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FACULDADE DE MEDICINA

CRISTINA PALMER BARROS

O IMPACTO DA NEUROTOXINA DERIVADA DE EOSINÓFILOS NO DIAGNÓSTICO DA ESOFAGITE EOSINOFÍLICA EM PACIENTES PEDIÁTRICOS

DOUTORADO EM CIÊNCIAS DA SAÚDE

2019

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Medicina da Universidade Federal de Uberlândia, como parte dos requisitos para obtenção do título de doutor em Ciências da Saúde.

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Orientador: Prof.º Dr. Luiz Ricardo Goulart Filho

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Reuniu-se no anfiteatro do bloco 4K, Campus Umuarama, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências da Saúde, assim composta: Professores Doutores: Ana Paula Beltran Moschione Castro (FMUSP), Cristina Helena Targa Ferreira (UFCSPA), Carlos Henrique Martins da Silva (UFU), Jair Pereira da Cunha Júnior(UFU) e Luiz Ricardo Goulart Filho (UFU) orientador do candidato.

Iniciando os trabalhos o presidente da mesa, Dr. Luiz Ricardo Goulart Filho, apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público, e concedeu a Discente a palavra para a exposição do seu trabalho. A duração da apresentação da Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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Presidente da banca: Prof.º Dr. Luiz Ricardo Goulart Filho

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Área de concentração: Ciências da Saúde

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Data da aprovação: Uberlândia, 02 de setembro de 2019

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Suplente Dra. Mariza Rodrigues de Faria

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*Dedico o esforço empreendido nesta obra a minha querida mãe **Eliani** que, onde quer que esteja, tenho a certeza que me acompanha e me sustenta.*

Mother
“Have you ever watched your old mother
making up the bed for you,
how she pulls, straightens, tucks in and smoothes the sheet
so you won't feel a single wrinkle?
Her breathing, the motion of her hands and palms
are so loving
that in the past they are still putting out the fire in Persepolis
and at this moment calming some future storm
off the China coast or in unknown seas”

Vladimír Holan

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“O amor me fere é debaixo do braço
de um vão entre as costelas
Atinge o meu coração é por essa via inclinada.
Eu ponho o amor no pilão com cinza
e grão roxo e soco. Macero ele,
faço dele cataplasma e ponho sobre a ferida.”

Adélia Prado

RESUMO

Introdução: A Esofagite Eosinofílica (EoE) como entidade clínica emergente apresenta desafios em sua condução clínica. A sobreposição dos sinais e sintomas com outros distúrbios gastrointestinais presentes na infância, com a doença do refluxo gastroesofágico (DRGE), dificulta a assertividade no diagnóstico e promove atrasos na abordagem terapêutica. **Objetivo:** O objetivo deste estudo foi identificar e validar um biomarcador para a EoE, através de amostras biológicas de pacientes pediátricos submetidos a endoscopia digestiva alta diagnóstica. **Métodos:** Paciente consecutivos foram incluídos em uma coorte endoscópica pediátrica. Escores de sintomas, endoscópico e histológico foram utilizados para a caracterização clínica. Amostras de tecido e muco esofágico foram obtidas durante o procedimento endoscópico. Os pacientes foram classificados em EoE, DRGE ou controles, durante o acompanhamento clínico. A proteômica foi aplicada para selecionar proteínas candidatas ao biomarcador. A imunohistoquímica (IHQ) no tecido e o ELISA no muco foram utilizados para validar o biomarcador em amostras de pacientes da coorte. **Resultados:** Cento e dez crianças elegíveis foram submetidas ao procedimento endoscópico, 3 foram perdidas durante o acompanhamento, 16 classificadas como EoE (14,5%), 14 como DRGE (12,5%) e 77 como controles (70%). A análise proteômica identificou a neurotoxina derivada de eosinófilos (EDN, RNase2) como o melhor biomarcador para EoE. As pontuações dos escores endoscópico, histológico e da IHQ do tecido diferiram os grupos EoE e controle (P <0,0001). Apenas o escore histológico diferiu os grupos EoE e DRGE (P = 0,0007). A presença da NDE no muco esofágico diferenciou os grupos EoE e DRGE (0,515 ± 0,402 vs 0,186 0,125, P = 0,0066), e EoE e controles (0,515 ± 0,402 vs 0,177 ± 0,194, P <0,0002). A NDE no muco foi altamente correlacionada com o pico de contagem de eosinófilos (PEC) no tecido esofágico. **Conclusão:** O diagnóstico da EoE foi significativamente otimizado pela detecção da NDE no tecido e no muco esofágico de pacientes pediátricos com acurácia de 90% e 78%, respectivamente. A avaliação da NDE em pacientes com escores endoscópicos e histológicos suspeitos para EoE pode ser considerada uma ferramenta útil no diagnóstico diferencial.

Palavras-chave: Esofagite eosinofílica. Proteômica. Biomarcador. Neurotoxina derivada de eosinófilos. Doença do refluxo gastroesofágico.

ABSTRACT

Introduction: Eosinophilic Esophagitis (EoE) as an emerging clinical entity presents challenges in its clinical management. Overlapping signs and symptoms with other gastrointestinal disorders present in childhood, such as gastroesophageal reflux disease (GERD), make diagnosis more difficult and delay therapeutic approach. **Objective:** The aim of this study was to identify and validate a biomarker for EoE through biological samples from pediatric patients undergoing diagnostic upper digestive endoscopy. **Methods:** Consecutive patients were included in a pediatric endoscopic cohort. Symptom, endoscopic and histological scores were performed for clinical characterization. Tissue and esophageal mucus samples were obtained during the endoscopic procedure. Patients were classified as EoE, GERD or controls during the clinical follow-up. Proteomics were applied to select candidate proteins for the biomarker. Tissue immunohistochemistry (IHC) and mucus ELISA were used to validate the biomarker in samples of the cohort. **Results:** One hundred and ten eligible children underwent the endoscopic procedure, 3 were lost during follow-up, 16 classified as EoE (14.5%), 14 as GERD (12.5%) and 77 as controls (70%). Proteomic analysis identified eosinophil-derived neurotoxin (EDN, RNase2) as the best biomarker for EoE. Endoscopic, histological, and tissue IHC scores differed between the EoE and control groups ($P < 0.0001$). Only the histological score differed between the EoE and GERD groups ($P = 0.0007$). The presence of NDE in esophageal mucus differentiated the EoE and GERD groups (0.515 ± 0.402 vs 0.186 0.125, $P = 0.0066$), and EoE and controls (0.515 ± 0.402 vs 0.177 ± 0.194, $P < 0.0002$). NDE in mucus was highly correlated with peak eosinophil count (PEC) in esophageal tissue. **Conclusion:** The diagnosis of EoE was significantly optimized by detecting NDE in tissue and esophageal mucus in pediatric patients with accuracy of 90% and 78%, respectively. The evaluation of NDE in patients with suspected endoscopic and histological scores for EoE can be considered a useful tool in the differential diagnosis.

Keywords: Eosinophilic esophagitis. Proteomics. Biomarker. Eosinophil-derived neurotoxin. Gastroesophageal reflux disease.

