bravo et al.[33] in 2013 had

already demonstrated this discrepancy, inferring the low relevance of DxWBS for patients with a low probability of distant disease.

Taking this into consideration, we imagine that in patients with metastatic disease, the ^{131}Iodine low dose used for DxWBS (generally 2 mCi) is very dispersed through a lot of metastatic tissue, making the detection performance ability unfeasible.

We next assessed the performance of sTg and DxWBS alone and in combination for the detection of distant metastasis. DxWBS alone was more specific than sTg, which in contrast showed better sensibility with a cutoff of 21.7 ng/ml. Despite this, the low performance in terms of sensitivity and NPV of DxWBS prevents us from recommending this method to advocate RIT. The use of the two methods together had a worse performance than individually. Thus, we alert that DxWBS in pediatric DTC may lead to an underdiagnosis of distant metastasis when sTg is already suggestive. Pacini et al.[34] also reported that there was no need for DxWBS in patients with low sTg levels. We were unable to find studies that jointly evaluated sTg and WBS as markers of distant metastasis in pediatric DTCs.

Among the limitations of this study are those inherent to all retrospective evaluations in addition to the limited number of patients, but this fact is conditioned by a rare disease and few studies about the subject. Few patients underwent DxWBS, with the majority in the past, but this did not prevent us from making the necessary statistical assessments. sTg values are influenced by TSH and Tg-Ab levels, but these factors were not significantly different between the studied groups. sTg must also be influenced by residual non-tumoral tissue, so patients who underwent low-risk stratification and who were not submitted to RIT could have higher titers not being a tumor marker. Because of that, we took care to exclude

these patients from sTg evaluation. We recommend additional studies on pediatric DTC that can validate our findings to build a consensus that is not extrapolated from data from the adult population.

Conclusions

sTg outperformed DxWBS in detecting distant metastases in pediatric DTC and could also be used as a prognostic factor of the treatment response if used with a specific pediatric cutoff. The disagreement between DxWBS and RxWBS suggests that their findings should be interpreted with caution in terms of the underdiagnosis and the possibly related undertreatment of metastatic disease, especially when sTg already suggests distant disease.

References

1. SEER. Cancer stat facts: thyroid cancer. Available at: https://seer.cancer.gov/statfacts/html/thyro.html.

2. Bernier MO, Withrow DR, Berrington de Gonzalez A, et al. Trends in pediatric thyroid cancer incidence in the United States, 1998-2013. Cancer. 2019;125(14):2497-2505.

3. Balmant NV, De Souza Reis R, De Oliveira Santos M, et al. Rare cancers in childhood and adolescence in Brazil: first report of data from 19 population-based cancer registries. *Cancer.* 2019;125:2638-2646.

4. Miranda-Filho A, Lortet-Tieulent J, Bray F, et al. Thyroid cancer incidence trends by histology in 25 countries: a population-based study. Lancet Diabetes Endocrinol. 2021 Apr;9(4):225-234.

5. De Souza Reis R, Gatta G, De Camargo B. Thyroid carcinoma in children, adolescents, and young adults in Brazil: a report from 11 population-based cancer registries. *PLoS One.* 2020;15:e0232416.

6. Thiesmeyer JW, Egan CE, Greenberg JA, et al. Prepubertal children with papillary thyroid carcinoma present with more invasive disease than adolescents and young adults. *Thyroid.* 2023;33:214-222.

7. Haugen BR, Alexander EK, Bible KC, et al. 2015 American thyroid association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American thyroid association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid.* 2016;26:1-133.

8. Francis GL, Waguespack SG, Bauer AJ, et al. Management guidelines for children with thyroid nodules and differentiated thyroid cancer. *Thyroid.* 2015;25:716-759.

9. Lebbink CA, Links TP, Czarniecka A, et al. 2022 European thyroid association guidelines for the management of pediatric thyroid nodules and differentiated thyroid carcinoma. *Eur Thyroid J.* 2022;11:e220146.

10. Nies M, Vassilopoulou-Sellin R, Bassett RL, et al. Distant metastases from childhood differentiated thyroid carcinoma: clinical course and mutational landscape. *J Clin Endocrinol Metab.* 2021;106:e1683-e1697.
11. Parisi MT, Eslamy H, Mankoff D. Management of differentiated thyroid cancer in children: focus on the American thyroid association pediatric guidelines. *Semin Nucl Med.* 2016;46:147-164.

12. Amin MB, Greene FL, Edge SB, et al. The eighth edition AJCC cancer staging manual: continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. *CA Cancer J Clin.* 2017;67:93-99.

13. Huang SH, O'Sullivan B. Overview of the 8th edition TNM classification for head and neck cancer. *Curr Treat Options Oncol.* 2017;18:40.

14. Webb RC, Howard RS, Stojadinovic A, et al. The utility of serum thyroglobulin measurement at the time of remnant ablation for predicting disease-free status in patients with differentiated thyroid cancer: a meta-analysis involving 3947 patients. *J Clin Endocrinol Metab.* 2012;97:2754-2763.

15. Chou R, Dana T, Brent GA, et al. Serum thyroglobulin measurement following surgery without radioactive iodine for differentiated thyroid cancer: a systematic review. *Thyroid.* 2022;32:613-639.

16. Campennì A, Ruggeri RM, Siracusa M, et al. Early preablation rhTSH-stimulated thyroglobulin predicts outcome of differentiated thyroid cancer (DTC) patients. *Eur J Nucl Med Mol Imaging.* 2021;48:2466-2475.

17. Livhits MJ, Pasternak JD, Xiong M, et al. Pre-ablation thyroglobulin and thyroglobulin to thyroid-stimulating hormone ratio may be associated with pulmonary metastases in children with differentiated thyroid cancer. *Endocr Pract.* 2016;22:1259-1266.

18. Tian T, Jiang L, Zhang X, et al. Association between clinical and tumor features with postoperative thyroglobulin in pediatric papillary thyroid cancer. *Surgery.* 2020;168:1095-1100.

19. Gulec SA, Ahuja S, Avram AM, et al. A joint statement from the American thyroid association, the European association of nuclear medicine, the European thyroid association, the society of nuclear medicine and molecular imaging on current diagnostic and theranostic approaches in the management of thyroid cancer. *Thyroid.* 2021;31:1009-1019.

20. Guleria P, Srinivasan R, Rana C, et al. Molecular landscape of pediatric thyroid cancer: a review. *Diagnostics (Basel).* 2022;12:3136.

21. Gallant JN, Chen SC, Ortega CA, et al. Evaluation of the molecular landscape of pediatric thyroid nodules and use of a multigene genomic classifier in children. *JAMA Oncol.* 2022;8:1323-1327.

22. Franco AT, Ricarte-Filho JC, Isaza A, et al. Fusion oncogenes are associated with increased metastatic capacity and persistent disease in pediatric thyroid cancers. *J Clin Oncol.* 2022;40:1081-1090.

23. Mollen KP, Shaffer AD, Yip L, et al. Unique molecular signatures are associated with aggressive histology in pediatric differentiated thyroid cancer. *Thyroid.* 2022;32:236-244.

24. Paulson VA, Rudzinski ER, Hawkins DS. Thyroid cancer in the pediatric population. *Genes (Basel).* 2019;10:723.

25. Rangel-Pozzo A, Sisdelli L, Cordioli MIV, et al. Genetic landscape of papillary thyroid carcinoma and nuclear architecture: an overview comparing pediatric and adult populations. *Cancers (Basel).* 2020;12:3146.

26. Da Silva Breder JRA, Alves PAG, Araújo ML, et al. Puberty and sex in pediatric thyroid cancer: could expression of estrogen and progesterone receptors affect prognosis? *Eur Thyroid J.* 2022;11:e210090.

27. Sisdelli L, Cordioli M, Vaisman F, et al. AGK-BRAF is associated with distant metastasis and younger age in pediatric papillary thyroid carcinoma. *Pediatr Blood Cancer.* 2019;66:e27707.

28. Vaisman F, Bulzico DA, Pessoa CH, et al. Prognostic factors of a good response to initial therapy in children and adolescents with differentiated thyroid cancer. *Clinics (Sao Paulo).* 2011;66:281-286.

29. Cistaro A, Quartuccio N, Garganese MC, et al. Prognostic factors in children and adolescents with differentiated thyroid carcinoma treated with total thyroidectomy and RAI: a real-life multicentric study. *Eur J Nucl Med Mol Imaging.* 2022;49:1374-1385.

30. Choudhury PS, Gupta M. Differentiated thyroid cancer theranostics: radioiodine and beyond. *Br J Radiol.* 2018;91:20180136.

31. Tavares C, Coelho MJ, Eloy C, et al. NIS expression in thyroid tumors, relation with prognosis clinicopathological and molecular features. *Endocr Connect.* 2018;7:78-90.

32. Kogai T, Brent GA. The sodium iodide symporter (NIS): regulation and approaches to targeting for cancer therapeutics. *Pharmacol Ther.* 2012;135:355-370.

33. Bravo PE, Goudarzi B, Rana U, et al. Clinical significance of discordant findings between pre-therapy ^{123}I and post-therapy ^{131}I whole body scan in patients with thyroid cancer. *Int J Clin Exp Med.* 2013;6:320-333.

34. Pacini F, Capezzone M, Elisei R, et al. Diagnostic 131-iodine whole-body scan may be avoided in thyroid cancer patients who have undetectable stimulated serum Tg levels after initial treatment. *J Clin Endocrinol Metab.* 2002;87:1499-1501.

Figure

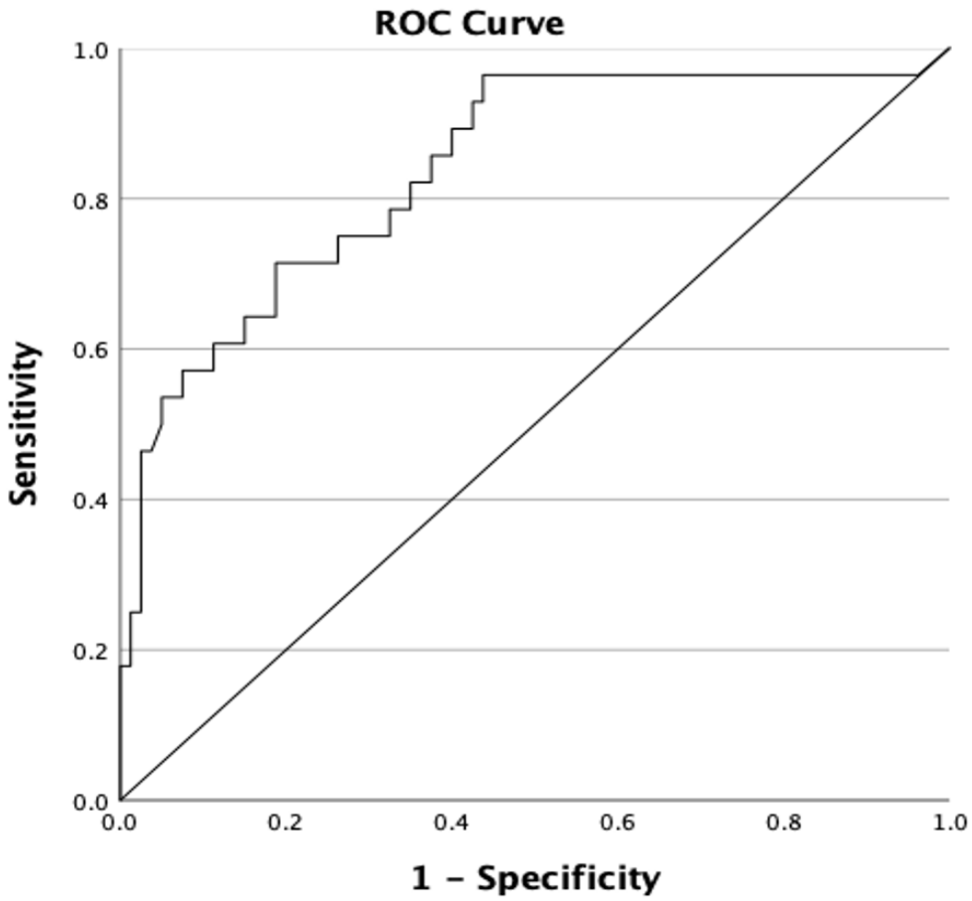

Figure 1. ROC curve of sTg in the detection of distant metastasis after thyroidectomy (sTg cutoff value 21.7 ng/ml: sensitivity 88%, specificity 60%)

Tables

Table 1. General characteristics of the population

	N or Med.	% or SD	MIN	MAX.
NUMBER OF PARTICIPANTS	142			
TIME OF FOLLOW-UP (YEARS)	9.5	± 7.2	0.6	30.9
SEX AT BIRTH				
FEMALE	102	72%		
MALE	40	28%		
AGE AT DIAGNOSIS (YEARS)	14.6	± 3.63	4.7	18.8
4-7	11	8%		
8-14	46	32%		
15-18	85	60%		
HISTOLOGICAL TYPE				
PAPILLARY	137	96%		
FOLLICULAR	5	4%		
TUMOR SIZE (CM)	2.7 (130)	± 1.6	0.5	11
METASTASIS TO LYMPH NODES	96	73%		
Nx	11	7%		
N0	35	25%		
N1a	35	25%		
N1b	61	43%		
DISTANT METASTASIS	35	25%		
ATA PEDIATRIC RISK CLASSIFICATION				
LOW RISK	39	27%		
INTERMEDIATE RISK	24	17%		
HIGH RISK	79	56%		
RESPONSE TO TREATMENT AT FINAL FOLLOW-UP				
EXCELLENT RESPONSE	68	49%		
INCOMPLETE BIOCHEMISTRY	12	8%		
INCOMPLETE STRUCTURAL	26	18%		
UNDETERMINED	36	25%		

N – Number of patients; Med. – median; SD – standard deviation; Min. – minimum; Max. – maximum; ATA – American Thyroid Academy.

Table 2. Evaluation of sTg according to the ATA pediatric risk criteria

ATA pediatric risk	**Low**	**Intermediate**	**High**	**p**
Median sTg value (ng/dl)	16.5 (± 31)	11.3 (± 47)	45 (± 1108)	**0.001**
Median TSH value for sTg	56.7 (± 29.4)	82 (± 34.7)	63.5 (± 32.4)	0.51
Presence of Tg-Ab	4 (10%)	8 (36%)	28 (36%)	0.89

ATA – American Thyroid Academy; sTg – stimulated thyroglobulin; Tg-Ab – antithyroglobulin antibody

Table 3. Evaluation of the performance of DxWBS and sTg in the prediction of disease persistence according to the ATA risk and post-thyroidectomy sTg evaluation according to the presence of an excellent response at the end of follow-up

ATA risk at diagnosis	**Persistence**	**No evidence of disease**	**p value**
Low risk with high sTg level Low risk with low sTg level	76.9% 80%	23.1% 20%	0.6
Intermediate risk with high sTg level Intermediate risk with low sTg level	60% 45%	40% 55%	0.49
High risk with high sTg level High risk with low sTg level	78.6% 25%	21.4% 75%	**0.002**
High risk with positive extracervical uptake on WBS High risk with positive cervical uptake only on WBS	75% 59.4%	25% 40.6%	0.6
End of follow-up	**Non-excellent response**	**Excellent response**	**p value**
Median sTg value (ng/dl)	48.9 (± 1187.9)	13 (± 135.6)	**0.004**

sTg – Stimulated thyroglobulin

Table 4. Evaluation of treatment with radioactive iodine

	N or Med.	% or SD	MIN	MAX
Patients undergoing adjuvant treatment	127	89%		
Mean dose in mCi (1st dose)	150	± 37	30	200
Mean dose in mCi (cumulative)	150	± 189.9	30	950
Patients with DxWBS	66	52%		
Patients with RxWBS	114	90%		
Patients with metastasis (on RxWBS)	29	25%		
Patients with Dx vs Rx-RIT WBS mismatch	28 (66)	42%		

N – Number of patients; Med. – Median; SD – Standard deviation; Min. – Minimum; Max. – Maximum, mCi – millicurie; RIT – Radioiodine treatment;WBS – Whole body scan; DxWBS – pre-RIT Whole-body scanning; RxWBS – Post-RIT WBS

Table 5. Evaluation of the performance of diagnostic WBS (DxWBS) and sTg in the detection of distant disease

	Sensitivity	Specificity	PPV	VPN
DxWBS	30%	100%	100%	74%
sTg > 21.7 ng/ml	88%	60%	39%	93%
DxWBS + sTg > 21.7 ng/ml	18%	62%	31%	45%

WBS – Whole-body scanning; DxWBS – Diagnostic WBS; sTg – Stimulated thyroglobulin; PPV – Positive predictive value; NPV- Negative predictive value

4.2 - Artigo 2

The Journal of Clinical Endocrinology & Metabolism

Distinct genetic profiles, puberty and sodium-iodide symporter (NIS) in pediatric differentiated thyroid carcinoma.