LISTA DE ABREVIAÇÕES

ALOX15	Arachidonate 15-lipoxygenase
DRGE	Doença do Refluxo Gastroesofágico
EDA	Endoscopia Digestiva Alta
EoE	Esofagite Eosinofílica
Eos/CGA	Eosinófilos por Campo de Grande Aumento
FLG	Filaggrin
IBP	Inibidor de Bomba de Prótons
Ig	Imunoglobulina
IL	Interleucina
NDE	Neurotoxina Derivada de Eosinófilos
PBP	Proteína Básica Principal
PCE	Proteína Catiônica Eosinofílica
POE	Peroxidase de Eosinófilos
SLURP1	Secreted LY6/PLAUR domain containing 1
TGF-β	*Transforming growth factor-β*
Th1	*T helper 1*
Th2	*T helper 2*
TSLP	Thymic Stromal Lymphopoientin

SUMÁRIO

1 INTRODUÇÃO

Esofagite Eosinofílica (EoE) é uma doença inflamatória crônica, mediada por antígenos imununológicos, e inflamação predominantemente eosinofílica (LIACOURAS et al., 2011). A doença é definida por sintomas de disfunção esofágica, presença de pelo menos 15 eosinófilos/campo de grande aumento (Eos/CGA) em biópsias esofágicas, e avalição clínica afastando outras causas que determinem a eosinofilia esofágica (DELLON et al., 2018). A prevalência global da EoE é de 34,4 casos por 100.000 habitantes (IC 95%, 23,1-47,5), maior em adultos (42,2; IC 95%, 31,1-55) do que em crianças (34; IC 95%, 22,3-49,2). As taxas de incidência também mostram a mesma tendência com 6,6/100.000 pessoas/ano (IC 95%, 3-11,7) em crianças, e 7,7/100.000 (IC 95%, 1,8-17,8) em adultos, sem diferenças significativas entre estudo norte-americanos e europeus (NAVARRO et al., 2019). Um levantamento em pacientes pediátricos relatou a prevalência agrupada de 3,7% (95% de intervalo de confiança [IC] 2,4-5,1) em endoscopias digestivas alta (EDA) por qualquer indicação, e 2,6% (95% IC 1,2-4,1) em EDAs indicadas por dor abdominal (SOON et al., 2013). Estudo brasileiro retrospectivo também analisando EDAs pediátricas, incluindo crianças até 14 anos de idade, publicou a incidência de 24,8/100.000 indivíduos acumulada em 10 anos (GONCALVES et al., 2018). Os relatos de incidência e prevalência da EoE apresentam claro aumento ao longo do tempo, se isto ocorre às custas do surgimento de novos casos, ou pelo maior reconhecimento do diagnóstico ainda é um tópico de debate.

A EoE é um distúrbio imuno mediado no qual antígenos alimentares e ambientais estimulam uma resposta inflamatória Th2, e citocinas importantes como as interleucinas (IL) IL-4, IL-5 e IL-13 estimulam a produção de eotaxina-3 na mucosa esofágica. A eotaxina-3 tem papel importante no recrutamento de eosinófilos teciduais. No entanto, descobertas recentes demonstram que a resposta Th2 na patogênese da EoE nem sempre está relacionada a produção sistêmica de IgE específica, isto porque sua ação parece estar mais direcionada a eventos locais. Já a produção de IgG4 específica está associada ao diagnóstico da EoE, e estudos recentes indicam que esta pode exercer um papel preditor (SCHUYLER et al., 2018). Os eosinófilos ativados secretam mediadores pró-inflamatórios e pro-fibróticos, causando danos teciduais, recrutando mastócitos adicionais e fibroblastos que

perpetuam a resposta inflamatória e, ao longo do tempo, resultam em fibrose e remodelamento tecidual (BLANCHARD et al., 2017).

O quadro clínico da EoE na criança varia em relação à idade. A presença de sintomas inespecíficos como vômitos, náuseas, dor abdominal, recusa alimentar, engasgos, baixo ganho ponderal e dificuldade na introdução de alimentos sólidos ocorre na faixa etária de lactente e pré-escolar. Sintomas mais específicos, semelhantes aos do adulto, são observados em crianças maiores e adolescentes, como disfagia e impactação de alimentos no esôfago, ocorrendo também os vômitos e a dor abdominal (CIANFERONI; SPERGEL, 2016). Evidências atuais em pacientes pediátricos demonstram que o tempo de progressão da doença, sem intervenção terapêutica adequada, pode remodelar o tecido esofágico por fibrose, o que se expressa nos sintomas de disfagia e impactação de alimentos no esôfago (MENARD-KATCHER et al., 2017).

A eosinofilia na mucosa esofágica, e os sintomas e as alterações endoscópicas decorrentes deste evento, não são específicos da EoE. Outras entidades clínicas como a doença do refluxo gastroesofágico (DRGE), reações adversas a medicamentos, Doença de Crohn, Doença Celíaca, infecção parasitária, vasculite alérgica e leiomiomatose esofágica podem se assemelhar (FURUTA; KATZKA, 2015). A apresentação clínica dos sintomas da EoE e da DRGE são as que mais se confundem na prática clínica, o que torna o diagnóstico diferencial entre as duas entidades um desafio. Pacientes pediátricos, que apresentam baixa resposta ao tratamento da DRGE, podem ser portadores de EoE em 5-10% dos casos (LIACOURAS et al., 2014). No entanto, a presença ou a ausência da DRGE, e a redução da eosinofilia esofágica em resposta ao tratamento com medicações inibidoras de bomba de prótons (IBPs) não são mais critérios excludentes de EoE, pois as duas doenças podem coexistir (LIACOURAS et al., 2011; LUCENDO et al., 2017; PAPADOPOULOU et al., 2014). Os achados endoscópicos descritos como edema (aparência opacificada da mucosa), anéis fixos ou transitórios, placas ou exsudato esbranquiçado, linhas ou sulcos verticais, estreitamento do calibre ou estenoses esofágicas, e fragilidade mucosa com ruptura a passagem do aparelho endoscópico também não são sinais específicos da EoE (HIRANO, 2014). E ainda, a ausência de alterações endoscópicas em pacientes com EoE pode ocorrer em 17% dos casos em estudos retrospectivos, e em 7% dos pacientes avaliados em estudos prospectivos (KIM et al., 2012).

O tratamento da doença visa à melhora dos sintomas, especialmente os relacionados às dificuldades alimentares, o risco de impactação de alimentos no esôfago, a melhora da qualidade de vida, e a resolução da eosinofilia esofágica por meio de terapias dietéticas e/ou farmacológicas. A dieta elementar que consiste na eliminação completa de antígenos alimentares e no uso de fórmulas baseadas em aminoácidos, mostra eficácia em até 97% dos pacientes com EoE (MARKOWITZ et al., 2003; MOLINA-INFANTE, LUCENDO, 2018). Este tratamento pode apresentar dificuldades de adesão devido à ampla restrição dietética e a necessidade de se ingerir grandes volumes de fórmula para atender às necessidades calóricas (LIEBERMAN; CHEHADE, 2012). Uma alternativa à dieta elementar é a eliminação de apenas alguns alérgenos alimentares principais da dieta. Isto requer a monitorização clínica dos sintomas e a realização de endoscopias seriadas, para avaliação prospectiva do impacto de cada alérgeno testado na ativação ou remissão da EoE (SPERGEL et al., 2012). Quando os sintomas são controlados, os alimentos individuais podem ser reintroduzidos, melhorando a qualidade de vida dos pacientes e prevenindo deficiências nutricionais. Outras modalidades de tratamento incluem corticosteróides orais e tópicos, agentes biológicos como anti-IL-5 e dilatação esofágica em procedimentos endoscópicos (DELLON et al., 2018).

As agendas científicas dos comitês de estudo em doenças eosinofílicas apontam áreas necessárias de aprofundamento das pesquisas, com o intuito de aprimorar a definição diagnóstica e acompanhamento da doença. A avaliação histopatológica isolada não tem sido suficiente para atingir a assertividade no diagnóstico, na caracterização dos diferentes grupos fenotípicos de pacientes, e na escolha das modalidades de tratamento, bem como o seu acompanhamento.