--Manuscript Draft--

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Article Type:	Clinical Research Article
Full Title:	Distinct genetic profiles, puberty and sodium-iodide symporter (NIS) in pediatric differentiated thyroid carcinoma.
Corresponding Author:	Fernanda Vaisman, MD, PhD Instituto Nacional de Cancer Rio de Janeiro, BRAZIL
Corresponding Author's Institution:	Instituto Nacional de Cancer
Order of Authors:	Paulo Alonso Garcia Alves Junior
	Mario Lucio Araújo
	Priscila Valverde
	Pedro Nicolau-Neto
	Sheila Coelho Soares Lima
	Luis Felipe Ribeiro Pinto
	Yasmin Paz Christiano
	Luiza Sisdelli
	Janete Maria Cerutti
	Fernanda Vaisman, MD, PhD
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Manuscript Classifications:	Thyroid; Follicular thyroid cancer; Papillary thyroid cancer; Pediatric endocrinology; Well differentiated thryoid cancer
Abstract:	Pediatric differentiated thyroid carcinoma (DTC) behaves more aggressively. The molecular landscape and puberty status appear to impact clinical scenarios and responses to treatment. The aim of this study was to evaluate the association between genetic profile and puberty with clinical presentation, response to treatment, and expression of the sodium-iodide symporter (NIS) in pediatric DTC. 97 patients under 19 years of age diagnosed with DTC were selected from the clinical presentation, puberty status, response to treatment, molecular profile, and NIS expression using immunohistochemistry. There was a difference in the proportion of males and females diagnosed with DTC according to puberty status ($p < 0.001$). Complete puberty presented with less aggressive disease (p 0.003). The molecular landscape was associated with puberty status and aggressiveness. NTRK and AGK::BRAF fusions were more frequent in patients without complete puberty, and RET/PTC and BRAF_V600E were more frequent among those with complete. NIS expression levels were low (26%) throughout the entire cohort and were not associated with puberty or molecular profiles. 36% of impubescent patients and 23% of complete puberty patients had incomplete structural responses at the end of the follow-up. In conclusion, distinct molecular genetic profiles were observed between different pubertal statuses in pediatric DTC patients. This could explain the differences seen in clinical presentations and therapeutic responses. Further studies are needed to understand the mechanisms by which these genetic alterations impact iodine uptake and responses to therapy and the role of sex hormones in this scenario.
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Distinct genetic profiles, puberty and sodium-iodide symporter (NIS) in pediatric differentiated thyroid carcinoma.

Paulo Alonso Garcia Alves Junior [1,2], Mario Lucio Araújo [3] , Priscila Valverde [3], Pedro Nicolau-Neto [4], Sheila Coelho Soares Lima [4] , Luis Felipe Ribeiro Pinto [4], Yasmin Paz Christiano [5], Luiza Sisdelli [5], Janete Maria Cerutti [5] ,Fernanda Vaisman [1,2]

1 - Endocrinology Department, Instituto Nacional de Câncer - INCA, Rio de Janeiro, Rio de Janeiro, Brazil.

2 - Endocrinology Department, Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

3 - Pathology Department, Instituto Nacional de Câncer - INCA, Rio de Janeiro, Rio de Janeiro, Brazil.

4 - Molecular Carcinogenesis Program, Instituto nacional de Câncer -INCA, Rio de Janeiro, Rio de Janeiro, Brazil.

5 - Genetic Bases of Thyroid Tumors Laboratory, Division of Genetics, Department of Morphology and Genetics, Universidade Federal de São Paulo/EPM, São Paulo SP 04039-032, Brazil

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Corresponding author: Fernanda Vaisman, Endocrinology Department, Instituto Nacional de Câncer, Praça Cruz Vermelha, 23, Centro, Rio de Janeiro, -RJ, Brazil, Phone: +552125126474, e-mai: vaismanfe@gmail.com

Abstract

Pediatric differentiated thyroid carcinoma (DTC) behaves more aggressively. The molecular landscape and puberty status appear to impact clinical scenarios and responses to treatment. The aim of this study was to evaluate the association between genetic profile and puberty with clinical presentation, response to treatment, and expression of the sodium-iodide symporter (NIS) in pediatric DTC. 97 patients under 19 years of age diagnosed with DTC were selected from the clinical presentation, puberty status, response to treatment, molecular profile, and NIS expression using immunohistochemistry. There was a difference in the proportion of males and females diagnosed with DTC according to puberty status ($p < 0.001$). Complete puberty presented with less aggressive disease (p 0.003). The molecular landscape was associated with puberty status and aggressiveness. *NTRK* and *AGK::BRAF* fusions were more frequent in patients without complete puberty, and *RET/PTC* and *BRAF_V600E* were more frequent among those with complete. NIS expression levels were low (26%) throughout the entire cohort and were not associated with puberty or molecular profiles. 36% of impubescent patients and 23% of complete puberty patients had incomplete structural responses at the end of the follow-up. In conclusion, distinct molecular genetic profiles were observed between different pubertal statuses in pediatric DTC patients. This could explain the differences seen in clinical presentations and therapeutic responses. Further studies are needed to understand the mechanisms by which these genetic alterations impact iodine uptake and responses to therapy and the role of sex hormones in this scenario.

Introduction

Differentiated thyroid carcinoma (DTC) in the pediatric population is rare (1-2) and presents in a more aggressive form due to greater lymph node involvement, distant metastases, and higher chances of persistence, despite extremely low mortality rates when compared to adults (3-6). Other intriguing characteristics particular to childhood DTC are changes in the ratio between male and female proportions (7), and the puberty process is associated with better presentation and outcomes (8-12).

An increased risk of recurrence among those patients with nodal involvement or distant metastasis is the reason for the indication of adjuvant therapy with radioactive iodine treatment (RIT) with iodine-131 (I-131) (1,13). The effectiveness of RIT depends, among other factors, on the uptake of I-131 by the tumor cell through sodium-iodide symporter (NIS) (14-16). The tumor genetic landscape seems to be associated with different expression levels of NIS, which may be one of the causes of therapeutic failure (iodorefractoriness) (17-19). There are few reports on NIS expression in pediatric DTC tumor cells (20-21).

In addition to clinical characteristics, pediatric DTC has a different molecular signature compared to adult tumors (22-23), and it could be associated not only with age but also with the puberty (24). Moreover, these factors could influence NIS expression in tumor cells and seem to be key for tailoring treatment on an individual basis (25).

Therefore, this study aimed to evaluate the molecular genetic landscape and puberty status of pediatric DTC patients and its possible associations with clinical presentation and NIS expression in tumor cells.

Materials and methods

Patients diagnosed with DTC, followed from 1976 to 2022, at the main cancer reference center in Brazil (Instituto Nacional de Câncer - INCA - Brazil) were selected to participate in the study.

After approval by the local Ethics Committee (CEP/CONEP 2.146.715 and CAAE 66569517.8.0000.5257), searches were carried out for pediatric patients (< 19 years) diagnosed with DTC in the Institution's physical and computerized medical records.

This study included a series of 165 pediatric cases seem between 1976 and 2022. Cases with a confirmed histological diagnosis of DTC, with clinical data and a formaldehyde fixed-paraffin embedded (FFPE) archived tumor sample available at the institution were selected. Those with follow-up of less than six months, who did not wish to participate, or with insufficient tumor quantity or poor quality for analysis were excluded. Therefore, the study included 97 out of 165 DTC cases.

Patients were divided into two groups based on puberty status: Group 1 - pre- or peri-pubertal patients and Group 2 – complete puberty patients. For these criteria, as we were unable to obtain standardized information on the pubertal staging of all patients, we considered the category “complete” when the age at diagnosis of DTC was greater than the date of menarche in girls or greater/equal to 16 years of age in boys.

The immunohistochemistry (IHC) analysis for NIS and molecular evaluation of the DTC were carried out at different times, independently and “blindly”. Care was taken to ensure that the material was not exhausted at the time of processing for the proposed analyses.

Molecular analysis

Among 97 cases, 59 were submitted to screening for the main pathogenic variants and fusions described in pediatric DTC.

DNA and total RNA were isolated from 30 µm thick FFPE sections and quantified. RNA from the tumor samples were submitted to specific molecular identification, capture, and enrichment of the target region. Next-generation sequencing (NGS) was performed using the NextSeq500 or NovaSeq6000 platform (Illumina)

NIS expression by immunohistochemistry analysis.

The immunohistochemistry (IHC) analysis was performed on two consecutive days. Commercial slides previously treated with fillers (immunoSlide-Easy Path) containing 3-micron sections were immersed in 3 baths of 5 minutes in xylol, followed by quick baths in 100%, 90%, 80%, and 70% alcohol. Excess alcohol was removed in running water for 3 minutes. Antigenic retrieval was performed in Trilogy Buffer (Cell Marque) at a temperature of 98°C using the steam process for 30 minutes. Peroxidase blocking and protein blocking were performed using the NovoLink Max Polymer Detection kit (Leica Microsystems) for 5 minutes each. Incubation with rabbit polyclonal against NIS at a 1:2800 dilution was carried out overnight at 4°C. On the second day of the technique, the slides were incubated with the secondary antibody for 30 minutes. Sections were further incubated with the Novolink polymer detection system for 30 minutes and, DAB chromogen for 3 minutes. Counterstaining was performed with hematoxylin (Haris) for 30 seconds. After removing excess hematoxylin in running water, the slides were dehydrated in 70%, 80%, 90%, and 100% alcohol and xylene baths. Balsam was used to assemble the slides, which were analyzed under an optical microscope to observe cytoplasmic/membrane NIS staining.

Statistical analysis

Statistical analysis was evaluated using GraphPad Prism 8.0 (Graphpad Software Inc., San Diego, CA, USA). Categorical variables were analyzed by absolute and relative frequency. Continuous variables were assessed for normality (parametric)

and are described as the mean/median, standard deviation/minimum, and maximum values and ranges. Statistical significance of continuous variables was assessed by Student's t test in case of nonparametric data or by one-way ANOVA when normally distributed. Chi-square (X^2) or Fischer's exact test was performed for categorical variables. A p value ≤ 0.05 was considered statistically significant.

Results

General features of the pediatric cases

Among the 165 patients under the age 19 diagnosed with DTC between 1976 and 2022, we identified 142 tumor samples stored at the files of the Institution. About 97 samples (68%) were selected according to the inclusion criteria described in material and methods section and presented at Figure 1. Although there were minor differences in the distribution of registered patients, there were no significant clinical differences among those 97 with analyzed tumor material and the 142 patients selected to participate. Table 1 provides more detailed information about the study populations, the subcategorization of patients by puberty status, and the results of comparative analyses.

The mean age of 97 patients at diagnosis was 14.9 ± 3.77 years. The mean follow-up period was 8.7 ± 5.9 years, and 68% of patients were female. When differentiated by puberty status, there was a predominance of males (59%) among those without complete puberty (Group 1). However, this trend was reversed in those defined as having completed puberty (Group 2), as this group had a predominance of females (84%). In relation to staging and aggressiveness data, 97% of the tumors were of the papillary type, with an average size of 2.7 ± 1.7 cm. Sixty-nine percent of patients

already had lymph node involvement at diagnosis, and 20% had distant metastases, all exclusively in pulmonary topography.

According to the *American Thyroid Association* Pediatric Guideline risk stratification system (1), over half (58%) of the patients were found to be at high risk of disease recurrence, while only 28% were at low risk and 13% at intermediate risk. However, at the end of the follow-up period, only 28% of patients had incomplete structural response, while 54% showed excellent response with no evidence of the disease. There were no records of deaths related to DTC during the follow-up period.

The assessment of puberty data revealed significant differences in sex distribution ($p < 0.001$), lymph node (p 0.02) or distant metastasis (p 0.003), risk of recurrence (p 0.01), and variation in the cumulative dose of RIT (p 0.02) between the two groups. In Group 1, there were more males and a higher rate of regional and distant metastases. Patients in Group 1 were also more likely to be classified as having a high risk of relapse/recurrence, indicating a greater aggressiveness in the presentation of the disease. Although the median cumulative dose of I-131 was the same in the two groups, the variation was different. Group 2 was subjected to lower cumulative doses.

Molecular genetic DTC sample results

Genetic alterations were indentified in 47 ou of 59 samples tested (80%). A total of 64 genetic alterations were identified, with 15 / 59 (25 %) of the samples presenting with more than one mutation in the same tumor. Eighty-four percent of positive mutations (54 / 64) were fusion-type. Among the genes with molecular changes, *RET, NTRK, PTC, BRAF, ETV, ALK, STRN*, and *AGK* were the most involved genes (Figure 2).

The *RET/PTC* fusions were the most prevalent molecular alteration, observed in 39% of positive samples (42% of the total number of patients evaluated). A total of 19

samples presented as *RET/PTC1,* and six presented as *RET/PTC3*. The NTRK fusions (*ETV6::NTRK3* and *SQSTM1::NTRK1)* were the second most common molecular change (26% of positive samples, 29% of total patients evaluated). The *ALK* fusion was found in 9% of the cases, being the *STRN* the most prevalent partner found. *PPP1R21::ALK and HMBOX1::ALK* were found in one case each.

The genetic alteration involving *BRAF* gene was found either as a AGK::BRAF fusion or BRAF_V600E point mutation, in 6% and 16 % of the cases respectively. Table 2 and Figure 2 summarizes the types and frequencies of molecular changes found in the studied population.

Some molecular DTC profiles were associated with the status of puberty. Although not statistically significant, the *RET/PTC* fusion and the *BRAF_V600E* mutation were more frequently present in Group 2 (OR 2.55 [0.74-8.26], p 0.15 and OR 4.2 [0.76-21.2], p 0.14 respectively). In contrast, *NTRK* fusions were more prevalent in Group 1, with 70% of *NTRK* fusions present in this group (OR 6.6 [1.6-24.5], p 0.005). Additionally, the *AGK::BRAF* fusions (four samples) was only observed in Group 1 (OR infinity [1.38-infinity], p 0.02). Table 2 presents a more detailed view of the molecular profile characteristics according to puberty status. Figure 3 summarize clinical and genetic findings according to puberty status in pediatric DTC.

NIS expression results

The results revealed that only 24 out of the 93 samples (26%) were positive for NIS, all of which were located on the membrane cell surface.

There was no significant difference between NIS expression and clinical presentation or puberty category. NIS expression was also not associated with different responses to treatment. The molecular landscape did not reveal different NIS expression patterns. Table 3 presents a detailed analysis of these findings.

Figure 4 summarizes all the main clinical findings of this study.

Discussion

Research on the development of tumors in children has uncovered both genetic and posttranscriptional mechanisms that can disrupt the intracellular signaling of proliferating cells (26-27). These findings may be the answer to distinct incidences and different responses to treatment of DTC patients with childhood onset than in patients with adulthood onset (28-31). In this new study focused on pediatric DTC, we highlighted the unique genetic signature associated with a more aggressive disease presentation compared to adults. We also investigated the integration of the puberty phenomenon with the molecular landscape and its role in modifying aggressiveness and sex frequency without interfering with NIS expression or mortality.

Studies on DTC have shown that the disease presents differently in children than in adults, particularly regarding its clinical presentation and mortality rates. In children and adolescents, DTC tends to be more aggressive and has a higher likelihood of regional and distant metastases. However, despite these characteristics, the mortality rate remains extremely low (1-7).

An interesting fact about DTC is the predilection for the female sex, which is different from most oncological diseases (32). When we examine the relationship between sexes more closely, we find that the proportion of males and females diagnosed before the age of 10 is almost identical. There are almost six times more women diagnosed with DTC than men in patients older than 15, and this trend continues into adulthood. This peculiar characteristic is briefly mentioned in the

American Thyroid Association Guidelines for the Management of Pediatric DTC (1) and supported by multiple studies conducted by other authors (9, 32-33).

In 1989, *Imai et al.* discovered a link between thyroid cancer and estrogen (34). More recently, *Joseph et al.* found a connection between thyroid cancer and breast cancer (35). These findings led us to believe that the reason for the higher incidence of DTC in females could be due to greater exposure to estrogen over time rather than just the age at which the disease is diagnosed, as impubescent patients have very little exposure to estrogen. In 2022, we examined estrogen and progesterone receptors in pediatric DTC (8). While we did observe differences related to puberty, we were unable to find an association between estrogen or progesterone receptor expression and pediatric DTC.

Here we highlights that although DTC is more common in females in adults, we found a significant difference between males and females that was not related to age but rather to the process of complete puberty. We observed a female to male ratio of 1 to 1.4 in Group 1 and a reversal of this pattern to 5.1 to 1 in Group 2 ($p < 0.001$). Additionally, we were able to identify a change in the molecular landscape associated with puberty. This finding suggests that unknown mechanisms associated with sex or puberty could be involved in the genetic expression of DTC and potentially affect the clinical presentation and response to treatment of the disease.

Regarding the presentation of the disease, a greater aggressiveness of thyroid cancer is described in children under 10 years of age or in impubescent individuals compared to adolescents (9-12). *Lazar et al.* (11) demonstrated higher rates of extrathyroidal extension, lymph node involvement and lung metastases among prepubertal children. *Thiesmeyer et al.* (12) conducted a recent study that showed that prepubertal status was associated with the presence of metastases despite long-term

survival rates. However, the underlying mechanisms behind these observations remain unclear.

Pediatric DTC exhibits molecular genetic alterations that are distinct from those found in adults. In pediatric cases of DTC, fusion-type mutations occur more frequently, especially those including *RET/PTC* and *NTRK*, while the mutations typically observed in adult DTC are point mutations, such as *BRAF_V600E (37)*.

Pediatric DTC research is needed to determine whether the degree of puberty or other clinical factors influence this genetic profile or their response to treatment. *Galuppini* and colleagues (9) found molecular alterations associated with some presentation conditions and responses to treatment. However, these alterations were not found to be independent of the age or puberty of the patients. In a different study, *Franco* and his team (38) classified 131 patients with thyroid cancer based on tumor genetic profiles. They categorized them as RAS, BRAF and RET/NTRK fusion types to determine the differences in clinical behavior and outcomes. Their research showed that patients with *RET/NTRK* fusions had a worse prognosis than those with other types.

In 2019, we evaluated the presence of the *AGK::BRAF* fusion as associated with the presence of distant metastases in younger patients (39). In a recent study, *Zhou et al*. (40) demonstrated that among 42 patients diagnosed with DTC, the point *BRAF_V600E* mutation was not associated with aggressive behavior in adolescent patients. The molecular landscape is a crucial factor in the development and behavior of thyroid tumors. This signature has been identified as a prognostic and conditioning factor for adult tumors and contributes to a high risk of recurrence. However, it has not been considered in the staging or prognosis of children and teenagers with DTC.

Here, we demonstrate that the molecular signature differs between pediatric patients based on their level of puberty. Patients with complete puberty had a similar

mutation profile to adults, while younger patients had a distinct mutation profile. Fusions predominated in Group 1, while point mutations were more frequent in Group 2. *NTRK* fusions, which are associated with worse outcomes, were more frequent in Group 1, the group with more aggressive presentations in this study. Expanding our genetic evaluation of the patients, we confirm that *AGK::BRAF* fusions was exclusively detected within Group 1. As mentioned, this group has previously been associated with more aggressive disease progression and distant metastases (39). However, the *BRAF_V600E* point mutation, which is more commonly associated with worse prognoses in adult tumors, was found mainly in patients who had undergone complete puberty.

The *RET/PTC* fusion was more prevalent in Group 2, but no statistical associations with puberty patterns were demonstrated. Therefore, we conclude that presentation and response to treatment are not solely associated with either puberty or molecular signatures but rather with both conditions.

Another goal of this study was to investigate how the molecular signature associated with puberty may affect NIS expression and impact the response to adjuvant treatment with RIT. This type of analysis is crucial in the age of precision medicine, as it can help identify personalized treatment options.

The function of the NIS is to capture iodine into the intracellular environment, which is essential for the synthesis of thyroid hormone (41-44). In the same way, this symporter facilitates the entry of I-131, which is used as an adjuvant therapy in patients at moderate to high risk of disease recurrence, provided that tumor cells maintain their NIS expression capacity (13). NIS is synthesized from the *SLC5A5* gene and incorporated into tumor cell membranes (45-48). Molecular mutations have been linked to tumors with low NIS expression, which makes them resistant to RIT (49-52). As an

example, *Riesco-Eizaguirre et al*. (51) demonstrated that the *BRAF_V600E* mutation was associated with reduced expression of NIS. New targeted therapies could silence genes and dedifferentiate tumor cells, allowing for retreatment with RIT in iodine-refractory patients (53-54).

During our evaluation of NIS expression in pediatric DTC, considering the molecular alterations observed and the phenomenon of puberty, we faced limitations in making major statistical inferences about the treatment response, possibly due to the small number of viable samples used. Here, we demonstrated a low expression of NIS (26% of pediatric tumor cells). Among those positive for NIS, we were not able to demonstrate an association between NIS expression and puberty or the pediatric DTC genetic landscape. New studies are needed to clarify the gene–protein interrelationship and its regulation and degradation.

Notably, none of our patients died due to the disease, regardless of their clinical or molecular prognostic factors. Although some subgroups of patients showed more aggressive presentations, only 36% of patients in Group 1 and 23% of patients in Group 2 had incomplete structural responses at the end of the follow-up. Furthermore, 75% of Group 1 and 48% of Group 2 were at high risk for recurrence, but they still showed a complete response. This suggests that pediatric DTC staging may benefit from incorporating new criteria such as molecular tumor analysis and puberty stage as prognostic factors.

There were limitations to this study that need to be considered. First, the use of retrospective data meant that the information in the records was not always standardized for all patients. Second, the molecular profiling was performed in 60% of the cases and, therefore, we were unable to perform other comparative analyses that we believe would have been useful because of the limited availability of preserved

material and the small number of tumor tissue samples related to a rare disease. We took great care to ensure that we did not exhaust the material stored at the institution. Our group believes that we can minimize these losses of inferences and contribute to the scientific literature through new studies and collaborations.

Conclusions

The molecular genetic landscape in pediatric DTC differs from that in adults and is associated with puberty patterns, aggressiveness, and risk of disease recurrence at presentation. During puberty, the prevalence in males and females changes, and different clinical presentations occur, despite a consistently undetectable mortality rate. Lower levels of NIS expression were observed in tumor cells, but they were not associated with puberty or a certain molecular profile. Additional studies are needed to evaluate the impact of NIS expression on treatment decisions. To better improve the prediction of treatment response in pediatric DTC, new staging criteria, such as puberty status and the molecular genetic profile, should be incorporated.

References

1 - Francis GL, Waguespack SG, Bauer AJ, *et al*.; American Thyroid Association Guidelines Task Force. Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2015;25(7):716-59.

2 - Haugen BR, Alexander EK, Bible KC, *et al*. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2016;26(1):1-133.

3 - Parisi MT, Eslamy H, Mankoff D. Management of Differentiated Thyroid Cancer in Children: Focus on the American Thyroid Association Pediatric Guidelines. Semin Nucl Med. 2016;46(2):147-64.

4 - Nies M, Vassilopoulou-Sellin R, Bassett RL, *et al*. Distant Metastases From Childhood Differentiated Thyroid Carcinoma: Clinical Course and Mutational Landscape. J Clin Endocrinol Metab. 2021;106(4):e1683-e1697.

5 - Lebbink CA, Links TP, Czarniecka A, *et al*. 2022 European Thyroid Association Guidelines for the management of pediatric thyroid nodules and differentiated thyroid carcinoma. Eur Thyroid J. 2022;11(6).

6 - Kim SY, Yun HJ, Chang H, *et al*. Aggressiveness of Differentiated Thyroid Carcinoma in Pediatric Patients Younger Than 16 years: A Propensity Score-Matched Analysis. Front Oncol. 2022;12:872130.

7 - Pires BP, Alves PA Jr, Bordallo MA, *et al*. Prognostic Factors for Early and Long-Term Remission in Pediatric Differentiated Thyroid Carcinoma: The Role of Sex, Age, Clinical Presentation, and the Newly Proposed American Thyroid Association Risk Stratification System. Thyroid. 2016;26(10):1480-1487.

8 - da Silva Breder JRA, Alves PAG, Araújo ML, *et al*. Puberty and sex in pediatric thyroid cancer: could expression of estrogen and progesterone receptors affect prognosis? Eur Thyroid J. 2022;11(2)

9 - Galuppini F, Vianello F, Censi S, *et al*. Differentiated Thyroid Carcinoma in Pediatric Age: Genetic and Clinical Scenario. Front Endocrinol (Lausanne). 2019 ;10:552.

10 - Kim SY, Yun HJ, Chang H, *et al*. Aggressiveness of Differentiated Thyroid Carcinoma in Pediatric Patients Younger Than 16 years: A Propensity Score-Matched Analysis. Front Oncol. 2022;12:872130.

11- Lazar L, Lebenthal Y, Steinmetz A, *et al*. Differentiated thyroid carcinoma in pediatric patients: comparison of presentation and course between pre-pubertal children and adolescents. J Pediatr. 2009;154(5):708-14.

12 - Thiesmeyer JW, Egan CE, Greenberg JA, *et al*. Prepubertal Children with Papillary Thyroid Carcinoma Present with More Invasive Disease Than Adolescents and Young Adults. Thyroid. 2023;33(2):214-222.

13 - Gulec SA, Ahuja S, Avram AM, *et al*. A Joint Statement from the American Thyroid Association, the European Association of Nuclear Medicine, the European Thyroid Association, the Society of Nuclear Medicine and Molecular Imaging on Current Diagnostic and Theranostic Approaches in the Management of Thyroid Cancer. Thyroid. 2021;31(7):1009-1019

14 - Filetti S, Bidart JM, Arturi F, *et al*. Sodium/iodide symporter: a key transport system in thyroid cancer cell metabolism. Eur J Endocrinol. 1999;141(5):443-57.

15 - Nagarajah J, Janssen M, Hetkamp P, *et al*. Iodine Symporter Targeting with $^{124}I/^{131}I$ Theranostics. J Nucl Med. 2017;58(Suppl 2):34S-38S.

16 - Carvalho DP, Ferreira AC. The importance of sodium/iodide symporter (NIS) for thyroid cancer management. Arq Bras Endocrinol Metabol. 2007;51(5):672-82.

17 - Lakshmanan A, Scarberry D, Shen DH, *et al*. Modulation of sodium iodide symporter in thyroid cancer. Horm Cancer. 2014;5(6):363-73.

18 - Castillo-Rivera F, Ondo-Méndez A, Guglielmi J, *et al*. Tumor microenvironment affects exogenous sodium/iodide symporter expression. Transl Oncol. 2021;14(1).

19 - Espadinha C, Santos JR, Sobrinho LG, *et al*. Expression of iodine metabolism genes in human thyroid tissues: evidence for age and BRAFV600E mutation dependency. Clin Endocrinol (Oxf). 2009;70(4):629-35.

20 - Castro P, Patiño E, Fierro F, *et al*. Clinical characteristics, surgical approach, BRAFV600E mutation and sodium iodine symporter expression in pediatric patients with thyroid carcinoma. J Pediatr Endocrinol Metab. 2020;33(11):1457-1463.

21 - Filbin M, Monje M. Developmental origins and emerging therapeutic opportunities for childhood cancer. Nat Med. 2019;25(3):367-376.

22 - Guo K, Qian K, Shi Y, *et al*. Clinical and Molecular Characterizations of Papillary Thyroid Cancer in Children and Young Adults: A Multicenter Retrospective Study. Thyroid. 2021;31(11):1693-1706.

23 - Alzahrani AS, Alswailem M, Alswailem AA, *et al*. Genetic Alterations in Pediatric Thyroid Cancer Using a Comprehensive Childhood Cancer Gene Panel. J Clin Endocrinol Metab. 2020;105(10).

24 - Nikita ME, Jiang W, Cheng SM, *et al*. Mutational Analysis in Pediatric Thyroid Cancer and Correlations with Age, Ethnicity, and Clinical Presentation. Thyroid. 2016;26(2):227-34.

25 - Lee YA, Lee H, Im SW, *et al*. NTRK and RET fusion-directed therapy in pediatric thyroid cancer yields a tumor response and radioiodine uptake. J Clin Invest. 2021;131(18).

26 - Venkataramany AS, Schieffer KM, Lee K, *et al*. Alternative RNA splicing defects in pediatric cancers: new insights in tumorigenesis and potential therapeutic vulnerabilities. Ann Oncol. 2022;33(6):578-592.

27 - Sweet-Cordero EA, Biegel JA. The genomic landscape of pediatric cancers: Implications for diagnosis and treatment. Science. 2019;363(6432):1170-1175.

28 - Kattner P, Strobel H, Khoshnevis N, *et al*. Compare and contrast: pediatric cancer versus adult malignancies. Cancer Metastasis Rev. 2019;38(4):673-682.

29 - Day FR, Thompson DJ, Helgason H, *et al*. Genomic analyses identify hundreds of variants associated with age at menarche and support a role for puberty timing in cancer risk. Nat Genet. 2017;49(6):834-841.

30 - Natarajan R, Aljaber D, Au D, *et al*. Environmental Exposures during Puberty: Window of Breast Cancer Risk and Epigenetic Damage. Int J Environ Res Public Health. 2020;17(2):493.

31 - Biro FM, Deardorff J. Identifying opportunities for cancer prevention during preadolescence and adolescence: puberty as a window of susceptibility. J Adolesc Health. 2013;52(5 Suppl):S15-20.

32 - Shobab L, Burman KD, Wartofsky L. Sex Differences in Differentiated Thyroid Cancer. Thyroid. 2022;32(3):224-235.

33 - Hogan AR, Zhuge Y, Perez EA, *et al*. Pediatric thyroid carcinoma: incidence and outcomes in 1753 patients. J Surg Res. 2009;156(1):167-72

34 - Imai Y, Yamakawa M, Matsuda M, *et al*. Endogenous sex hormone and estrogen binding activity in thyroid cancer. Histol Histopathol. 1989;4(1):39-45.

35 - Joseph KR, Edirimanne S, Eslick GD. The association between breast cancer and thyroid cancer: a meta-analysis. Breast Cancer Res Treat. 2015;152(1):173-181.

36 - Thiesmeyer JW, Egan CE, Greenberg JA, *et al*. Prepubertal Children with Papillary Thyroid Carcinoma Present with More Invasive Disease Than Adolescents and Young Adults. Thyroid. 2023;33(2):214-222.

37 - Sweet-Cordero EA, Biegel JA. The genomic landscape of pediatric cancers: Implications for diagnosis and treatment. Science. 2019;363(6432):1170-1175.

38 - Franco AT, Ricarte-Filho JC, Isaza A, *et al*. Fusion Oncogenes Are Associated With Increased Metastatic Capacity and Persistent Disease in Pediatric Thyroid Cancers. J Clin Oncol. 2022;40(10):1081-1090.

39 - Sisdelli L, Cordioli MICV, Vaisman F, *et al*. AGK-BRAF is associated with distant metastasis and younger age in pediatric papillary thyroid carcinoma. Pediatr Blood Cancer. 2019;66(7).

40 - Zhou B, Lu X, Hei H, *et al*. Single BRAFV600E mutation is not associated with aggressive biological behavior in adolescent and pediatric papillary thyroid carcinoma. Cancer Cytopathol. 2023. doi: 10.1002/cncy.22746. Epub ahead of print.

41 - Dohán O, De la Vieja A, Paroder V, *et al*. The sodium/iodide Symporter (NIS): characterization, regulation, and medical significance. Endocr Rev. 2003;24(1):48-77.

42 - Riesco-Eizaguirre G, Santisteban P, De la Vieja A. The complex regulation of NIS expression and activity in thyroid and extrathyroidal tissues. Endocr Relat Cancer. 2021;28(10):T141-T165.

43- Darrouzet E, Lindenthal S, Marcellin D, *et al*. The sodium/iodide symporter: state of the art of its molecular characterization. Biochim Biophys Acta. 2014;1838(1 Pt B):244-53.

44 - Ravera S, Reyna-Neyra A, Ferrandino G, *et al*. The Sodium/Iodide Symporter (NIS): Molecular Physiology and Preclinical and Clinical Applications. Annu Rev Physiol. 2017;79:261-289.

45 - Lazar V, Bidart JM, Caillou B, *et al*. Expression of the Na+/I- symporter gene in human thyroid tumors: a comparison study with other thyroid-specific genes. J Clin Endocrinol Metab. 1999;84(9):3228-34.

46 - Carvalho DP, Ferreira AC. The importance of sodium/iodide symporter (NIS) for thyroid cancer management. Arq Bras Endocrinol Metabol. 2007;51(5):672-82.

47 - Morari EC, Marcello MA, Guilhen AC, *et al*. Use of sodium iodide symporter expression in differentiated thyroid carcinomas. Clin Endocrinol (Oxf). 2011;75(2):247-54.

48 - de Morais RM, Sobrinho AB, de Souza Silva CM, *et al*. The Role of the NIS (SLC5A5) Gene in Papillary Thyroid Cancer: A Systematic Review. Int J Endocrinol. 2018;2018:9128754.

49 - Oh JM, Ahn BC. Molecular mechanisms of radioactive iodine refractoriness in differentiated thyroid cancer: Impaired sodium iodide symporter (NIS) expression owing to altered signaling pathway activity and intracellular localization of NIS. Theranostics. 2021;11(13):6251-6277.

50 - Hou S, Xie X, Zhao J, *et al*. Downregulation of miR-146b-3p Inhibits Proliferation and Migration and Modulates the Expression and Location of Sodium/Iodide Symporter in Dedifferentiated Thyroid Cancer by Potentially Targeting MUC20. Front Oncol. 2021;10:566365.

51 - Riesco-Eizaguirre G, Rodríguez I, De la Vieja A, *et al*. The BRAFV600E oncogene induces transforming growth factor beta secretion leading to sodium iodide symporter repression and increased malignancy in thyroid cancer. Cancer Res. 2009;69(21):8317-25.

52 - Lakshmanan A, Scarberry D, Shen DH, *et al*. Modulation of sodium iodide symporter in thyroid cancer. Horm Cancer. 2014;5(6):363-73.

53 - Ullmann TM, Liang H, Moore MD, *et al*. Dual inhibition of BRAF and MEK increases expression of sodium iodide symporter in patient-derived papillary thyroid cancer cells in vitro. Surgery. 2020;167(1):56-63.

54 - Liu J, Liu Y, Lin Y, *et al*. Radioactive Iodine-Refractory Differentiated Thyroid Cancer and Redifferentiation Therapy. Endocrinol Metab (Seoul). 2019;34(3):215-225.