Um grupo de estudos com a participação de membros da *United European Gastroenterology (UEG)*, da *European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)*, da *European Academy of Allergy and Clinical Immunology (EAACI)*, e da *European Society of Eosinophilic Oesophagitis (EUREOS)* sugere campos de investigação nas sub-áreas do conhecimento, e aqui se destacam objetivos a serem atingidos na “Avaliação do diagnóstico e da atividade da doença” (LUCENDO et al., 2017):

a) definição de critérios padronizados para remissão e melhora da doença;
b) identificação precoce de fenótipos clínicos (inflamatório, fibroestenótico, ambos);

c) ferramentas diagnósticas não invasivas ou minimamente invasivas para monitoramento das doenças;
d) otimização de escores que avaliem a atividade da doença através de sintomas, achados endoscópicos e qualidade de vida;
e) melhorar a precisão do diagnóstico em pacientes com suspeita de EoE e <15 Eos/CGA ou indivíduos assintomáticos com ≥15 Eos/CGA.

O *National Institute of Health (NIH) Taskforce on the Research of Eosinophil-Associated Diseases* reforça a importância das abordagens “ômicas” (transcriptoma, proteoma, metaboloma, glycome e microbioma) correlacionadas aos achados clínicos, para responder perguntas ainda não esclarecidas no campo das doenças eosinofílicas, e no desenvolvimento de novas tecnologias de diagnóstico e acompanhamento destas doenças (KHOURY et al., 2018).

A utilização de escalas métricas para avaliação de sintomas, alterações endoscópicas e histológicas apresentam um papel preditor importante no diagnóstico e atividade da EoE. A utilização sistemática destes instrumentos poderá esclarecer valores de corte que identifiquem os grupos fenotípicos onde a aplicação de biomarcadores ocorrerá de forma específica e direcionada, aumentando a acurácia dos testes moleculares, e esclarecendo desafiadoras dúvidas nos processos diagnóstico e terapêutico. No entanto, estudos de validação são necessários para entender como os escores clínicos podem ser utilizados associados à aplicação dos biomarcadores moleculares.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Consensos e Definições

O termo Esofagite Eosinofílica (EoE) foi registrado pela primeira vez na base de dados PubMed ao final da década de 70 com a descrição de um paciente adulto com acalasia e infiltração eosinofílica na mucosa esofágica (LANDRES et al., 1978). No entanto, como entidade clínica foi descrita originalmente em 1981, através de um relato de caso de um adolescente de 16 anos que apresentava intensa disfagia (PICUS; FRANK, 1981). Mas foi nas últimas duas décadas que o que parecia uma condição rara associada a relatos isolados de casos se tornou uma causa importante de morbidade do trato gastrointestinal alto, gerando desde então, um crescente número de publicações científicas.

A presença de eosinófilos na mucosa esofágica de humanos não é habitual, portanto sua presença determina necessariamente uma condição patológica (Collins, 2008). A doença pode ser encontrada em qualquer idade e raça com predomínio ainda não esclarecido em brancos não hispânicos. O típico paciente portador de EoE é do sexo masculino (masculino/feminino, 3:1), com características atópicas e apresentação da doença na infância ou durante a terceira ou quarta década de vida (LIACOURAS et al., 2011).

Em 2007 foi publicado o primeiro consenso de recomendações para o diagnóstico e tratamento da EoE reunindo publicações de revisões sistemáticas e opiniões de especialistas das mais diversas áreas clínicas (FURUTA et al., 2007). A EoE foi definida como uma entidade clínico patológica primária do esôfago, caracterizada clinicamente por disfunção esofagiana, onde a eosinofilia esofágica era demonstrada pela presença de 15 (quinze) ou mais Eos/CGA, na área de maior densidade eosinofílica, como um critério necessário, porém não suficiente para o diagnóstico. O outro critério incluía a ausência de sinais sugestivos da DRGE pela falta de resposta ao tratamento com altas doses de medicação IBP ou estudo de pHmetria esofágica dentro dos limites de normalidade. Tais recomendações foram revisadas posteriormente por *experts* buscando atualizar os rápidos avanços na patogênese, epidemiologia, apresentação clínica, diagnóstico e tratamento da doença (DELLON et al., 2018; LIACOURAS et al., 2011; LUCENDO et al., 2017; PAPADOPOULOU et al., 2014).

Um aspecto amplamente discutido nas comunicações científicas é a limitação do diagnóstico da EoE realizado por contagem de eosinófilos em CGA. O critério histológico (≥15 Eos/CGA) ainda é vigente, mas apresenta claras desvantagens. Dentre elas ressalta-se a distribuição não uniforme da eosinofilia esofágica em lâminas de um mesmo paciente, a ausência de padronização do tamanho do CGA, variações no conceito de qual seria a localização exata do eosinófilo intraepitelial contável, e a dificuldade de se estabelecer valores de referência capazes de diferenciar a EoE da DRGE. Sendo assim, outros aspectos começaram a ser valorizados na descrição da lâmina histopatológica, como: a formação de micro abscessos eosinofílicos, a presença de distribuição estratificada dos eosinófilos, a liberação de grânulos eosinofílicos extracelulares, a hiperplasia da camada basal, o alongamento de papilas epiteliais, a presença de fibrose subepitelial da lâmina própria e o aumento de outras células inflamatórias como linfócitos e mastócitos (LIACOURAS et al., 2011).

Outro ponto chave na evolução dos consensos é o que se refere à relação diagnóstica entre EoE e a DRGE. Inicialmente, consideradas condições excludentes, hoje entende-se que ambas as doenças podem coexistir e se potencializar. A agressão mucosa decorrida pela ação ácido-péptica pode alterar a permeabilidade tecidual favorecendo a penetração de alérgenos que deflagram a patogênese da EoE. E em sentido reverso, a mucosa inflamada por estímulos imunogênicos pode ter sua motilidade alterada promovendo a dismotilidade, e a ocorrência da DRGE secundária à EoE (CHENG et al., 2014; DELLON et al., 2018). A correlação entre DRGE e EoE é complexa, não apenas pelas semelhanças dos sintomas e pela possiblidade de sobreposição dos mecanismos fisiopatológicos, mas também pela resposta à terapia com inibidor de bomba de prótons (IBP). Cerca de 50% dos pacientes com EoE são caracterizados como eosinofilia esofágica responsiva à IBP, alcançando remissão clínica e histológica com o uso desta terapia exclusiva (MOLINA-INFANTE et al., 2016). Além da capacidade de recuperar os danos teciduais causados pelo refluxo gastroesofágico, a medicação também exerce ação inflamatória bloqueando a produção de eotaxina 3, que é a responsável pelo recrutamento de eosinófilos (CHENG et al., 2013; ZHANG et al., 2012). Por esse motivo, a terapia com IBP não é mais um critério utilizado para fazer o diagnóstico diferencial entre EoE e DRGE. E pacientes respondedores ao uso de IBP, com característica atópicas e sem evidências de outras doenças que possam explicar a eosinofilia esofágica, são classificados

como EoE (DELLON et al., 2018). Este grupo de pacientes apesar de possuir uma resposta terapêutica particular ao uso de IBP, quando não tratados, apresentam semelhança molecular com os pacientes EoE não resposivos a IBP, compartilhando a mesma via Th2 da resposta imune. O evento marcante é que após o tratamento com IBP apresentam resposta clínica, histológica e molecular, retornando a expressão de transcriptos para níveis normais próximos a expressão de controles não alérgicos. A compreensão deste mecanismos pode revelar opções terapêuticas significativas (WEN et al., 2015).

2.2 Caracterização clínica da Esofagite Eosinofílica – Escalas métricas

A percepção clínica de que os pacientes portadores de EoE podem apresentar características fenotípicas distintas, apoiadas por descobertas de estudos de genômica, incentivou o desenvolvimento de escalas métricas de caracterização.