Figures

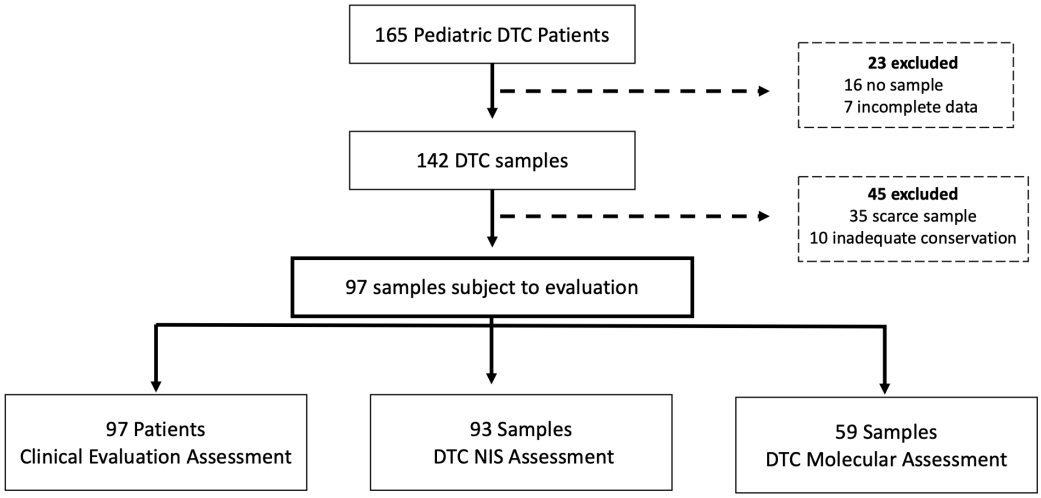

Figure 1 – Flow diagram describing the patients and samples exclusions criteria.

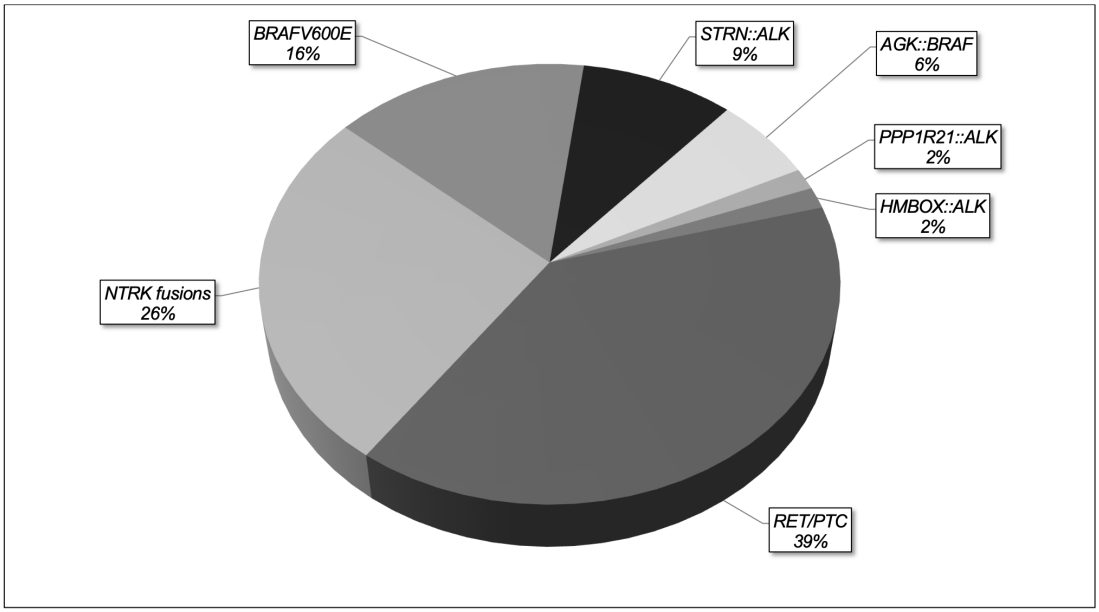

Figure 2 – Frequency of molecular genetic alterations in 59 tumor samples. Genetic alterations were identified in 47 out of 59 samples tested with 64 genetic alterations identified. 25% of the samples presented with more than one mutation in the same tumor.

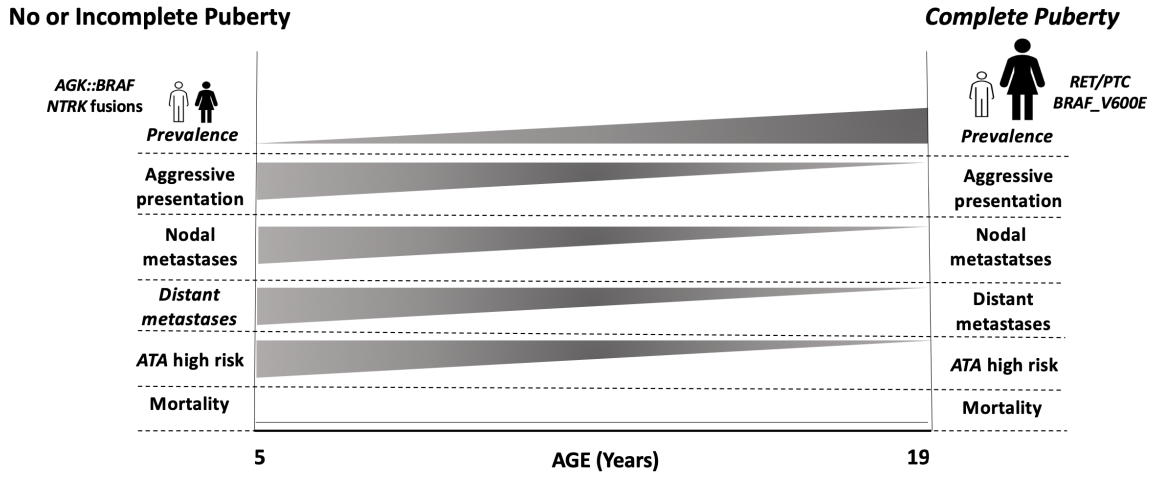

Figure 3 – Summary of clinical and genetic findings according to puberty status in pediatric DTC. Patients with complete puberty are associated with a higher female to male ratio, better tumor presentation and a different tumor genetic landscape compared to patients with no or incomplete puberty, although no mortality in both groups.

Tables

Table 1 – General population and puberty category characteristics. Evaluation of clinical profile, risk of recurrence, radioiodine treatment, and response to treatment.

				GROUP 1	GROUP 2	
CATEGORY	**Entire population**	**Population with viable tumor sample**		**PRE- OR PERI-PUBERTAL POPULATION**	**COMPLETE PUBERTY POPULATION**	
	N or Med. (% or s.d.)	**N or Med. (% or s.d.)**	**p**	**N or Med. (% or s.d.)**	**N or Med. (% or s.d.)**	**p**
NUMBER OF PATIENTS	142	97 (68%)		36	61	
FOLLOW-UP (YEARS)	9,5 (± 7,2)	8.7 (±5,9)	0.57	11.4 (±5,8)	8.3 (±5,9)	0.06
FEMALE SEX	102(72%)	66 (68%)	0.47	15 (41%)	51(84%)	**<0.001**
AGE AT DIAGNOSIS (YEARS)	14.6 (± 3,63)	14.9 (±3,77)	0.71	11.0 (±3,3)	16.7 (±2,0)	**<0.001**
PAPILLARY TYPE	137 (96%)	95 (97%)	0.70	35 (97%)	60(98%)	>0.9
TUMOR SIZE (CM)	2.7 (± 1,6)	2.7 (±1,7)	>0.9	3.0 (± 1,7)	2.3 (± 1,7)	0.09
NODAL METASTASIS	96 (73%)	67 (69%)	0.88	30 (83%)	37 (61%)	**0.02**
DISTANT METASTASIS	35 (25%)	19 (20%)	0.43	13 (36%)	6 (1%)	**0.003**
ATA PEDIATRIC RISK GROUP						
LOW	39 (27%)	28 (28%)	0.88	6 (17%)	22 (36%)	0.06
INTERMEDIARY	24 (17%)	13 (13%)	0.58	3 (8%)	10 (16%)	0.36
HIGH	79 (56%)	56 (58%)	0.79	27 (75%)	29 (48%)	**0.01**
I-131 MEDIAN CUMULATIVE DOSE	150 (± 193)	150(± 189)	0.29	150 (± 187)	150 (± 159)	**0.02**
TREATMENT RESPONSE AT THE END OF *FOLLOW-UP*						
EXCELLENT RESPONSE	68 (49%)	52 (54%)	0.43	17 (47%)	35 (57%)	0.40
INCOMPLETE STRUCTURAL RESPONSE	26 (18%)	27 (28%)	0.11	13 (36%)	14 (23%)	0.17

ATA - American Thyroid Association, I-131 - Iodide-131, N. - Number, Med. - Median, s.d. - Standard deviantion

Table 2 – Description and evaluation of the molecular profile of differentiated thyroid carcinoma in the general population and by puberty category.

		GROUP 1	GROUP 2		
CATEGORY	**General population (n)**	**PRE- OR PERI-PUBERTAL (n)**	**COMPLETE PUBERTY (n)**	**ODDS RATIO (OR)**	**p**
MOLECULAR ASSESSMENT	59/97	25/36	34/61	-	-
NUMBER OF MUTATIONS	64	30	34	-	-
PATIENTS WITH MUTATIONS	47	20	27	-	>0.999
PATIENTS WITHOUT MUTATIONS	12	5	7		
PATIENTS WITH 1 MUTATION	32	11	21	2.86 [0.85-9.02]	0.12
PATIENTS WITH > 1 MUTATION	15	9	6		
FUSION MUTATIONS	54	29	25	4.6 [0.66-58.4]	0.19
POINT MUTATIONS	10	2	8		
RET/PTC	25	8	17	2.55 [0.74-8.26]	0,15
ETV6::NTRK3/SQSTM1::NTRK1	17	12	5	6.6 [1.6-24.5]	**0.005**
BRAF_V600E	10	2	8	4.2 [0.76-21.2]	0.14
STRN::ALK	6	3	3	-	>0.999
AGK::BRAF	4	4	0	Inf. [1.38-inf.]	**0.02**
PPP1R21::ALK	1	0	1	**-**	*
HMBOX1::ALK	1	1	0	-	*

Table 3 – Evaluation of NIS expression according to clinical categorical assessments, response to treatment and main molecular alterations.

Clinical analysis	Category	NIS + (% grand total)	NIS – (% grand total)	p
Sex	Male	6.4	25.8	0.45
	Female	19.3	48.4	
Puberty	Complete	18.3	44.1	0.46
	No complete	7.5	30.1	
Nodal metastasis	Yes	17.6	57.7	0.39
	No	8.2	16.5	
Distant metastasis	Yes	3.5	18.8	0.37
	No	22.3	55.3	
ATA high risk	Yes	13.4	51.2	0.11
	No	13.4	21.9	
Excellent response	Yes	16.3	39.1	0.48
	No	9.8	34.8	
Incomplete structural response	Yes	3.3	25.0	**0.06**
	No	22.8	48.9	
Multiple I-131 dose	Yes	3.5	20.9	0.26
	No	20.9	54.6	
Molecular analysis	**Mutation present**	**NIS + (% grand total)**	**NIS – (% grand total)**	**p**
ETV6::NTRK6	Yes	9.1	29.5	0.74
	No	18.2	43.2	
RET/PTC	Yes	17.4	38.1	0,18
	No	82.6	61.9	
BRAF_V600E	Yes	6.8	13.6	0,68
	No	20.4	59.1	
STRN::ALK	Yes	6.8	6.8	0.32
	No	20.4	65.9	
AGK::BRAF	Yes	0	6.8	0.55
	No	27.3	65.9	

ATA - American Thyroid Association, I-131 - Iodide-131, NIS - Sodium-Iodide symporter

4.3 - Artigo 3

Revisiting the expression of the Sodium-Iodide Symporter (NIS) through the *SLC5A5 gene* in pediatric differentiated thyroid carcinoma. A study on the assessment of NIS and its association with molecular profile and treatment in pediatric thyroid cancer.

Paulo Alonso Garcia Alves Junior [1.2] , Priscila Valverde [3] , Pedro Nicolau-Neto [4] , Luis Felipe Ribeiro Pinto [4] , Sheila Coelho Soares Lima [4] , Mario Lucio Araújo [3] , Janete Maria Cerutti [5] , Fernanda Vaisman [1.2]

1 - Endocrinology Department, Instituto Nacional de Câncer (INCA), Rio de Janeiro, R.J., Brazil.

2 - Endocrinology Department, Medicine School, Universidade Federal do Rio de Janeiro, R.J., Brazil.

3 - Pathology Department, Instituto Nacional de Câncer (INCA), Rio de Janeiro, R.J., Brazil.

4 - Molecular Carcinogenesis Program, Instituto Nacional de Câncer (INCA), Rio de Janeiro, R.J., Brazil.

5 - Genetic Bases of Thyroid Tumors Laboratory, Division of Genetics, Department of Morphology and Genetics, Universidade Federal de São Paulo/EPM, São Paulo, S.P., Brazil.

Corresponding author: Fernanda Vaisman, Endocrinology Department, Instituto Nacional de Câncer, Praça Cruz Vermelha, 23, Centro, Rio de Janeiro, -RJ, Brazil, Phone: +552125126474, e-mai: vaismanfe@gmail.com

Abstract

Introduction

The Sodium-Iodide Symporter (NIS) is the gateway to the adjuvant treatment of differentiated thyroid carcinoma (DTC). Due to its rarity, few studies have evaluated NIS expression in pediatric DTC, despite a more aggressive presentation and a peculiar genetic signature.

Purpose

To describe the expression of the *SLC5A5 gene* and the NIS protein in pediatric DTC cells and evaluate possible associations with adjuvant treatment, response to treatment at the end of follow-up, and the tumor genetic signature.

Materials and methods

The expression of NIS was evaluated by Immunohistochemistry and its coding gene *SLC5A5* by qPCR in thyroid tumor tissue samples from patients under 19 years of age and looked for associations with clinical and histological characteristics, response to treatment and the tumor mutational profile by NGS.

Results

In 72 samples capable of evaluation, despite the association between gene and protein, 25% showed NIS expression although 54% showed to *SLC5A5*. Patients with higher NIS expression required less retreatment (p 0.05) and possibly lower doses of 131-I (p 0.08). The *STRN::ALK* fusion was related to higher expression of *SLC5A5*. NIS expression did not determine different responses at the end of treatment.

Conclusion

Possibly intrinsic factors determine a lower expression of NIS compared to *SLC5A5*. Patients with higher NIS expression require less retreatment with 131-I. The

molecular profile of pediatric thyroid tumors was not associated with a different expression of NIS, despite being more expressed in *STRN::ALK* fusions.

Introduction

Adjuvant treatment of differentiated thyroid carcinoma (DTC) is based on radioactive iodine therapy (RIT) after thyroidectomy. Patients with nodal and distant metastases have an increased risk of recurrence of the tumor, with treatment with Iodine-131 (I-131) being an important therapeutic strategy adopted to facilitate follow-up and reduce the chance of recurrence or mortality associated with the disease.[1]

The sodium-iodide symporter, widely known as NIS, is a glycoprotein found mainly in the basolateral membrane of thyroid follicular cells, being encoded by the gene *SLC5A5* (solute carrier family 5 member 5) which is located on chromosome 19 (19p13.11). Its main function is to mediate the active uptake of iodine from the bloodstream into the cell through a sodium-iodide pump (2 Na^+/ I^-) and enable the synthesis of thyroid hormones in the thyroid gland.[2] In thyroid tumors that maintain their expression, the NIS represents the gateway for the uptake of I-131, leading to the destruction of the tumor cell due to its ionizing activity and β particle emission. [3.4]

Some studies have demonstrated failure in the expression of NIS in thyroid tumor cells,[5] determining resistance to adjuvant treatment associated with higher rates of recurrence or even mortality.[6-8] Dedifferentiation therapies that would reincorporate NIS into tumor cells are currently being studied with the aim of enabling retreatment with I-131 in patients previously considered to have iodorefractory metastatic disease. [9-11]

Due to its rarity, few studies have evaluated the expression of NIS or its coding gene - *SLC5A5* in DTC tumor cells of the pediatric age group.[12-14] This is important because pediatric DTC presents more aggressively, with high rates of cervical and distant metastases compared to adults, requiring adjuvant treatment in more than 70% of cases.[15-18] Furthermore, the tumor genetic profile of children and

adolescents could modify NIS expressions and determine different doses or even responses to treatment.[19-20]

Thus, the aim of this work was to describe the expression of the *SLC5A5 gene* and NIS protein in pediatric DTC cells and evaluate possible associations with RIT, response to treatment at the end of follow-up, and the tumor genetic signature.

Materials and methods

Patients diagnosed with DTC, followed from 1976 to 2022, at the main cancer reference center in Brazil (National Cancer Institute – INCA – Ministry of Health - Brazil) were selected to participate in the study.

After approval by the local Ethics Committee (CEP/CONEP 2.146.715 and CAAE 66569517.8.0000.5257), searches were carried out for pediatric patients diagnosed with DTC contained in the Institution's physical and computerized records.

The study included patients under 19 years of age with a confirmed revisited histological diagnosis of DTC, with clinical data capable of interpretation and with tumor material stored at the Institution. Those with follow-up of less than 6 months, who did not wish to participate, or with tumor material in insufficient quantity or quality for analysis were excluded.

The sample of tumor cells was obtained from the primary tumor tissue originating from thyroidectomy surgery that was stored in the Institution's Pathology Service. Care was taken to ensure that the material was not exhausted at the time of processing for the proposed analyses.

SLC5A5 gene expression evaluation. RNA was extracted from FFPE samples, using one section of 10 µm per sample, with *Purelink* FFPE RNA kit (*Invitrogen*) following the instructions provided by the manufacturer. The RNA samples were

quantified in the Nanodrop (*Thermo*), evaluating the purity ratios 260 nm / 280 nm and 260 nm / 230 nm. cDNA was synthesized from 1.0 µg RNA of each sample by SuperScript IV Reverse Transcriptase (*Invitrogen*). qPCR was performed in 10.0 µL reaction volume with Quantinova SYBR Green PCR kit (*Qiagen*) on a Rotor-Gene thermal cycler (*Qiagen*). Samples were analyzed in triplicate. NIS mRNA relative quantification was analyzed by the ΔCt method using GAPDH as a housekeeping gene. The sequences of primers used in this project were: NIS-F 5' ACCTCATCAAACCTCGGCTG-3'; NIS-R 5'- GATCCGTAGATGAGTGAGAGC-3'; GAPDH-F 5'- CAACAGCCTCAAGATCATCAGCAA-3'; GAPDH-R 5' AGTGATGGCATGGACTGTGGTCAT-3'.

Tumor molecular evaluation. Molecular evaluation of pediatric thyroid tumors was based on the extraction and fragmentation of RNA from FFPE sample, followed by specific molecular identification, capture, and enrichment of the target region with a customized kit. Next-generation sequencing (NGS) was performed using the *NextSeq500* or *NovaSeq6000* platforms (*Illumina*).

NIS protein expression evaluation. The immunohistochemistry (IHC) technique was performed on two consecutive days. Commercial slides, previously treated with fillers (immunoSlide -*Easy Path*) containing 3-micron sections, were immersed in 3 baths of 5 minutes in xylol, followed by quick baths in 100%, 90%, 80%, and 70% alcohol. Excess alcohol was removed in running water for 3 minutes. Antigenic retrieval was performed in *Trilogy Buffer* (*Cell Marque*), at a temperature of 98°C, using the steam process, for 30 minutes. Peroxidase blocking and protein blocking were performed using the NovoLink Max Polymer Detection kit, (*Leica Microsystems*), for 5 minutes each. Incubation with polyclonal rabbit antibody *SLC5A5* at a 1:2800 dilution was carried out overnight, in the refrigerator. On the

second day of the technique, the slides were incubated with the post-primary antibody and with the polymer (*Novolink*), both for 30 minutes. To reveal the reaction, DAB chromogen was used for 3 minutes. Counterstaining was performed with hematoxylin for 30 seconds. The DAB from the *Novolink* kit and Haris Hematoxylin were used. After removing excess hematoxylin in running water, the slides were immersed in 70%, 80%, 90%, and 100% alcohol and xylene baths. The balsam was used to assemble the slides, which were analyzed under an optical microscope, observing cytoplasmic/membrane staining.

All collected data and results were plotted in Microsoft Excel v.16.0 (*Microsoft, Redmond, Washington, USA*). Statistical analysis was evaluated using GraphPad Prism 8.0 (*Graphpad Software Inc., San Diego, CA, USA*). Categorical variables were analyzed by absolute and relative frequency. Continuous variables were assessed for normality (parametric) and described as mean/median, standard deviation, minimum, maximum and range values. Test of statistical significance of continuous variables was obtained by *Student's t-test* in case of non-parametric data or by *One-Way ANOVA* when normally distributed; *Chi-square* (X^2) or *Fischer's exact test* was performed for categorical variables. A p-value ≤ 0.05 was considered statistically significant.

Results

Of 142 patients under 19 years old with DTC diagnosis and tissue samples stored at the institution between 1976 and 2022, 72 samples (50.7% of the total) were found suitable for the proposed analyses. The patients were predominantly female (65%), with a median age of 15 years, more than half of whom had completed puberty at diagnosis and were followed for an average of 8.6 years.

Regarding the primary tumor, 97% were of the papillary type, with a median size of 2.5 cm. At diagnosis, 71% of patients had metastases to cervical lymph nodes and 22% had distant metastases (all lung metastases), giving 64% a high risk for disease recurrence and 26% low risk, according to the Pediatric *ATA* criteria.[15] Despite this aggressive presentation, at the end of follow-up, only 28% had evidence of structural disease, while more than half (55%) had no evidence of tumor disease (excellent response).

Regarding adjuvant treatment, the average cumulative dose with I-131 was 150 mCi, ranging from 30 to 800 mCi. 16 patients (22%), all high-risk according to the *Pediatric ATA*,[15] underwent more than one treatment with RIT. Table 1 provides clinical and histological information about the study population.

Tumor molecular profile

Regarding the genetic profile of the tumor, the molecular analysis could be evaluated in 41 samples from the 72 patients. A total of 50 mutations were found (5 patients did not demonstrate mutations recognized by the panel), with 12 patients demonstrating more than one mutation in the same sample. As expected, the number of fusions prevailed (86% of the samples), with 19 *RET/PTC* fusions, 15 associated with *NTRK*, 4 *STRN::ALK*, 3 *AGK::BRAF* and another 2 associated with *ALK* (*PPP1R21::ALK*; *HMBOX::ALK*). Point mutations - all BRAF_V600E type, were found in 7 samples. Figure 2 demonstrates in detail the frequency of mutations found and their distribution among patients.

Assessment of NIS expression

The NIS protein evaluated by IHC was present in only 18 of the 72 samples evaluated (25%), all located in the cytoplasmic membrane. The expression of the gene *SLC5A5* was positive in a greater number of samples, 39 of 72 (54%). No

significant differences were found between NIS expression and the clinical parameters evaluated. As expected, protein positivity in tumor cells was associated with *SLC5A5* expression. Figure 1 and Table 2 detail the association findings evaluated.

Regarding the evaluation of NIS or gene expression *SLC5A5* according to the molecular genetic profile presented by the study population, no statistical significance was found with the *RET/PTC, NTRK* fusions*, AGK::BRAF*. There was a significant difference in the median of the quantitative *SLC5A5/GAPDH* relationship between those carrying the *STRN::ALK fusion* (p 0.002). No difference was observed in NIS expression in patients carrying *BRAF_V600E* point mutation. Table 3 and Figure 1 summarize the findings and statistical significance found.

Treatment response assessment

Regarding treatment, it was seen that patients who received only one dose of RIT expressed more NIS protein in tumor cells compared to those who required multiple doses (p 0.05). This difference was also seen for the expression of the gene *SLC5A5*, but without reaching the significance threshold (p 0.08).

Regarding the response at the end of the follow-up, there was no difference between the expression of NIS or its coding gene and the excellent or incomplete structural response. Among the 16 patients who required multiple doses of RIT, 8 (50%) remained with structural disease at the end of follow-up, with *NTRK* fusion found in 4 of them. Of the 56 patients with a single dose of RIT, only 12 (21.4%) maintained structural disease status, with *NTRK* fusion being found in 2 patients. Other individual characteristics can be seen in more detail in Figure 2 details.

Discussion

In this new work, we show the expression of NIS in differentiated thyroid tumors of children and adolescents, this time evaluating, in addition to the protein, its coding gene *SLC5A5*. We noticed here, despite an association between gene and protein (p 0.02), a higher expression of the *SLC5A5 gene* (54% of samples) in relation to NIS protein (25% of samples), justifying theories that intracellular factors could modulate nuclear expression. To better evaluate whether clinical conditions or the molecular signature could be factors for this modulation, we were unable to provide decisive answers through this work, but we noticed a lower need for retreatment with 131-I in those with higher expression of NIS.

Pediatric DTC is a rare disease representing only 1.9% of all thyroid cancer cases.[15,21] This means that few studies evaluate the peculiarities of thyroid cancer in the child population, generally being restricted to large academic or research centers. Despite these few studies, the differentiated tumor behavior compared to adults, through a more aggressive presentation but with a more favorable response to treatment, is already well-established and widespread.[22] The molecular profile of the pediatric tumor is also known as peculiar, with a greater pattern of gene fusions such as *RET/PTC* and *NTRK*.[23-24] Therefore, understanding the characteristics of tumor behavior related to clinical or mutational parameters is fundamental to improving patient staging and ensuring targeted and optimized therapeutic proposals.

We currently use the *Pediatric ATA Guideline*[15] to differentiate patients at risk of recurrence and indicate adjuvant treatment in those at moderate or high risk of recurrence. RIT is a fundamental “weapon” in the war against iodine-capturing tumor cells,[4] however, some cancers become resistant to this treatment due to an under expression of NIS.

Numerous studies have investigated the expression of NIS in adult thyroid cancer, most of them are described by de *Morais et. al.*[5] in their systematic review. Associations of NIS expression with genetic profiles, such as the *BRAF_V600E* mutation, with methylations in promoter regions or even with prognostic factors, were seen. *Tavares* et.al.[7] in 2018 demonstrated that reduced expression of the gene *SLC5A5* was related to a lower detection of NIS in the plasma membrane of DTC and this factor was associated with greater aggressiveness of the disease and with mutations typical of adult tumors such as *BRAF, RAS, TERT*.

Here, using the pediatric population as a standard, we were also able to notice a lower expression of NIS (only 25% of the samples), which could lead to the understanding that few of these children would respond to adjuvant treatment with 131-I. At the end of our study's follow-up, it was surprising to find that a larger number of patients (55%) were free from disease compared to our initial estimate. Only 28% of the patients were found to have identifiable disease after approximately 8 years of follow-up. It is worth mentioning that there were no deaths related to the disease, despite the initial risk of recurrence being estimated in more than half of the patients (64%). This leads to the thought that, or the initial staging of patients overestimates the chance of recurrence in children, and we need to add new criteria to improve the performance of the initial assessment, or that the high-risk pediatric population is more sensitive to treatment and may not need intensive treatments.

We try to better understand the role of the NIS in this context of prognostic relationship. *Morari et al.*[25] cited 13 adult patients who died from DTC and had NIS negativity in their tumor cells. In one of the few child studies, *Patel et. al.* [26] demonstrated, despite a much smaller number of patients than this and using a different staging system than that recommended by the *ATA*[15], a lower risk of

recurrence in differentiated thyroid tumors was seen in that who carried positive expression for NIS by IHC. In a recent study, *Lee et. al.*[27] demonstrated a dramatic 131-I reuptake response following targeted therapy targeting *NTRK* and *RET/PTC fusion* in patients with metastatic disease previously considered iodorefractory.

In this study, we were unable to determine the relationship between NIS expression and the patients' final response to the treatment, but we were able to notice that those who had positive expression for NIS were less exposed to retreatment with I-131 (p 0.05). Possibly these patients could also receive lower doses of RIT, but due to the retrospective nature of the study with patients diagnosed on dates prior to the ATA Pediatric *Guideline*[15], where higher doses of 131-I were used for metastatic or even low-risk patients, it was not possible to find significance relating NIS to cumulative 131-I doses (p 0.39). We, therefore, suggest new studies that can evaluate the expression of NIS associated with other clinical or laboratory parameters (such as stimulated thyroglobulin) to determine the ideal dose of RIT in metastatic patients and reduce complications related to ionizing radiation.

In our previous study, we were able to demonstrate the relationship between the patient molecular signature and clinical parameters, associating greater severity of presentation with some mutational profiles and characteristics related to puberty. In that study, we were unable to link the NIS protein with these important clinical factors or molecular profiles. So, we choose to revisit the expression of NIS, this time, through its coding gene *SLC5A5* to better understand possible associations not found.

In this study, we were also unable to demonstrate an association between *SLC5A5* expression and the molecular signature of pediatric DTC, except with the gene *STRN::ALK,* which maintained a higher expression in all patients who carried

the mutation. Studies that attempt to relate this association generally use the mutational profile of adults. *Romei et. al.*[28] demonstrated that the *BRAF_V600E mutation* (the most common mutation found in adult tumors) leads to lower expression of NIS, while the same does not occur with the *RET/PTC fusion)*. *Tavares et. al.*[29] *revealed genetic profiles associated* with lower NIS expression, finding *BRAF_V600E mutations* and those that carry *RAS* and *TERT* as associated with SLC5A5 underexpression. Here, we were unable to demonstrate that the *BRAF_V600E mutation* was associated with lower NIS expression, but it is worth reporting that only 7 patients presented this mutation of whom 4 of these also presented other mutations in the same tumor (1 was associated with the *NTRK* fusion and 3 with the *RET/PTC*). Of those with a single *BRAF_V600E mutation, 2 did not demonstrate SLC5A5* expression and 1 was positive.

Once again, we demonstrate that there is no impact of NIS or *SLC5A5* expression on the final response to treatment of children and adolescents CDT. This is an important point of view since at the current time new target-drugs capable to redifferent tumor cells that were previously iodine refractory are in process of development. A recent study published by *Nikitski et.al*.[30] demonstrated the restoration of radioiodine uptake in patients who had inhibition of *ALK* signaling upon NIS downregulation induced by the *STRN::ALK fusion*. Previously, Hong et. al.[9], Liu et. al.[10], and Van Nostrand et. al.[11] had also published their research on differentiation therapy in thyroid tumors. There are no studies covering the pediatric population on this topic, but a better understanding of how the NIS is expressed and the molecular profile in pediatric DTC can determine the profile of patients who benefit from the development of target drugs such as these.

Regarding the limitations of this study, a low sample size of childhood thyroid tumors limits further evaluations, but given a rare tumor in childhood and few studies, we understand the importance of the entire evaluation carried out here. Retrospective studies like this one lead to a lack of data standardization. Although we were careful to exclude those with insufficient information, data such as those related to the dose administered to metastatic or even low-risk CDT pediatric patients may have limited the finding of greater statistical relevance.

Conclusions

Pediatric DTC expresses the NIS protein in smaller quantities in relation to its coding gene *SLC5A5*, determining that intrinsic factors interfere with tumor gene expression. Patients with higher expression of NIS require less retreatment with RIT and possibly lower doses, which can be used as a factor for deciding on adjuvant treatment. The molecular profile of pediatric thyroid tumors was not associated with a different expression of NIS, different from that demonstrated for mutations in adults. New studies using the pediatric population are necessary to improve staging and management of DTC, in addition to stimulate research into specific target drugs.

References

1 - Haugen BR, Alexander EK, Bible KC, *et al.* 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2016;26(1):1-133.

2 - Darrouzet E, Lindenthal S, Marcellin D, *et al*. The sodium/iodide symporter: state of the art of its molecular characterization. Biochim Biophys Acta. 2014;1838(1PtB):244-53.

3 - Carvalho DP, Ferreira AC. The importance of sodium/iodide symporter (NIS) for thyroid cancer management. Arq Bras Endocrinol Metabol. 2007;51(5):672-82.

4 - Gulec SA, Ahuja S, Avram AM, *et al*. A Joint Statement from the American Thyroid Association, the European Association of Nuclear Medicine, the European Thyroid Association, the Society of Nuclear Medicine and Molecular Imaging on Current Diagnostic and Theranostic Approaches in the Management of Thyroid Cancer. Thyroid. 2021;31(7):1009-1019

5 - de Morais RM, Sobrinho AB, de Souza Silva CM, *et al*. The Role of the NIS (SLC5A5) Gene in Papillary Thyroid Cancer: A Systematic Review. Int J Endocrinol. 2018;2018:9128754.

6 - Castillo-Rivera F, Ondo-Méndez A, Guglielmi J, *et al*. Tumor microenvironment affects exogenous sodium/iodide symporter expression. Transl Oncol. 2021;14(1):100937.

7 - Tavares C, Coelho MJ, Eloy C, *et al*. NIS expression in thyroid tumors, relation with prognosis clinicopathological and molecular characteristics. Endocr Connect. 2018;7(1):78-90.

8 - Ward LS, Santarosa PL, Granja F, *et.al*. Low expression of sodium iodide symporter identifies aggressive thyroid tumors. Cancer Lett. 2003;200(1):85-91.

9 - Hong CM, Ahn BC. Redifferentiation of Radioiodine Refractory Differentiated Thyroid Cancer for Reapplication of I-131 Therapy. Front Endocrinol (Lausanne). 2017;8:260.

10 - Liu J, Liu Y, Lin Y, *et.al*. Radioactive Iodine-Refractory Differentiated Thyroid Cancer and Redifferentiation Therapy. Endocrinol Metab (Seoul). 2019;34(3):215-225.

11 - Van Nostrand D, Veytsman I, Kulkarni K, *et al*. Redifferentiation of Differentiated Thyroid Cancer: Clinical Insights from a Narrative Review of Literature. Thyroid. 2023;33(6):674-681.

12 - Patel A, Jhiang S, Dogra S, *et al*. Differentiated thyroid carcinoma that expresses sodium-iodide symporter has a lower risk of recurrence for children and adolescents. Pediatr Res. 2002;52(5):737-44.

13 - Cordioli MI, Moraes L, Alves MT, *et al*. Thyroid-Specific Genes Expression Uncovered Age-Related Differences in Pediatric Thyroid Carcinomas. Int J Endocrinol. 2016;2016:1956740.

14 - Castro P, Patiño E, Fierro F, *et al*. Clinical characteristics, surgical approach, BRAFV600E mutation and sodium iodine symporter expression in pediatric patients with thyroid carcinoma. J Pediatr Endocrinol Metab. 2020;33(11):1457-1463.

15 - Francis GL, Waguespack SG, Bauer AJ, *et al*; American Thyroid Association Guidelines Task Force. Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2015;25(7):716-59.

16 - Lebbink CA, Links TP, Czarniecka A, *et al*. 2022 European Thyroid Association Guidelines for the management of pediatric thyroid nodules and differentiated thyroid carcinoma. Eur Thyroid J. 2022;11(6).