Na avaliação dos sintomas pediátricos duas escalas foram publicadas. A primeira, uma escala métrica denominada *Symptom scoring tool (SST)* usada anteriormente para desordens ácido-pépticas e modificada para a avaliação de pacientes com EoE. Quando aplicada na diferenciação de pacientes com GERD e EoE apresentou distinção dos grupos nos sintomas de disfagia e anorexia com saciedade precoce (ACEVES et al., 2009). Em publicação mais recente uma escala PRO *(patient-reported outcome)* aplicada a pais e crianças com EoE (*Pediatric Eosinophilic Esophagitis Symptom Score - PEESS v2.0*) foi desenvolvida para avaliação dos sintomas de disfagia, sinais de DRGE, náuseas/vômitos e dor (FRANCIOSI et al., 2011). O instrumento gera um escore numérico capaz de mensurar os sintomas no momento do diagnóstico e no acompanhamento do tratamento. Sua aplicação em um estudo de validação objetivou correlacionar os sintomas aos achados histológicos e à biomarcadores moleculares, apresentando resultados promissores (MARTIN et al., 2015). O instrumento foi recentemente traduzido e adaptado culturalmente para a língua portuguesa do Brasil abrindo novas perspectivas para pesquisas nacionais (SANTOS et al., 2017).

Na caracterização métrica endoscópica da EoE foi publicada uma escala (*EREFS – Endoscopic Reference Score*) capaz de gerar também um escore numérico, com potencial de predizer o diagnóstico, a resposta ao tratamento, e caracterizar fenotípica a doença nos padrões inflamatório e/ou fibroestenótico (HIRANO et al.,

2013; SINGLA et al., 2015). Os achados endoscópicos graduados em presença ou intensidade incluem a descrição de anéis concêntricos fixos e/ou transitórios, exsudato granular, sulcos ou estrias verticais, edema com apagamento da trama vascular, estreitamento do calibre esofágico, estenoses e fragilidade da mucosa tipo "papel crepom". Estudo recente de validação em crianças demonstra boa acurácia com o uso da escala para o diagnóstico e avaliação da atividade da doença pós tratamento (WECHSLER et al., 2018).

No campo da avaliação histopatológica, uma escala de avaliação histológica denominada *Histology scoring tool (HST)* avalia o número de eosinófilos por campo de grande aumento, a hiperplasia da camada zona basal, a dilatação dos espaços intercelulares, a descamação epitelial, a presença de abscessos eosinofílicos, a degranulação de eosinófilos, e a presença de fibrose na lâmina própria (ACEVES et al., 2007; ACEVES et al., 2009). Outra escala recentemente publicada (*EoE specific histologic scoring system - EoEHSS*) utiliza critérios semelhantes, porém além da avaliação da intensidade dos achados (grau) avalia também sua extensão (estágio) (COLLINS et al., 2017).

A composição dos escores pode ser utilizada na tentativa de se montar painéis fenotípicos que auxiliem a delinear os perfis da doença. Mulder et al. (2013) propuseram um sistema baseado em escores clínicos e endoscópicos sugerindo que pacientes do sexo masculino, com disfagia, história de impactação de alimentos, ausência de dor retroesternal e pirose, linhas transversais e pápulas brancas no esôfago constituem um grupo com características fortemente sugestivas para o diagnóstico de EoE. A análise conjunta dos escores endoscópicos, histológicos e de um painel com 96 transcriptos para EoE gerou a definição de 3 endotipos que individualizam pacientes com características clínicas e evoluções distintas. Esta caracterização dá início a uma abordagem individualizada no diagnóstico e tratamento da EoE (SHODA et al., 2018).

2.3 Caracterização imunopatológica da Esofagite Eosinofílica - Biomarcadores

Dellon (2013) descreveu a atuação de mastócitos, fibroblastos, células epiteliais e linfócitos na determinação do processo inflamatório coadjuvante a ação clássica dos eosinófilos. Em particular, os eosinófilos induzem a remodelagem esofágica via ativação TGF-β resultando em fibrose subepitelial, transição epitélio-

mesenquimal, disfunção do músculo liso e aumento da proliferação epitelial esofágica. Os mastócitos estão aumentados na EoE quando comparados a grupos controle e com DRGE, por provável resposta Th2 via ação da IL-9. Em alguns casos o número de mastócitos está mais elevado que o número de eosinófilos. Os fibroblastos também apresentam papel decisivo na deposição de colágeno e nas cicatrizes esofágicas responsáveis por muitas das manifestações clínicas. As células epiteliais são unidas por junções fortes, junções adesivas e desmossomos que contém proteínas como claudina-1 e -4, E-caderina e desmogleína. Na EoE observa-se quebras nesses mecanismos de junção. A expressão da filagrina também se apresenta reduzida na EoE. Estes defeitos alteram a função de barreira do epitélio permitindo a penetração de antígenos sensibilizadores.

A ação dos linfócitos está representada pela resposta Th2 a estímulos provenientes de alérgenos alimentares e aero alérgenos. A IL-13 e IL-5 produzidas pelos linfócitos estimulam o epitélio esofágico a produzir eotaxina-3, uma potente citocina que recruta e ativa eosinófilos. Outro fator relacionado é o TSLP *(thymic stromal lymphopoientin)* que representa uma citocina importante (IL-7 like) na deflagração da resposta alérgica. As moléculas co-estimuladoras de células T também parecem estar envolvidas neste processo (ZHANG et al., 2013).

Wen et al. (2013) desenvolveram um painel molecular para ser usado como teste diagnóstico na EoE. O teste teria como vantagens a rapidez, acurácia e baixo custo oferecendo, através da análise do transcriptoma da EoE, um biomarcador capaz de avaliar as intervenções terapêuticas através da extração de uma única biópsia esofágica.

Matoso et al. (2013) identificaram um conjunto de biomarcadores capazes de avaliar os pacientes com EoE virgens de tratamento, tratados, com DRGE e controles normais. Observou-se um aumento na expressão de ALOX15 e uma baixa expressão de FLG e SLURP1 apresentando alta sensibilidade e especificidade como marcadores diagnósticos.

Uma citocina mais recentemente estudada da família da IL-1, denominada IL-33, possui a capacidade de desenvolver uma resposta Th2, por ativação das células dendríticas e recrutamento de eosinófilos, mastócitos e fibroblastos, com supressão da resposta Th1. Sua presença no homem foi detectada em células epiteliais de brônquios, vias aéreas de fino calibre, fibroblastos e músculo liso sugerindo um potencial envolvimento nos mecanismos de imunidade da mucosa. Estudos iniciais

demonstram a relação desta IL com a evolução da EoE. A inibição pela IL-33 da função das células T reguladoras do esôfago pode induzir a perda de tolerância antigênica, o que explicaria o mecanismo que favorece a evolução da doença (JUDD et al., 2016).

As proteínas de degranulação eosinofílica estão obviamente relacionadas aos eventos patogênicos da EoE e são fortes candidatas para biomarcadores de diagnóstico e atividade da doença. Existem quatro proteínas de degranulação eosinofílica descritas: a proteína básica principal (PBP), a proteína catiônica eosinofílica (PCE), a peroxidase de eosinófilos (POE), e a neurotoxina derivada de eosinófilos (NDE). Elas são potentes toxinas citolíticas e apresentam duração prolongada no tecido. A NDE atua via ativação das células dendríticas e exerce um papel importante na permeabilidade tecidual e na patogênese da doença alérgica. Mesmo com um baixo número de eosinófilos intactos contados, no diagnóstico ou controle do tratamento, não é raro se encontrar pacientes que apresentem quadro clínico e alterações endoscópicas típicas da EoE. Estudo avaliando pacientes com anéis esofágicos, sintomáticos, após tratamento com IBP, e baixo número de Eos/CGA apresentaram altos níveis de NDE e PBP1 teciduais indicando atividade da doença (PETERSON et al., 2015).

3 OBJETIVOS

3.1 Objetivo geral

Identificar e validar um biomarcador para a EoE, através de amostras biológicas de pacientes pediátricos submetidos a endoscopia digestiva alta diagnóstica.