17 - Pires BP, Alves PA Jr, Bordallo MA, *et al*. Prognostic Factors for Early and Long-Term Remission in Pediatric Differentiated Thyroid Carcinoma: The Role of Sex, Age, Clinical Presentation, and the Newly Proposed American Thyroid Association Risk Stratification System. Thyroid. 2016;26(10):1480-1487.

18 - Kim SY, Yun HJ, Chang H, *et al*. Aggressiveness of Differentiated Thyroid Carcinoma in Pediatric Patients Younger Than 16 years: A Propensity Score-Matched Analysis. Front Oncol. 2022;12.

19 - Sweet-Cordero EA, Biegel JA. The genomic landscape of pediatric cancers: implications for diagnosis and treatment. Science. 2019;363(6432):1170-1175.

20 - Lee YA, Lee H, Im SW, *et al*. NTRK and RET fusion-directed therapy in pediatric thyroid cancer yields a tumor response and radioiodine uptake. J Clin Invest. 2021;131(18).

21 - Moleti M, Aversa T, Crisafulli S, *et al*. Global incidence and prevalence of differentiated thyroid cancer in childhood: systematic review and meta-analysis. Front Endocrinol (Lausanne). 2023;14:1270518.

22 - Thiesmeyer JW, Egan CE, Greenberg JA, *et al*. Prepubertal Children with Papillary Thyroid Carcinoma Present with More Invasive Disease Than Adolescents and Young Adults. Thyroid. 2023;33(2):214-222.

23 - Sweet-Cordero EA, Biegel JA. The genomic landscape of pediatric cancers: implications for diagnosis and treatment. Science. 2019;363(6432):1170-1175.

24 - de Sousa MSA, Nunes IN, Christiano YP, *et al*. Genetic alterations landscape in pediatric thyroid tumors and/or differentiated thyroid cancer: Systematic review. Rev Endocr Metab Disord. 2023. Epub ahead of print.

25 - Morari EC, Marcello MA, Guilhen AC, *et al*. Use of sodium iodide symporter expression in differentiated thyroid carcinomas. Clin Endocrinol(Oxf). 2011;75(2):247-54.

26 - Patel A, Jhiang S, Dogra S, *et al*. Differentiated thyroid carcinoma that expresses sodium-iodide symporter has a lower risk of recurrence for children and adolescents. Pediatr Res. 2002;52(5):737-44.

27 - Lee YA, Lee H, Im SW, *et al*. NTRK and RET fusion-directed therapy in pediatric thyroid cancer yields a tumor response and radioiodine uptake. J Clin Invest. 2021;131(18):e144847.

28 - Romei C, Ciampi R, Faviana P, *et al*. BRAFV600E mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer. Endocr Report Cancer. 2008;15(2):511-20.

29 - Tavares C, Coelho MJ, Eloy C, *et al*. NIS expression in thyroid tumors , relation with prognosis clinicopathological and molecular characteristics . Endocr Connect. 2018;7(1):78-90.

30 - Nikitski AV, Condello V, Divakaran SS, *et al*. Inhibition of ALK-Signaling Overcomes STRN-ALK-Induced Downregulation of the Sodium Iodine Symporter and Restores Radioiodine Uptake in Thyroid Cells. Thyroid. 2023;33(4):464-473.

Tables

Table 1 – Clinical and histological characteristics of 72 patients with stored sample.

CATEGORY	N OR MED. (% OR S.D.)	MINIMUM	MAXIMUM
NUMBER OF PATIENTS	72	-	-
FOLLOW-UP (YEARS)	8.6 (±5.9)	0.6	23
FEMALE GENDER	47 (65%)	-	-
AGE AT DIAGNOSIS (YEARS)	15.0 (± 3.7)	4.7	18.8
COMPLETE PUBERTY	43 (60%)	-	-
PAPILLARY TYPE	70 (97%)	-	-
TUMOR SIZE (CM)	2.5 (± 1.6)	0.8	5.6
NODAL METASTASIS	51 (71%)	-	-
DISTANT METASTASIS	16 (22%)	-	-
ATA PEDIATRIC RISK GROUP			
LOW	19 (26%)	-	-
INTERMEDIARY	7 (10%)	-	-
HIGH	46 (64%)	-	-
I-131 MEDIAN CUMULATIVE DOSE (mCi)	150 (± 160)	30	800
PATIENTS WITH MULTIPLE (> 1) RIT	16 (22%)	1	5
TREATMENT RESPONSE AT THE END OF *FOLLOW-UP*			
EXCELLENT RESPONSE	40 (55%)	-	-
INCOMPLETE BIOCHEMICAL RESPONSE	3 (4%)	-	-
INDETERMINATE RESPONSE	9 (13%)	-	-
INCOMPLETE STRUCTURAL RESPONSE	20 (28%)	-	-

ATA - American Thyroid Association, I-131 - Iodide-131, mCi - millicurie, RIT – RadioIodine treatment
N. – Number, Med. – Median, S.D. – Standard deviation.

Table 2 – Results of NIS expression associated with clinical and response to therapy.

	Expression			*Expression*		
Category	**SLC5A5 -**	**SLC5A5 +**	***p***	**NIS -**	**NIS +**	***p***
Total population	33 (46%)	39 (54%)	-	54 (75%)	18 (25%)	-
NIS+	5 (7%)	13 (18%)	0.10	-	-	-
NIS -	28 (39%)	26 (36%)		-	-	
Female gender	22 (31%)	25 (35%)	>0.99	34 (47%)	13 (18%)	0.57
Male gender	11 (15%)	14 (19%)		20 (28%)	5 (7%)	
Complete puberty	21 (28%)	22 (31%)	0.10	31 (43%)	12 (17%)	0.58
Un or Incomplete puberty	12 (17%)	17 (24%)		23 (32%)	6 (8%)	
Distant Metastasis Yes	7 (10%)	9 (12%)	>0.99	14 (19%)	2 (3%)	0.32
Distant metastasis No	26 (36%)	30 (42%)		40 (56%)	16 (22%)	
ATA High Risk Yes	22 (31%)	24 (33%)	0.80	36 (50%)	10 (14%)	0.41
ATA High Risk No	11 (15%)	15 (21%)		18 (25%)	8 (11%)	
Multiples (> 1) RIT	11 (15%)	5 (7%)	0.08	15 (21%)	1 (1%)	**0.05**
Unique RIT	22 (31%)	34 (47%)		39 (54%)	17 (24%)	
Dose < 150 mCi	8 (11%)	14 (19%)	0.32	15 (21%)	7 (10%)	0.39
Dose ≥150 mCi	25 (35%)	25 (35%)		39 (54%)	11 (15%)	
Excellent response No	13 (18%)	19 (26%)	0.48	25 (35%)	7 (10%)	0.78
Excellent response Yes	20 (28%)	20 (28%)		29 (40%)	11 (15%)	
Structural incomplete response No	26 (36%)	26 (36%)	0.30	37 (51%)	15 (21%)	0.36
Structural incomplete response Yes	7 (10%)	13 (18%)		17 (24%	3 (4%)	

NIS - Sodium-Iodide symporter, ATA - American Thyroid Association, RIT - RadioIodine treatment, mCi - miliCurrie

Table 3 – Results of NIS expression associated with genetic molecular profile.

	Expression			***Expression***		
Category	**SLC5A5 -**	**SLC5A5 +**	***p***	**NIS -**	**NIS +**	***p***
Total population	13 (36%)	23 (64%)	-	25 (69%)	11 (31%)	-
***RET/PTC* fusion No**	4 (11%)	13 (36%)	0.61	10 (28%)	7 (19%)	0.28
***RET/PTC* fusion Yes**	9 (26%)	10 (28%)		15 (42%)	4 (11%)	
***NTRK* fusions No**	10 (28%)	11 (31%)	0.15	14 (39%)	7 (19%)	0.73
***NTRK* fusions Yes**	3 (8%)	12 (33%)		11 (31%)	4 (11%)	
***AGK::BRAF* fusion No**	13 (36%)	20 (56%)	0.53	22 (61%)	11 (31%)	0.54
***AGK::BRAF* fusion Yes**	0	3 (8%)		3 (8%)	0	
***BRAF_V600E* No**	10 (28%)	19 (53%)	0.67	21 (58%)	8 (23%)	0.65
***BRAF_V600E* Yes**	3 (8%)	4 (11%)		4 (11%)	3 (8%)	
***STRN::ALK* fusion No**	13 (36%)	19 (53%)	0.27	23 (63%)	9 (25%)	0.57
***STRN::ALK* fusion Yes**	0	4 (11%)		2 (6%)	2 (6%)	

NIS - Sodium-Iodide symporter

Figures

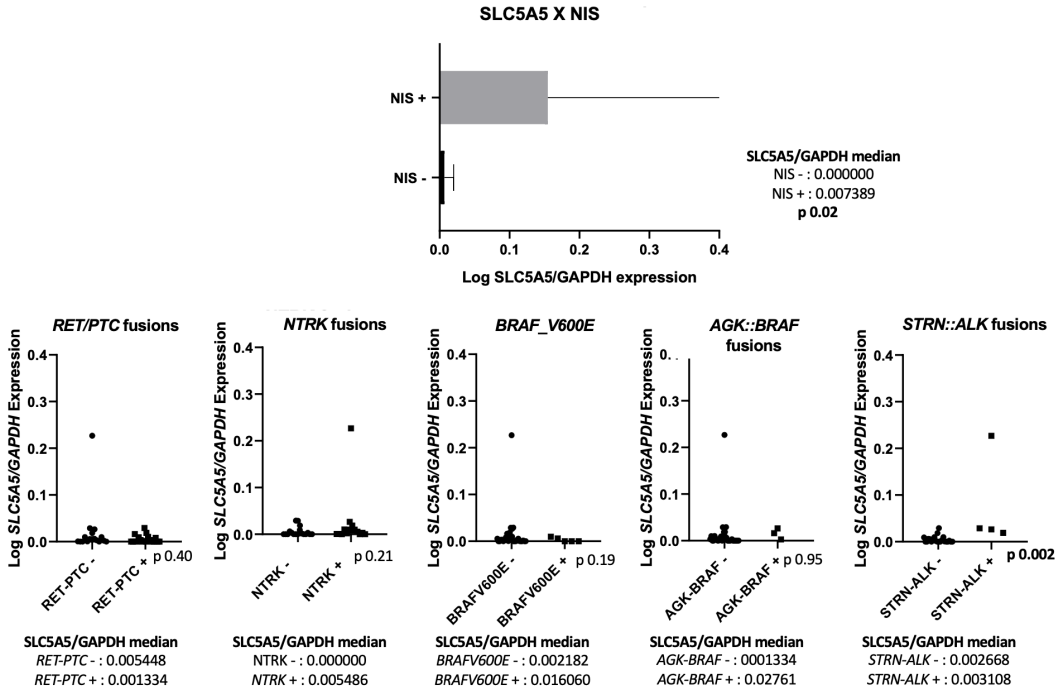

Figure 1 – Comparable of SLC5A5 / GAPDH median expression.

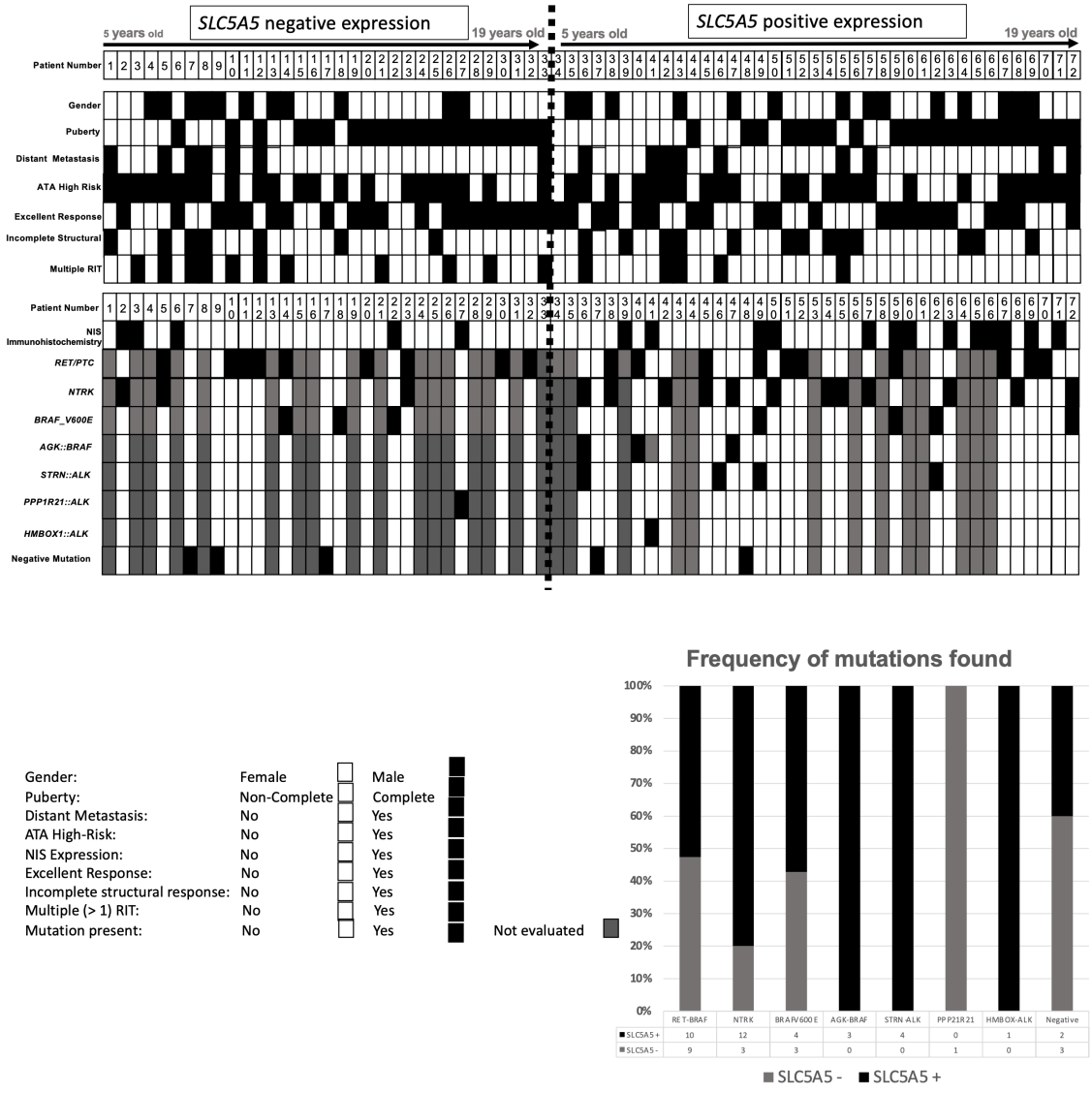

Figure 2 – Individual analysis of 72 patients ordered by *SLC5A5* expression and chronological age and frequency of mutations found.

5- Comentários e discussão geral

Nesta tese, ao usarmos o CDT pediátrico como modelo, percebemos a complexidade de se tentar definir um tumor maligno apenas por sua histologia, forma de apresentação ou resposta ao tratamento proposto. Mesmo ao analisarmos mais a fundo, levando em consideração as alterações moleculares representativas de uma classe de câncer, muitas vezes falhamos em prever seu comportamento. Hoje, cada vez mais entendemos que mecanismos genéticos e pós-transcricionais estão atuando em conjunto associados à toda a maquinaria celular para modificar mecanismos proliferativos ou anti-apoptóticos.[30]

Para elaborar estudos e condutas sobre o câncer usando a população pediátrica, não basta apenas selecionar pacientes abaixo de uma determinada idade e aplicar as mesmas orientações destinadas à adultos. Devemos saber que estaremos diante de uma doença rara, com diversos fatores peculiares a estes indivíduos como, por exemplo, o processo de crescimento, a complexidade do fenômeno da puberdade ou mesmo um perfil genético diferenciado que podem isoladamente ou de forma associada, atuar como modificadores de incidência, apresentação e respostas ao tratamento.[26,31] A criança não é um “adulto pequeno”.

Exemplificando este cenário, estudos sobre pacientes com CDT diagnosticados antes dos 18 anos de idade revelam uma apresentação bem diferente da população adulta.[8] Além disso, dentro do subgrupo representado pela própria população pediátrica, existem características dicotômicas, em que pacientes com idade maior que 10 anos de idade se associam à uma apresentação mais branda da doença quando comparado ao diagnóstico de pacientes em idade mais jovem.[9-11]

Um dado interessante relacionado ao CDT de forma geral é o maior acometimento de pessoas do sexo feminino, algo raro na oncologia quando excluídos os canceres ginecológicos e de mama.[32] Fato curioso é que quando analisamos somente crianças, algo ocorre mais uma vez perto dos 10 anos de idade a ponto de modificar a relação de prevalência entre os sexos. Entre os mais jovens há um maior diagnóstico da doença no sexo masculino e a partir dos 10 anos de idade esta relação se inverte, com predomínio bem maior do sexo feminino,[33] seguindo assim por toda a vida adulta.[7] Associado a este fato, também chama a atenção um pico de incidência do CDT pediátrico na adolescência, principalmente

em meninas de 15 anos, representando o segundo câncer mais diagnosticado em meninas dessa faixa etária.[1]

Foi a partir deste cenário que começamos a cogitar uma possível influência da puberdade implicada no processo de desenvolvimento do câncer de tireoide pediátrico. Acreditávamos que possivelmente a atuação de esteroides sexuais, prioritariamente o estrogênio, poderia ser um gatilho para a gênese do tumor, visto que antes dos 10 anos de idade a exposição a este hormônio seria mínima, com um aumento gradual na puberdade e maior entre aqueles do sexo feminino. Os estudos de *Imai et.al.*[34] e *Joseph et.al.*[35] descrevendo possíveis conexões de estrogênio com o tumor tireoidiano e a associação de câncer de mama com o câncer de tireoide tornaram-se incentivadores para que avaliássemos a hipótese da puberdade e do estrogênio como associados ao CDT pediátrico.

Revimos nossos dados publicados em 2016 que confirmavam a prevalência do sexo feminino a partir dos 10 anos[10] e objetivamos então avaliar em 2022[36] a associação da doença com receptores de estrogênio e progesterona. Neste último estudo citado, não conseguimos confirmar nossa hipótese de relação direta entre estrogênio e o CDT pediátrico, talvez pela técnica adotada, mas muito possivelmente pelo baixo número de amostras representativas de uma doença rara.

Nesta tese voltamos a estudar o sexo feminino e a puberdade atrelados ao CDT pediátrico. Aqui, demonstramos a mudança da relação de prevalência da doença entre sexos masculino e feminino, mas agora não mais atrelada à idade, mas ao fenômeno de puberdade completa. Também fomos capazes de confirmar uma maior agressividade da doença, categorizada como um maior número de metástases, em pacientes com puberdade não-completa. Estes resultados estão mais bem detalhados em um dos artigos resultantes desta tese intitulado "*Distinct genetic profiles, puberty and sodium-iodide symporter (NIS) in pediatric differentiated thyroid carcinoma*".

Uma outra característica relacionada ao CDT pediátrico que nos estimula a aprofundar nosso conhecimento é o fato de uma doença maligna com apresentação mais agressiva possuir taxas de mortalidade extremamente baixas, principalmente compara à adultos.[1,8]

Este foi um questionamento que se seguiu após as análises preliminares e revisão da literatura. O fato de existirem poucos estudos na área dificultavam este entendimento. A existência de praticamente um único *Guideline (ATA,* 2015*)*[1], que

refere entender as peculiaridades da população pediátrica, mas mantém muitas recomendações baseadas na população adulta poderia estar nos levando a possíveis vieses ou mesmo excesso de tratamento para pacientes menores de 18 - 20 anos.

Assim, entender melhor o estadiamento, os métodos utilizados para classificar os pacientes em risco de recidiva e principalmente a resposta ao tratamento utilizado passaram a ser o cerne desta pesquisa. De forma mais objetiva, como poderíamos aperfeiçoar as recomendações do *Guideline* pediátrico?

Optamos inicialmente por rever a orientação da *ATA* Pediátrica de utilizar a PCI-diagnóstica para o estadiamento de pacientes pediátricos. Em nossa experiência, havíamos percebido discrepâncias de achados de imagem entre a PCI-diagnóstica e a PCI pós-tratamento. Em 2013, *Bravo et.al.*[37] demonstrava este fato, mas o *Guideline* de 2015 seguiu sugerindo a utilização da PCI diagnóstica como norteador de avaliação de metástases. Nossa interpretação é que a aplicação de baixa atividade (geralmente são instituídos 1 a 2 mci de radioatividade) de Iodo-131 na PCI-diagnóstica poderia estar levando a resultados falso-negativos, principalmente entre aqueles com muita doença metastática em que o iodo precisar se distribuir em diversas células tumorais.

Avaliamos então os estudos por imagens funcionais (PCI) com Iodo-131 em uma coorte de 127 pacientes submetidos à RIT, tendo 66 deles realizado a PCI-diagnóstica. Foi confirmado neste estudo que a PCI-diagnóstica teve uma baixa sensibilidade em detectar metástase à distância e um valor preditivo negativo de apenas 74%. Um dos principais destaques desta primeira fase da pesquisa foi o encontro de uma discrepância na detecção de doença metastática em 48% entre a PCI-diagnóstica e a PCI-pós-tratamento, confirmando assim muitas imagens falso-negativas na PCI-diagnóstica e que determinaram o emprego de atividade de Iodo-131 abaixo do ideal para o tratamento. Portanto, a PCI-diagnóstica condicionava um subestadiamento da doença em quase metade dos pacientes, com necessidade de retratamento com RIT em alguns destes.

Neste primeiro estudo também pudemos comparar a performance da PCI e da Tgs em detectar doença metastática. Revelamos primeiramente que a mediana dos níveis de Tgs em nossos pacientes onde não seria indicado tratamento com RIT (16,5 ng/dl naqueles classificados como baixo risco de recorrência) foram mais elevados que os descritos no *Guideline*[1] (> 10 ng/dl) para indicar tratamento com

RIT independente do encontro de metástases. Este dado demonstra que muitos dos nossos pacientes (aqueles com Tgs > 10 ng/dl) receberiam RIT, apesar de representarem um baixo risco de recorrência. Também foi demonstrado que os valores de Tgs encontrados após a tireoidectomia estão associados a um risco diferenciado de recorrência (baixo 16,5 ng/dl; intermediário 11,3 ng/dl; alto 45ng/dl; p 0,001) e a uma resposta excelente (Tgs 13 ng/dl) ou não-excelente (48,9 ng/dl) ao final do seguimento (p 0,004), além de possuir uma melhor performance quando comparados à PCI-diagnóstica.

O artigo relacionado a esta tese intitulado "*Stimulated thyroglobulin and diagnostic 131-iodine whole-body scan as a predictor of distant metastasis and association with response to treatment in pediatric thyroid cancer patients*" detalha os achados acima descritos e conclui que a PCI-diagnóstica pode ser dispensável e que a Tgs pode ser um importante fator prognóstico.

A PCI com Iodo-131 como um exame funcional na procura de metástases depende da manutenção da proteína NIS na célula tumoral. O Iodo-131, portanto, possui uma propriedade dita teragnóstica, pois atua tanto diagnosticando, como servindo de tratamento para células tireoidianas tumorais.[12] Acontece que algumas das células tumorais perdem a capacidade de expressar o NIS, dificultando assim a ação radioativa do Iodo-131.[15-17] Esta incapacidade de expressão vem sendo mais amplamente estudada com o desenvolvimento de terapias-alvo que permitem uma reexpressão do NIS em tumores antes considerados iodo-refratários, favorecendo um novo ataque às células tumorais com RIT.[18-20]

Alguns estudos demonstram que o perfil genético do tumor está relacionado com detecções diferenciadas de NIS ou de expressões seu gene codificante *SLC5A5*.[38-39] Como exemplo, *Romei et. al.*[39] demonstrou que tumores que carreiam a mutação *BRAF_V600E* parecem expressar menos o NIS, diferentemente daqueles que expressavam a fusão *RET/PTC*. Em um dos principais estudos sobre o assunto, *Tavares et al.*[16] revelou uma menor detecção e expressão de NIS nos CDT e associou a expressão com a resposta ao tratamento e com o perfil molecular do tumor.

Poucos estudos avaliaram o NIS no CDT de crianças e adolescentes.[21-23] Entendemos que este é um assunto extremamente relevante uma vez que possuem uma apresentação com mais metástases, conferindo a necessidade de tratamento adjuvante em mais de 70 % dos pacientes e por possuírem uma assinatura

molecular diferenciada de adulto, o que poderia determinar também expressões alteradas do NIS, como antes mencionado. Além disso, a otimização da atividade de Iodo-131 ou números de retratamento poderiam sofrer influência desta expressão, podendo ser aventado uma menor atividade naqueles com maior expressão de NIS, reduzindo riscos de complicações associadas a terapia ionizante.

Assim, desenvolvemos como objetivo principal desta tese a avaliação da detecção da proteína NIS e expressão do gene *SLC5A5* no CDT pediátrico e avaliamos em conjunto, o perfil molecular, a apresentação clínica e a resposta ao tratamento ao final do seguimento.

Inicialmente achamos que a avaliação da detecção proteica através da imuno-histoquímica poderia responder nossas dúvidas, mas ao avaliarmos uma baixa detecção da proteína NIS nas células tumorais a despeito de uma excelente resposta ao tratamento no final do seguimento, entendemos que deveríamos avaliar a relação gene-proteína (*SLC5A5* - NIS) e possíveis moduladores desta expressão, como puberdade e a assinatura molecular do tumor.

Aqui é importante ser citado que a assinatura genética tumoral já era associada a diferenças prognósticas e que o perfil de crianças e adolescentes é bem diferente daquele descrito para adultos. Um maior número de rearranjos no lugar de mutações pontuais é mais encontrado nos CDT pediátricos. A fusão *RET/PTC* é a mais comum, além de genes como *NTRK*, *AGK*, *ALK*, *BRAF*. Em relação às relações prognósticas, *Franco e colaboradores* [40] demonstraram um pior prognóstico entre os CDT pediátricos que carreavam as fusões com *RET* e *NTRK*. Em 2019 demonstramos que a fusão *AGK::BRAF* esteve mais associada com metástases à distância.[41]

Nesta tese pudemos demonstrar que o perfil genético tumoral se associou também com a puberdade. Crianças sem puberdade completa estavam mais associados com fusões tipo *NTRK* ou *AGK::BRAF* e ao mesmo tempo mais relacionados a uma pior apresentação da doença, diferentes daqueles com puberdade completa, em que encontramos mutações mais parecidas com a de adultos jovens como *BRAF_V600E* e uma maior prevalência do *RET/PTC*, mas clinicamente associado a menores taxas de metástases. Assim, justificamos que a apresentação e a resposta ao tratamento do CDT pediátrico não estão associadas à puberdade ou assinatura molecular de maneira isolada, mais sim a condições mutuamente inclusivas.

Independente deste perfil molecular dicotômico, não houve registro de óbitos entre os pacientes e o número daqueles com evidência de doença (Resposta Estrutural Incompleta) ao final do seguimento foi bem menor (18 – 28% da população avaliada) que o previsto ao utilizar o sistema de estadiamento ao diagnóstico (56 – 58 %). Isto nos leva a pensar que possamos estar errando ao classificar os pacientes pediátricos no risco de recorrência (detalhado no início desta discussão) ou existem outros fatores associados a uma melhor resposta ao tratamento, podendo ser uma maior sensibilidade ao RIT e não o perfil molecular isoladamente.

Avaliamos então a detecção da proteína NIS e posteriormente a expressão do gene *SLC5A5* nas células tumorais tireoidianas de crianças e adolescentes.

Neste estudo, percebemos um baixo número de amostras com detecção da proteína NIS, o que poderia determinar piores respostas ao tratamento adjuvante, apesar de termos demonstrado excelentes taxas de resposta e mortalidade igual a zero. Avaliando então a expressão de seu gene codificante, como esperado, houve uma associação da proteína com o gene (p 0,02), porém com o NIS sendo detectado em menos amostras (25%) que a expressão do gene *SLC5A5* (54%). Isto é mais um indicador de que fatores pós-transcricionais estão atuando sobre a incorporação do NIS na membrana das células tumorais.

Cabe relatar que para a sua atuação, o Iodo-131 necessita, além da síntese proteica do NIS, da sua incorporação em uma localização específica na célula que é a membrana plasmática. Entender então possíveis fatores transcricionais e pós-transcricionais e como eles atuam é fundamental na otimização do tratamento do CDT pediátrico.

Das análises realizadas, apenas identificamos a expressão do *SLC5A5* associada com a fusão *STRN::ALK (p 0,002)*. Não conseguimos de maneira significativa associar a expressão do NIS com puberdade, sexo, apresentação da doença, resposta ao tratamento ou outros perfis mutacionais. Um dado encontrado e significativo foi a maior expressão naqueles que fizeram menores números de RIT (p 0,05) e possivelmente entre aqueles com menores doses acumulativas (p 0,08), sugerindo que a avaliação do NIS pode otimizar o tratamento com RIT com necessidade de menores doses de Iodo-131 naqueles com maior expressão.

O artigo "*Revisiting the expression of the Sodium-Iodide Symporter (NIS) through the SLC5A5 gene in pediatric differentiated thyroid carcinoma. A study on the*

assessment of NIS and its association with molecular profile and treatment in pediatric thyroid cancer." trata das análises realizadas para o NIS e o gene *SLC5*A5. A figura 2 aqui replicada a partir deste artigo (Artigo 3) facilita a avaliação das principais alterações individuais encontradas, podendo ser visto o paciente em sua integralidade e como subgrupo para expressão de *SLC5A5*. A figura 3 traz um resumo dos achados clínicos associados ao estágio puberal e principais alterações moleculares encontradas neste estudo.

Quanto às principais limitações deste estudo temos aquelas relacionadas a análise retrospectiva, como por exemplo a interrupção da utilização da PCI-diagnóstica de maneira sistemática em nosso serviço desde 2015, levando a um baixo número de pacientes com este dado para análise. Ainda assim, conseguimos demonstrar sua não necessidade.

A falta de uma padronização para avaliação de puberdade em prontuários, principalmente entre os mais antigos, foi mais um fator relacionado ao caráter retrospectivo. Minimizamos esta análise ao utilizar o critério "puberdade completa" adotado na literatura e descrito em nosso artigo[36] que descrevem a mediana de idade de meninos no Brasil com puberdade completa (G5) de 15,8 anos de idade. Ainda assim, optamos por usar o ponto de corte de 16 anos de idade no sexo masculino para garantir que realmente estávamos diante uma população com puberdade completa, além de usarmos o marcador de menarca entre as meninas, este mais estabelecido.

Também entendemos que o número total de amostras tumorais possa ter sido pequeno. Isto fica claro quando atingíamos valores estatístico de *p* próximo ao de significância. Cabe aqui relatar que usamos como modelo uma doença rara e que a nobreza representada pelo material tumoral de uma tireoidectomia é único e não podia ser esgotado. Fizemos questão de tomar este cuidado de forma que em alguns pacientes não pudemos fazer toda a análise pretendida.

O custo de pesquisas translacionais também se torna impactante e este foi um dos motivos de a tese ter sido feita em etapas.

Em relação às perspectivas futuras, entendemos a necessidade de novos estudos sobre a atuação dos esteroides sexuais no câncer de tireoide para respondermos definitivamente se há uma atuação do estrogênio na gênese tumoral, principalmente na população pediátrica. Estes dados podem favorecer uma mudança no tratamento do CDT, assim como ocorreu no câncer de mama ao se

utilizar inibidores de ação do estrogênio. Também sugerimos o desenvolvimento de outros estudos que validem os valores séricos mais altos de Tgs na população pediátrica e sua incorporação no estadiamento da doença. Outro ponto importante, ao entendermos que fatores pós-transcricionais estão associados ao desenvolvimento do câncer, necessitamos nos aprofundar mais sobre pesquisas em proteômica e metabolômica para melhor entender a relação gene-proteína.

6 – Conclusões

As alterações moleculares tipo rearranjo/fusão estão mais presentes nos tumores pediátricos, sendo a *RET/PTC* a mais encontrada. O NIS foi detectado em 25% das células tumorais de crianças e adolescentes com CDT, apesar de uma maior expressão (55%) de seu gene codificante *SLC5A5*.

A PCI-diagnóstica possui uma baixa performance na detecção de metástase à distância e na modalidade prognóstica, podendo ser preferível os valores de Tgs como preditores de resposta ao tratamento.

A puberdade determina mudanças na prevalência entre os sexos e menor gravidade na apresentação da doença, mas não modificam a resposta ao tratamento.

As fusões com *NTRK* e o *AGK::BRAF* estão mais presentes em crianças menores, grupo que apresenta maior gravidade de apresentação da doença, enquanto aqueles com puberdade completa apresentam mutações mais parecidas com a de adultos jovens (*BRAF_V600E* e *RET/PTC*), grupo mais associado a menores taxas de metástases. Apesar deste perfil dicotômico, a assinatura genética não interfere na resposta ao tratamento, mantendo a população pediátrica com altas taxas de sobrevida.

A proteína NIS se associou com menores necessidades de tratamento com RIT, e apesar de estar associada ao seu gene codificante *SLC5A5,* não revelou expressões diferenciadas associadas à puberdade, critérios clínicos, resposta ao tratamento ou a um perfil molecular específico, exceto uma maior expressão entre aqueles que carreiam a fusão *STRN::ALK*.

7 - Bibliografia

1. Francis GL, Waguespack SG, Bauer AJ, *et al*. Management guidelines for children with thyroid nodules and differentiated thyroid cancer. *Thyroid.* 2015;25:716-759.

2. Lebbink CA, Links TP, Czarniecka A, *et al*. 2022 European thyroid association guidelines for the management of pediatric thyroid nodules and differentiated thyroid carcinoma. *Eur Thyroid J.* 2022;11:e220146.

3. SEER. Cancer stat facts: thyroid cancer. Available at: https://seer.cancer.gov/statfacts/html/thyro.html.

4. Miranda-Filho A, Lortet-Tieulent J, Bray F, *et al*. Thyroid cancer incidence trends by histology in 25 countries: a population-based study. *Lancet Diabetes Endocrinol.* 2021;9(4):225-234.

5. Instituto Nacional de Câncer (Brasil). Estimativa 2023: Incidência de câncer no Brasil / Instituto nacional de Câncer – Rio de Janeiro:INCA. 2022.

6. De Souza Reis R, Gatta G, De Camargo B. Thyroid carcinoma in children, adolescents, and young adults in Brazil: a report from 11 population-based cancer registries. *PLoS One.* 2020;15:e0232416.