3.2 Objetivos específicos

a) caracterizar clinicamente pacientes pediátricos de uma coorte endoscópica;
b) comparar os achados clínicos, endoscópicos, histológicos e bioquímicos entre pacientes com EoE, DRGE e controles;
c) testar a correlação entre os achados clínicos, histológicos, histológicos e bioquímicos;
d) avaliar o comportamento do biomarcador em biópsia esofágica e em muco esofágico.

4 ARTIGOS CIENTÍFICOS

4.1 Artigo 1 – Título: *"Identification and Validation of RNase 2 as a Major Biomarker for Eosinophilic Esophagitis Diagnosis in a Pediatric Endoscopic Cohort*

Parecer do CEP consubstanciado (ANEXO A)

Normas de submissão para autores - *The American Journal of Gastroenterology* (ANEXO B)

Title: Identification and Validation of RNase 2 as a Major Biomarker for Eosinophilic Esophagitis Diagnosis in a Pediatric Endoscopic Cohort

Short running title: RNase 2 in Eosinophilic Esophagitis Diagnosis

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Conflict of interest:

All authors declare no conflict of interests.

ABSTRACT

Objective: Overlapping endoscopic symptoms and clinical findings in pediatric patients with eosinophilic esophagitis (EoE), gastroesophageal reflux disease (GERD), or functional gastrointestinal disorders represent a challenge for medical diagnosis. This study aimed to identify and validate a diagnostic biomarker capable of distinguishing pediatric EoE patients from other gastrointestinal diseases with similar clinical manifestations.

Methods: A prospective study was performed in a pediatric endoscopic cohort with incident diagnosis of EoE. Symptom scoring tool (SST), endoscopic reference score (EREFS) and epithelial histological score (EHS) were used to clinically characterize patients. One hundred and seven children undergoing diagnostic endoscopic procedures were included. The follow-up stratified patients into 16 EoE (14.5%), 14 GERD (12.5%) and 77 control (70%) patients. After identification of Eosinophil-derived neurotoxin (EDN, RNase2) proteomic analysis of esophageal tissue samples, we have performed immunohistochemistry in esophageal tissues and ELISA in mucus to validate this biomarker. (ClinicalTrials.gov NCT03069573)

Results: The EREFS, EHS, and EDN Immunohistochemistry scores differed EoE group from control (P<0.0001). Only EHS differed EoE group from GERD (P=0.0007). The EDN validation in esophageal mucus by ELISA differed EoE from GERD (0.515±0.402 vs 0.186±0.125, P =0.0066) and EoE from controls (0.515±0.402 vs 0.187±0.194, P<0.0002). EDN was highly correlated with peak eosinophil count (PEC).

Conclusion: EoE diagnosis was significantly improved by EDN detection in both esophageal tissues and mucus of patients with accuracies of 90% and 78%,

respectively, and may become a useful tool prior pathological analysis. EREFS and EHS were clinical predictors of the disease.

Key words: Eosinophilic esophagitis, Proteomic, Molecular biomarker, Eosinophil derived neurotoxin, Gastroesophageal reflux disease.

INTRODUCTION

Eosinophilic esophagitis (EoE) is an inflammatory esophageal disease characterized by symptoms related to esophageal dysfunction and eosinophilic tissue infiltration. Diagnostic criteria require the presence of at least 15 eosinophils per high power field (hpf) in esophageal mucosal biopsies obtained by esophagogastroduodenoscopy (EGD), excluding stomach and duodenum eosinophilia. (1, 2) Finally, after these criteria are met, other non-EoE-related disorders that cause or potentially contribute to esophageal eosinophilia should be ruled out. (3)

EoE is a chronic disease with increasing incidence and prevalence at all ages. (4-6) Diagnosis and follow-up based on endoscopic procedures and histopathological evaluation are costly and burdensome, especially for children requiring anesthetic sedation. Systematic assessment of clinical manifestations and endoscopic findings has shown a good correlation with eosinophil infiltration, constituting a guiding tool for the investigation and a predictor for the diagnosis and remission of the disease. (7-10) However, the esophageal biopsies could not be replaced in their diagnostic and treatment evaluation role.

The complexity of the EoE diagnosis lies in its phenotypic variability, along with clinical overlaps with other gastrointestinal disorders. There is a wide variety in the presentation of symptoms according to age and disease stage. Heartburn, dysphagia,

and food impaction in adults; and vomiting, abdominal pain, feeding difficulties, and failure to thrive, in children, are frequent symptoms of EoE. However, they are also present in functional disorders and gastroesophageal reflux disease (GERD). (11, 12) Symptom progression seems to express decreased esophageal distensibility, beginning with inflammatory character in children and progressing to fibrosis remodeling in adulthood. (13) Endoscopic findings also show the same pathway towards esophageal remodeling. Inflammatory features such as edema, exudates, and furrows are more expected in children than fixed rings, strictures, narrowing of the caliber and shearing mucosa, as in adults. (14) Some of those endoscopic features are also present in GERD and other inflammatory esophageal disorders.

Peak eosinophil count (PEC) on esophageal histological biopsy slides is a gold standard criterion for evaluating EoE diagnoses. This criterion has known limitations as it cannot express the reality of the entire organ and is unable to determine the activation state of eosinophils. Extracellular deposition of eosinophilic degranulation proteins such as Eosinophil derived neurotoxin (EDN), Eosinophil cationic protein (ECP), Eosinophil granule major basic protein 1 (eMBP1), and Eosinophil peroxidase (EPX) show strong correlation with symptoms and endoscopic alterations (15-17), and furthermore featuring high levels in patients with typical EoE phenotype without esophageal eosinophilia. (15) However, the evaluation of these molecular biomarkers and other cytokines in serum blood samples has conflicting results. (18, 19) Another breakthrough in molecular diagnosis of EoE was the description of its transcriptome and the development of EoE diagnostic panel (EDP), with 94 selected representative genes capable to distinguished EoE patients in remission from GERD, and controls. (20) A multicenter cross-sectional study recently proposed an EoE endotype classification based on EDP expression associated with endoscopic and histological

scores, with a focus on an individualized diagnosis and targeted therapeutic choice. (21) However, these remarkable advances have not yet changed the reality of EoE diagnoses in clinical practice.

Outstanding questions in the EoE area include the lack of a feasible biomarker for distinguished the overlaps with other gastrointestinal diseases and to predict the development of esophageal eosinophilia in suspected patients. The National Institute of Health (NIH) Taskforce on the Research of Eosinophil-Associated Diseases (TREAD) reinforces the importance of “omics” approach (transcriptome, proteome, metabolome, glycome, and microbiome) to answer unmeet questions and in the development of new diagnostic technologies. (22) Hence, this study aimed to identify diagnostic biomarkers, able to distinguish EoE patients and other similar gastrointestinal disorders investigated in a pediatric endoscopic cohort. To achieve this propose we used proteomic analyses to select a set of biomarkers; clinical scores to characterize the symptoms, endoscopic and histopathological findings, and immunohistochemistry and ELISA analyses to validate the chosen biomarker in the same cohort subjects.

METHODS

Patient Population

Eligibility criteria were consecutive pediatric patients (age bellow 18 y) undergoing a diagnostic EGD for investigating some general gastrointestinal complaint in pediatric gastroenterology clinic at the Federal University of Uberlândia (UFU), MG, Brazil, between January 2015 and August 2016. The subjects were screening upon the enrollment. Exclusion criteria were receiving acid suppressed therapy and corticoids

in the last 4 weeks, congenital or acquired esophageal stenosis, and previous diagnoses related to eosinophilia. Baseline information such as demographics (age, sex), presence of atopy (allergic rhinitis, asthma, eczema, food allergies), and the duration of the symptoms was collected at enrolment by the investigators. All parents or guardians, and adolescents up to 12 y provided written informed consent. The institutional review board approved this study under the protocol number CAAE 36787714.0.0000.5152.