7. Haugen BR, Alexander EK, Bible KC, *et al*. 2015 American thyroid association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American thyroid association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid.* 2016;26:1-133.

8. Jarzab B, Handkiewicz-Junak D. Differentiated thyroid cancer in children and adults: same or distinct disease? *Hormones (Athens)*. 2007;6(3):200-9.

9. Kim SY, Yun HJ, Chang H, *et al*. Aggressiveness of Differentiated Thyroid Carcinoma in Pediatric Patients Younger Than 16 years: A Propensity Score-Matched Analysis. *Front Oncol*. 2022;12:872130.

10. Pires BP, Alves PA Jr, Bordallo MA, *et al*. Prognostic Factors for Early and Long-Term Remission in Pediatric Differentiated Thyroid Carcinoma: The Role of Sex, Age, Clinical Presentation, and the Newly Proposed American Thyroid Association Risk Stratification System. *Thyroid.* 2016;26(10):1480-1487.

11. Lazar L, Lebenthal Y, Steinmetz A, *et al*. Differentiated thyroid carcinoma in pediatric patients: comparison of presentation and course between pre-pubertal children and adolescents. *J Pediatr.* 2009;154(5):708-14.

12. Gulec SA, Ahuja S, Avram AM, *et al*. A Joint Statement from the American Thyroid Association, the European Association of Nuclear Medicine, the European Thyroid Association, the Society of Nuclear Medicine and Molecular Imaging on Current Diagnostic and Theranostic Approaches in the Management of Thyroid Cancer. *Thyroid.* 2021;31(7):1009-1019.

13. Darrouzet E, Lindenthal S, Marcellin D, *et al*. The sodium/iodide symporter: state of the art of its molecular characterization. *Biochim Biophys Acta.* 2014;1838(1 Pt B):244-53.

14. Carvalho DP, Ferreira AC. The importance of sodium/iodide symporter (NIS) for thyroid cancer management. *Arq Bras Endocrinol Metabol*. 2007;51(5):672-82.

15. Castillo-Rivera F, Ondo-Méndez A, Guglielmi J, *et al*. Tumor microenvironment affects exogenous sodium/iodide symporter expression. *Transl Oncol*. 2021;14(1).

16. Tavares C, Coelho MJ, Eloy C, *et al*. NIS expression in thyroid tumors, relation with prognosis clinicopathological and molecular characteristics. *Endocr Connect*. 2018;7(1):78-90.

17. Ward LS, Santarosa PL, Granja F, *et al*. Low expression of sodium iodide symporter identifies aggressive thyroid tumors. *Cancer Lett*. 2003;200(1):85-91.

18. Hong CM, Ahn BC. Redifferentiation of Radioiodine Refractory Differentiated Thyroid Cancer for Reapplication of I-131 Therapy. *Front Endocrinol (Lausanne)*. 2017;8:260.

19. Liu J, Liu Y, Lin Y, *et al*. Radioactive Iodine-Refractory Differentiated Thyroid Cancer and Redifferentiation Therapy. *Endocrinol Metab (Seoul)*. 2019;34(3):215-225.

20. Van Nostrand D, Veytsman I, Kulkarni K, *et al*. Redifferentiation of Differentiated Thyroid Cancer: Clinical Insights from a Narrative Review of Literature. Thyroid. 2023;33(6):674-681.

21. Patel A, Jhiang S, Dogra S, *et al*. Differentiated thyroid carcinoma that expresses sodium-iodide symporter has a lower risk of recurrence for children and adolescents. *Pediatr Res*. 2002;52(5):737-44.

22. Cordioli MI, Moraes L, Alves MT, *et al*. Thyroid-Specific Genes Expression Uncovered Age-Related Differences in Pediatric Thyroid Carcinomas. *Int J Endocrinol*. 2016;2016:1956740.

23. Castro P, Patiño E, Fierro F, *et al*. Clinical characteristics, surgical approach, BRAFV600E mutation and sodium iodine symporter expression in pediatric patients with thyroid carcinoma. *J Pediatr Endocrinol Metab*. 2020;33(11):1457-1463.

24. Vogelstein B, Papadopoulos N, Velculescu VE, *et al*. Cancer genome landscapes. *Science*. 2013;339(6127):1546-58.

25. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell*. 2014;159(3):676-90.

26. Sweet-Cordero EA, Biegel JA. The genomic landscape of pediatric cancers: Implications for diagnosis and treatment. *Science*. 2019;363(6432):1170-1175.

27. Guo K, Qian K, Shi Y, *et al*. Clinical and Molecular Characterizations of Papillary Thyroid Cancer in Children and Young Adults: A Multicenter Retrospective Study. *Thyroid*. 2021;31(11):1693-1706.

28. Alzahrani AS, Alswailem M, Alswailem AA, *et al*. Genetic Alterations in Pediatric Thyroid Cancer Using a Comprehensive Childhood Cancer Gene Panel. *J Clin Endocrinol Metab*. 2020;105(10).

29. Nikita ME, Jiang W, Cheng SM, *et al*. Mutational Analysis in Pediatric Thyroid Cancer and Correlations with Age, Ethnicity, and Clinical Presentation. *Thyroid*. 2016;26(2):227-34.

30. Filbin M, Monje M. Developmental origins and emerging therapeutic opportunities for childhood cancer. *Nat Med*. 2019;25(3):367-376.

31. Venkataramany AS, Schieffer KM, Lee K, *et al*. Alternative RNA splicing defects in pediatric cancers: new insights in tumorigenesis and potential therapeutic vulnerabilities. *Ann Oncol*. 2022;33(6):578-592.

32. -Shobab L, Burman KD, Wartofsky L. Sex Differences in Differentiated Thyroid Cancer. *Thyroid*. 2022;32(3):224-235.

33. Hogan AR, Zhuge Y, Perez EA, *et al*. Pediatric thyroid carcinoma: incidence and outcomes in 1753 patients. *J Surg Res*. 2009;156(1):167-72.

34. Imai Y, Yamakawa M, Matsuda M, Kasajima T. Endogenous sex hormone and estrogen binding activity in thyroid cancer. *Histol Histopathol*. 1989;4(1):39-45.

35. Joseph KR, Edirimanne S, Eslick GD. The association between breast cancer and thyroid cancer: a meta-analysis. *Breast Cancer Res Treat*. 2015;152(1):173-181.

36. da Silva Breder JRA, Alves PAG, Araújo ML, *et al*. Puberty and sex in pediatric thyroid cancer: could expression of estrogen and progesterone receptors affect prognosis? *Eur Thyroid J*. 2022;11(2)

37. Bravo PE, Goudarzi B, Rana U, *et al*. Clinical significance of discordant findings between pre-therapy (123)I and post-therapy (131)I whole body scan in patients with thyroid cancer. *Int J Clin Exp Med*. 2013;6(5):320-33.

38. Nikitski AV, Condello V, Divakaran SS, *et al*. Inhibition of ALK-Signaling Overcomes STRN-ALK-Induced Downregulation of the Sodium Iodine Symporter and Restores Radioiodine Uptake in Thyroid Cells. *Thyroid*. 2023;33(4):464-473.

39. Romei C, Ciampi R, Faviana P, *et al*. BRAFV600E mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer. *Endocr Relat Cancer*. 2008;15(2):511-20.

40. Franco AT, Ricarte-Filho JC, Isaza A, *et al*. Fusion Oncogenes Are Associated With Increased Metastatic Capacity and Persistent Disease in Pediatric Thyroid Cancers. *J Clin Oncol*. 2022;40(10):1081-1090.

41. Sisdelli L, Cordioli MICV, Vaisman F, *et al*. AGK-BRAF is associated with distant metastasis and younger age in pediatric papillary thyroid carcinoma. *Pediatr Blood Cancer*. 2019;66(7).

8 - Anexos

Tabelas

Tabela 1 – Sistema de estadiamento inicial para o carcinoma diferenciado de tireoide pediátrico.

CATEGORIA DE RISCO DA ATA PEDIÁTRICA	DEFINIÇÃO
BAIXO	Doença confinada à tireoide com ausência ou desconhecimento de acometimento de linfonodo ou pacientes com doença linfonodal incidental em nível cervical VI.
INTERMEDIÁRIO	Doença linfonodal extensa em nível cervical VI ou mínimo acometimento linfonodal cervical fora do nível VI.
ALTO	Doença linfonodal extensa fora do nível cervical VI ou doença localmente invasiva, com ou sem metástase à distância.

Adaptado do *Guideline* da ATA 2015[1].

Tabela 2 – Classificação de resposta ao tratamento para o carcinoma diferenciado de tireoide.

CATEGORIA DE RESPOSTA	ACHADOS
EXCELENTE	Imagem negativa e Tg < 0,2 ng/ml ou Tgs < 1 ng/ml
BIOQUÍMICA INCOMPLETA	Imagem negativa e Tg ≥ 1 ng/ml ou Tg ≥ 10 ng/ml ou Anticorpos anti-Tg em elevação
ESTRUTURAL INCOMPLETA	Evidência de doença estrutural ou funcional com qualquer nível de Tg, independente do anticorpo anti-Tg
INDETERMINADO	Achados inespecíficos nos estudos de imagem, PCI com captação no leito tireoidiano, Tg detectável, mas < 1 ng/ml ou Tgs < 10 ng/ml ou anticorpos anti-Tg estáveis ou em declínio na ausência de doença estrutural ou funcional

Adaptado do Guideline de adulto da ATA.[7] Tg - Tireoglobulina, Tgs – Tireoglobulina estimulada, Anti-Tg – Anticorpo anti-tireoglobulina, PCI – Pesquisa de corpo inteiro.

Figuras

UFRJ - HOSPITAL UNIVERSITÁRIO CLEMENTINO FRAGA FILHO DA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Carcinoma diferenciado de tireóide na infância e adolescência;Análise comparativa entre as faixas pré e pós puberais.
Pesquisador: Fernanda Vaisman
Área Temática:
Versão: 2
CAAE: 66569517.8.0000.5257
Instituição Proponente: HOSPITAL UNIVERSITARIO
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.146.715

Apresentação do Projeto:
Protocolo 077-17 do grupo III. Notificação recebida em 20.6.2017.

As informações colocadas nos campos denominados "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram retiradas do documento intitulado "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_880545.pdf" (submetido na Plataforma Brasil em 20/06/2017).

Introdução:O câncer de tireoide não é comum na infância correspondendo apenas a 1,5-3% das patologias malignas nessa faixa etária nos EUA e na Europa. Idade, história familiar de câncer de tireóide , exposição a radiação são fatores de risco importantes. (1) A presença de um nódulo palpável em crianças tem 5 vezes mais chances de ser um tumor maligno quando comparado aos adultos (2). Assim como nas outras faixas etárias o subtipo mais comum é o carcinoma papilífero clássico, entretanto a apresentação clinica do tumor em crianças e adolescentes parece ter algumas características diferentes. Tumores maiores de 4cm são encontrado em 36% dos pacientes menores de 20 anos e apenas em 15% dos adultos entre 20-50 anos, por sua vez, tumores menores que 1 cm são encontrados em 9% das crianças e adolescentes e em 22% dos

Endereço: Rua Prof. Rodolpho Paulo Rocco Nº255 Sala 01D-46
Bairro: Cidade Universitária **CEP:** 21.941-913
UF: RJ **Município:** RIO DE JANEIRO
Telefone: (21)3938-2480 **Fax:** (21)3938-2481 **E-mail:** cep@hucff.ufrj.br

Figura 1 – Folha de capa da aprovação do estudo pelo CEP.

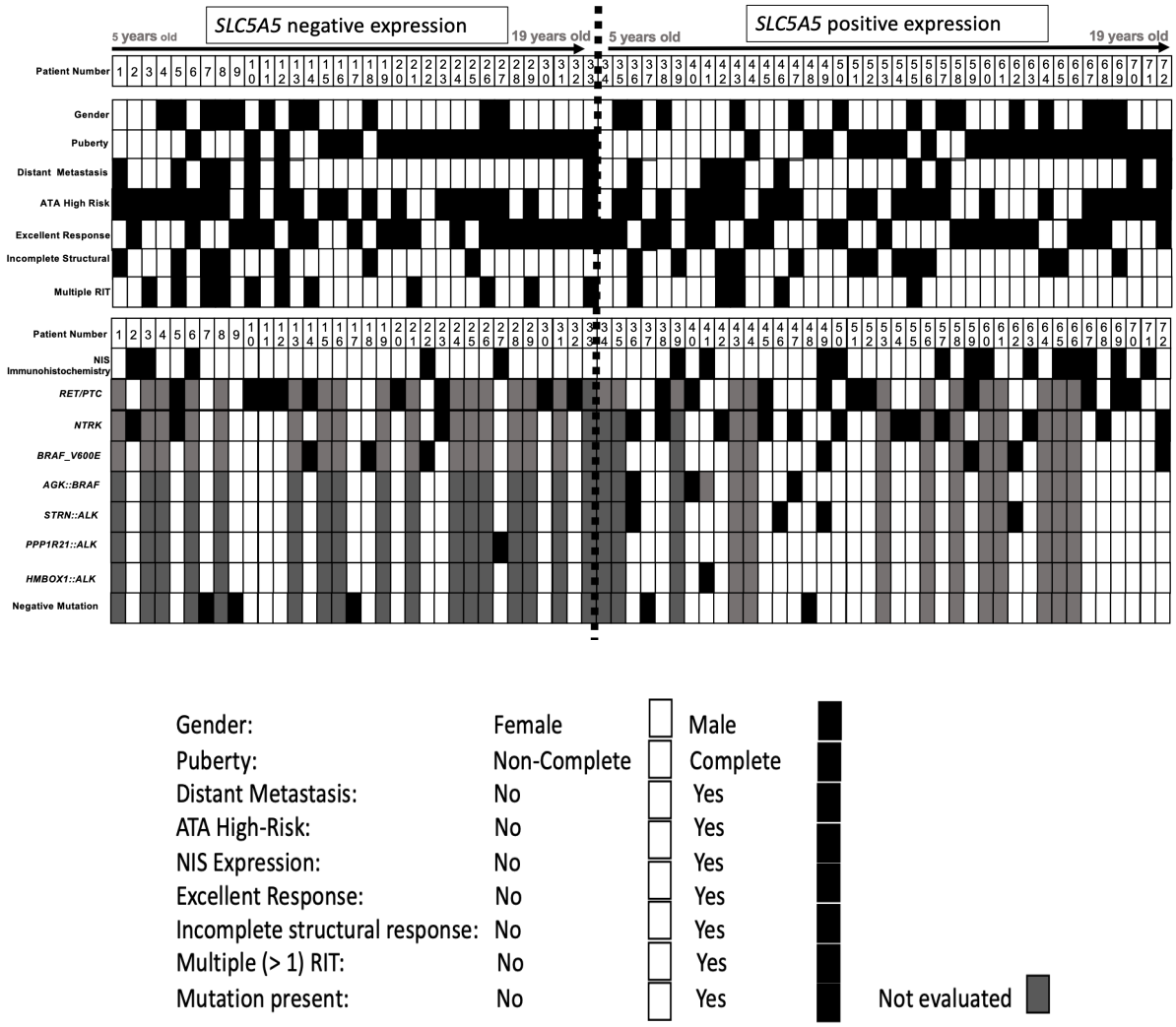

Figura 2 – Análise individual dos 72 pacientes com avaliação de NIS e *SLC5A5* em mesma amostra, ordenados por idade cronológica e divididos por expressão de *SLC5A5*.

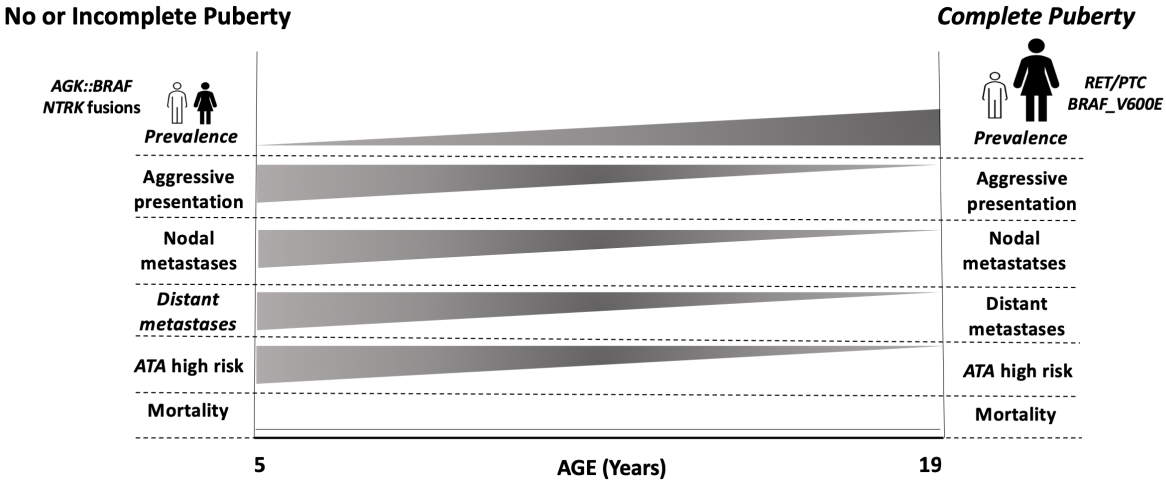

Figura 3 – Resumo dos achados clínicos associados à puberdade.