Endoscopic procedures and specimens collection

Immediately before the EGD, the parents or guardians, and their children were invited to full-field the Symptom scoring tool (SST), a simple metric tool previously used for acid peptic disorders and modified for EoE patients evaluation. This score included 7 categories (heartburn/regurgitation, abdominal pain, nocturnal awakening, nausea/vomiting, anorexia/early satiety, dysphagia/odynophagia, and GI bleeding), each scoring between 0 and 2 points (maximum, 14 points). Zero points were awarded if symptoms were absent, 1 if the symptoms were mild and did not disrupt daily activities, and 2 if the symptoms were severe enough to disrupt daily activities. Previous GI bleeding was considered mild (1 point) if there was no associated anemia or hemodynamic changes and considered severe (2 points) if it occurred more than once, caused anemia, or required a blood transfusion. (9)

Two investigators, pediatric endoscopists (CPB, LBM), regularly performed all EGD. They scored the findings upon consensus based on the EREFS (Endoscopy reference system) using a slide with original pictures, immediately after the procedure. The EREFS consist of severity classification of some findings – fixed rings (trachealization) (0–3), exudates (0–2), furrows (0–2), edema (0–2), and the presence of other –

strictures (0–1), transient rings (feline esophagus) (0-1), narrow caliber esophagus (0–1), and crepe paper esophagus (0–1), scoring between zero to 13 points. (23) The inflammation score was calculated by the sum of exudates, furrows, transient rings, and edema ranging from 0 to 7 points, and fibrostenotic score was the sum of fixed rings and any strictures manifestation (strictures, narrow caliber, and crepe paper esophagus) ranging from 0 to 6 points.

Esophageal mucus specimens were collected under direct endoscopic visualization, from distal to proximal esophagus using standard cytology brushes (Olympus BC-202D-5010) before obtaining esophageal biopsies. The brushes were dipped and stirred into the PBA tube, transferred in ice to the Laboratory for storage and further analysis. Four mucosal biopsies were taken from duodenum and stomach, and four from mid-proximal and distal levels. The duodenum, stomach, and four esophagus biopsies were kept in formalin for histologic analyses. The other four esophagus samples (two mid-proximal and two distal) were held in PBS and frozen for laboratorial analyses.

Histopathological analyses

Mucosal biopsies were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin (H&E). All biopsies were evaluated by two pathologists (TMA, TT) to full-filled the EoE histologic epithelial score upon consensus.(24) The mid-proximal and distal PEC (peak eosinophil count) were analyzed. The epithelium histological score (EHS) consists of the analysis of 5 histological features: peak eosinophil count (0 = 0 Eos/hpf, 1 = 1-10 Eos/hpf, 2 = 10-20 Eos/hpf, 3 = 21-40 Eos/hpf, 4 = 41-60 Eos/hpf, 5 > 61 Eos/hpf), basal zone hyperplasia (0 < 20% of epithelial thickness, 1 = 21-51%, 2 = 51-75%, 3 > 75%), the presence of dilated intercellular spaces (0–1), epithelial desquamation (0–1), eosinophil clusters/abscesses (0–1), and

degranulated eosinophils (0–1). The lamina propria score (LPS) were the level of PEC (0 = 0 Eos/hpf, 1 = 1-5 Eos/hpf, 2 = 6-20 Eos/hpf, 3 > 20 Eos/hpf) and lamina propria fibrosis including fibroblast and collagen bundles (0 = absent, 1 = mild, 2 = moderate, 3 = severe). The EHS ranging from 0 to 12, the LPS from 0 to 6 and final sum ranged from 0 to 18 points. The PEC for each biopsy specimen was defined as the maximum number of eosinophils in any single hpf. Olympus BX50 microscope with a field diameter of 0.535mm and an hpf area (40 objective; 400 total magnification) of 0.225mm^2 was used for analyses.

Criteria for EoE and GERD diagnoses at the follow-up

All enrolled patients were followed up at the pediatric gastroenterology outpatient clinic until completing the diagnoses of EoE, GERD, or control, under the current guidelines recommendations. (25-28) The anthropometric data were obtained at the first appointment.

The criteria used to define the groups were:

- EoE – the presence of any symptom related to esophageal dysfunction with at least one esophageal biopsy with 15 or more eos/these procedures, and their outcome did not reveal any criteria of esophageal infection or other eosinophilic disorder (e.g., Eosinophilic gastroenteritis, Celiac disease, IBD, Barrett's esophagus, Connective disease, hypereosinophilic syndrome). The number of Eo on the stomach e duodenum mucosa was usual.

The PPI therapy was applied for 8 weeks (1-2mg/kg/day), and the responders were noted but were not excluded from the EoE group. (29)

- GERD – the presence of any symptoms related to reflux disease considering the age of the patient, associated with erosion esophagitis or abnormal

esophageal pH monitoring study for infants and children under the age of 8. For older children and adolescents, the same criteria were used, adding heartburn improvement upon PPI therapy. (27)

- Controls – consisted of patients whose esophageal epithelium was unremarkable, without esophageal eosinophilia, and their outcome did not reveal eosinophilic disorders or GERD.

Biomarker selection by proteomic analysis

The esophageal samples selected from well-defined 3 EoE, 3 GERD and 8 controls patients were utilized for proteomic analysis in the search for a possible biomarker. Esophageal tissue samples were digested using the ProteoExtract® All-In-One Trypsin Digestion Kit (Calbiochem) according to the protocol for tissue samples. Digested peptides were then de-salted using C18 ZipTips (Millipore). Peptides were identified by chromatography using a PepSwiX monolithic column coupled to a Q-Exactiv hybrid quadrupole-orbitrap™ mass spectrometer (Thermo Fisher Scientific). The resulting fragmented spectra were processed using the Proteome Discoverer and PEAKS Studio 7.5, and and analyzed using human UniProtkB database.

Immunohistochemistry based on Polyclonal Anti-Eosinophil-Derived Neurotoxin (EDN) detection

One glass slide from mid-proximal and distal esophagus from all patients, initially coded by clinical histopathology laboratory personnel, was prepared upon the following EDN-pAb–based immunohistochemistry protocol by lab research investigators (APS, AN, TA).

Paraffin-embedded tissue sections were deparaffinized, rehydrated, and incubated with ten percent sodium hydroxide in 95% ethanol for removal of formalin pigment. The

tissue sections were rinsed seven times in distilled water and then submitted to antigen retrieval with citrate buffer + Tween-20 buffer (10mM, pH 6.0 using a microwave oven for three cycles of 5 min). Endogenous avidin and biotin-binding activities were blocked according to the method of Miller et al., and endogenous peroxidase activity was blocked with three 10-min washes with 3% H_2O_2. Subsequently, the sections were incubated with the primary antibody, polyclonal rabbit anti-human EDN (1:100, LS-B12991, LSBio, INC., USA) in a humidifying chamber for 2 h t 25°C. After that, slides were washed two times in TRIS-HCl (pH 7.4) for 2 min each followed by incubation with HRP-conjugated goat anti- IgG (1:500, A24537, Thermo Fisher, EUA) for 1 h at 25°C. After a washing step with TRIS-HCl, peroxidase activity was visualized by incubation in 3, 3'- diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, USA) for 5 min at room temperature and counterstained with Harris hematoxylin. As a negative control, the primary antibodies were replaced by TRIS-HCl buffer. Fragments of human colon tissues were utilized as controls.

Histopathologic Score based on Polyclonal Anti-Eosinophil-Derived Neurotoxin (EDN) detection

Evaluation of the immunohistochemical staining was performed by four investigators in pairs (CBP, APS, AN, or TA), under a senior pathologist supervision (TMA). The investigators created a newly IHC histologic scoring system for reading the slides, its parameters and levels are described in **Table 1.** Pictures showed well-characterized EDN extracellular deposition levels from patients of this study. IHC total score is the sum of four listed parameters, and ranged from 0 to 12 points.

An Olympus BX50 microscope with a field diameter of 0.535mm and an hpf area (40 objective; 400 total magnification) of 0.225mm2 was used for analyses.

EDN detection by ELISA in mucus

For ELISA using mucus from 71 patients, ELISA plates were coated with 4 µg of protein in 50 µl/well in duplicate of the soluble fraction of the mucus diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6), incubating overnight at 4ºC. After washing three times with PBS containing 0.1% Tween 20 (PBST), the plates were blocked for nonspecific sites with 270 µL of 5% BSA in PBST, incubating for two hours at 37ºC. The plates were then rewashed six times with PBST. The rabbit polyclonal IgG to human EDN (LSBio, LS-B12991) was diluted 1:1000 in PBST with 5% BSA in 50 µl/well. After incubation, for 2h at 37ºC, the plates were washed six times with PBST, and 50µl/well of secondary antibody (HRP-conjugated anti-rabbit IgG, Invitrogen, G21234) was diluted 1:1000 using 5% BSA in PBST was added. After one hour at 37 ºC the plates were washed six times with PBST, and the reaction was revealed by addition of 50 uL of TMS substrate solution (Marca) at RT for 15 min. Finally, the reaction was stopped using 2M H_2SO_4, and plates were read at 450 nm using a microplate reader **(**Thermo Plate/TP-reader)

Statistical analysis

The baseline characteristics are described using percentages for categorical variables, and medians, ranges, and percentiles for continuous variables. Body mass index (BMI) are reported by medians z-scores. Comparative analyses across groups were performed using Binomial Proportions Test for atopic history and gender, and non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test for other demographic data, medians scores, and EDN mucus levels. Predictive epidemiological parameters of diagnostic efficiency were measured by sensitivity, specificity, positive,

and negative predictive values; and Receiver operating characteristic (ROC) curves at different cut-off points. Spearman's rank correlation coefficient followed by Bonferroni adjustment was used for correlation matrix across scores and PEC, and for correlation between EDN mucus levels and PEC. The GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, was used for the analyses.

RESULTS

Diagnostic classification and cohort clinical-demographic characterization

A total of 110 subjects, from 390 consecutive pediatric endoscopies, were included in this study at UFU Clinical Hospital, between January 2015 and August 2016. The flow-chart of the study describes the steps of the diagnostic process classification and distribution of samples in analysis procedures **(Figure 1)**. Three subjects were lost to follow-up, they did not return to clinical appointments to complete the diagnostic process. During the cohort follow-up, 16(14.5%) subjects were classified as EoE, 14 (12.7%) as GERD, and 77(70%) as controls. EoE cases compared with controls were more likely to be a man, and have atopic disorders as food allergy and atopic dermatitis. **(Table 2)**. The population characterization by symptoms at baseline, endoscopic and histopathological features were described using the SST, EREFS and EHS parameters **(Table 3)**.

Identification of the best protein biomarkers for EoE diagnosis

The clinical and laboratory characteristics of fourteen subjects selected for proteomic analysis are shown in **Table 4**. Only control subjects had no eosinophils in their biopsies. The best 17 protein hits identified by proteomics were plotted in a heat map. For EDN identification of E1, E2 and E3 patients, it was found 10, 13 and 9 peptide

matches with a coverage of 57, 40 and 65%, respectively. All the other controls and GERD patients presented no hits. **(Figure 2-A)** EDN was selected as the principal biomarker due to the ability to distinguish the three groups. **(Figure 2-B/C)** Native EDN identified and analyzed by mass spectrometry with its regions covered by identified peptides are shown in **Figure 2-D.**

Scores description – Symptom scoring tool, Endoscopic reference score, Epithelial Histology Score, and EDN IHC score

The comparison of symptom score means and endoscopic, histopathological, and EDN IHC scores medians of the groups, demonstrated that only the symptom score did not differ EoE group from the other groups. The EREFS, EHS, and EDN IHC scores differed EoE group from control (P<0.0001). Only EHS differed EoE group from GERD (P=0.0007). **(Table 5)** All comparisons using EDN IHC score and EHS used the mean between mid-proximal and distal scores, because scores from both sites correlated in histological and IHC analyses (EDN IHC score mid-proximal x distal, r=0.69, P<0.0001 and EHS mid-proximal x distal, r=0.55, P<0.0001). The eosinophil count from mid-proximal and distal esophagus also showed significant correlation (r=0.57, P<0.0001). The Spearman rank correlation coefficients between PEC and all scores are shown in **Table 6**. There is a positive and significant correlation between EHS, EREFS and IHC EDN score and PEC, although SST did not correlated with any other score. The best correlation with PEC occurred with EHS and IHC EDN score, since they take into account the presence of eosinophil.

Application of EDN ELISA in esophageal mucus as a biomarker for EoE diagnosis

ELISA EDN analyzes were performed in esophageal mucus samples for 51 controls, 9 GERD and 11 EoE patients. The mean of EDN levels in esophageal mucus for EoE, GERD, and control groups were respectively 0.515±0.402, 0.186±0.125, and 0.187±0.194 (EoE vs. GERD, P=0.0066; control vs. EoE, P<0.0002; control vs. GERD, P>0.9999) (**Figure 3A**). The Spearman coefficient showed a strong and significant correlation between EDN levels at esophageal mucus, in EoE patients with the PEC by H&E (the current gold standard for EoE diagnosis). **(Figure 3B)** EDN levels in the mucus of EoE patients demonstrated that the protein used as a biomarker is able to express the same inflammation status observed in esophageal tissue.

Receiving operator characteristic (ROC) curve for EDN ELISA demonstrated a good ability to discriminate between patients with and without EoE with AUC (area under curve) = 0.774. **Figure 3C** shows the composition of all ROC curves calculated for all clinical and laboratorial scores, and EDN ELISA analyses in mucus.

Through the analysis of predictive values generated by ROC curves for EDN ELISA in mucus and EDN IHC score in esophageal tissue, the best *cut off* values were suggested for EoE diagnosis. Elected *cut-off* points are shown in the table of **Figure 3D** at center position (EDN ELISA>0.1598 and EDN IHQ score> 4.5). The description of the predictive diagnostic parameters for these *cut-offs* values, and one previous value and the next, are described at the table.

Considering the *cut-off* for EDN ELISA in mucus at 0.1598 point, and the standard deviation of the mean for each group, 6 outlier patients were identified (1 EoE, 1 GERD, and 4 controls). **Table 7** describes clinical and laboratory characteristics for each outlier subject with their respective EDN ELISA levels in esophageal mucus.

DISCUSSION

Although many markers have been attempted to improve EoE characterization, diagnosis is still based on a clinical consensus (1, 30) using peak eosinophil counts (PECs) on esophageal histological biopsies in endoscopic procedures, which besides the high cost and intrinsic risks involved, the method still lacks specificity. In pediatric settings, this becomes even more necessary due to the need for anesthetic sedation to perform these procedures, besides the overlapping symptoms with other frequent childhood diseases. Although numerous studies have searched for serum biomarkers, results are conflicting (18, 31) and lack specificity due to the presence or influence of other allergic diseases. In order to supplant this unmet need, we have successfully investigated esophageal tissues and mucus from patients with EoE and GERD, using proteomics, immunohistochemistry, and ELISA, which resulted in the validation of EDN as a major marker for EoE diagnosis.

Detection of increased levels of degranulation with eosinophilic proteins demonstrates a relationship with disease activity. (16, 32) However, eosinophil counts alone may overestimate or underestimate the actual inflammatory status of esophageal tissue. (33) It is not uncommon to observe during the treatment, remission of symptoms with the maintenance of high levels of PEC in endoscopic analyses. (34) In this sense, diagnostic tools that seek to evaluate eosinophil activation, and not only the number of eosinophils infiltrated in the epithelium, tend to increase reliability and broaden the predictive values of the test. (35) Although EDN is not an eosinophil-specific protein, it offers promising results in this regard. This ribonuclease was the protein that presented the best intensity and coverage results in the proteomic analysis, and its validation was statistically significant in esophageal tissue and mucus samples when comparing patients with and without EoE. The positive, strong and significant correlation between

esophageal mucus EDN values and PEC **(Figure 3B)** in patients with EoE suggests that the biomarker found within eosinophils counted in epithelial fields also expresses its activity in adjacent esophageal mucus. Smadi et al. conducted a similar prospective investigation in adults and children, and collected blind esophageal brushing using a nasogastric tube obtained with significant accuracy of EDN detection by ELISA (36); however, the potential limitation of nasogastric sampling is the contamination with secretions with other allergic diseases, which may have altered EDN results.

The use of biomarkers for EoE diagnosis should be understood as an auxiliary resource applied to the differential diagnosis of the disease. Therefore, as a predictive feature, the test should have balanced specificity and sensitivity. Endoscopic (EREFS) and histological (EHS) scores showed good predictive capacity and should continue to be used as initial tools to guide the diagnostic investigation. (7) The systematic use of these tools will allow a better rationale of tests with greater assertiveness and cost optimization.

Favorable results regarding the analysis of symptoms were not observed, the predictive values were not capable of stratifying groups. Due to non-specificity and overlap of symptoms with other functional and inflammatory gastrointestinal diseases, metric assessment of symptoms has not yet become a useful tool for disease screening. However, the publication and validation of PRO *(patient-reported outcome)* questionnaires applied to the adult, and pediatric population presents promising results in monitoring disease remission. The PEESS v2.0, as a pediatric instrument, applied to parents and children over 8 years that has shown moderate results in a publication that sought to correlate symptoms with biomolecular markers of disease activity.(10) The metric tool was translated and adapted linguistically by this group and presents new perspectives for symptom assessment, especially in multicenter studies. (37)

A prominent point of our study was the inclusion of incident cases of EoE, GERD, and control cases with similar symptoms. At the beginning of the sample collection, no patient had a diagnostic definition, and the investigation was carried out under the control of researchers applying defined and uniform protocols. The metric instruments were also used systematically and standardized among the researchers. However, a clear point of our study was the reduced number of patients with EoE, and the non-availability of the same amount of esophageal mucus samples when compared to tissue. The impossibility of processing all mucus might have happened due to the liability of esophageal samples.

This study identified and validated EDN as a biomarker of esophageal tissues and mucus in EoE pediatric patients. Besides, it demonstrated that metric evaluation of endoscopic and histopathological findings could be used as good predictors of the disease. Our results also open perspectives for multicentric studies to validate EDN as a biomarker of esophageal mucus samples, which is a less invasive procedure with reduced costs that may become an important auxiliary tool for EoE diagnosis.

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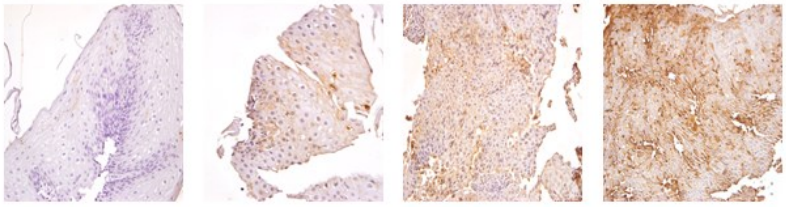
Table 1 – Parameters for calculation of EDN-pAb-based Immunohistochemistry diagnostic scoring

EDN-pAb staining parameters	Numeric score			
Eosinophil infiltration				
Maximum single focus*	0 (<5)	1 (5-10)	2 (10-15)	3 (>15)
Average of five designated foci**	0 (<5)	1 (5-10)	2 (10-15)	3 (>15)
Degranulation				
Percent area of all biopsies affected by degranulation	0 (<10%)	1 (10-39%)	2 (40-69%)	3 (70-100%)
Level of degranulation observed in maximally affected biopsy*	0 (negative)	1 (minimal)	2 (moderate)	3 (confluent)



*40x, 0.22 mm² field of view, **40x, 1.1 mm² field of view

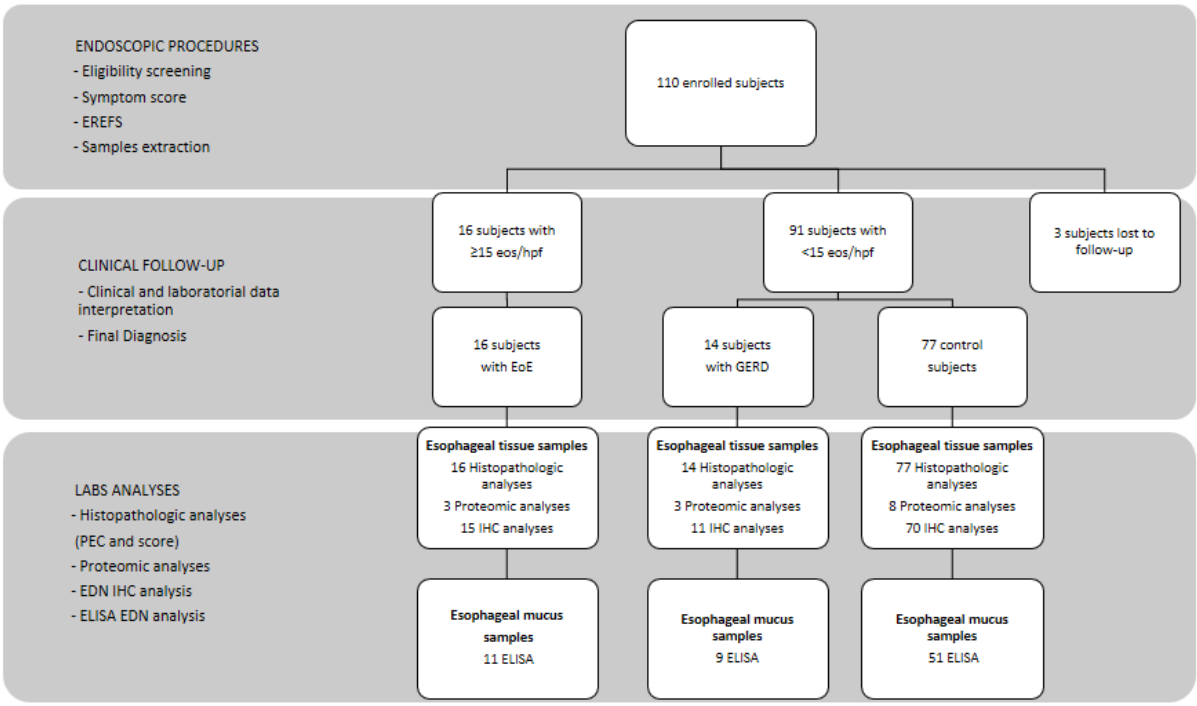


FIGURE X: The flow-chart of the study with the description of procedures and analyses in each step.
EREFS, endoscopic reference score; IHQ, immunohistoqchimistry; EDN, eosinophil derived neurotoxin

Table 2 – Demographics and clinical features of the cohort groups

Demographics/Clinical features	Control	EoE	GERD	p-value		
				Control X EoE	Control X GERD	EoE X GERD
N (%)	77 (70.0)	16 (14.5)	14 (12.7)			
Gender (male) n (%)	36 (46.8)	12 (75)	7 (50)	0.0415[b]*	>0.9999[b]	00768[b]
Age – median (range)	9 (0-16)	8.5 (1-17)	10 (5-12)		0.5120[a]	
BMI z-score - median [P25,P75]	0.03 [-0.32, 1.14]	0.51 [-0.77, 0.51]	0.09 [-1.06, 1.47]		0.9350[a]	
Time from onset symptoms to diagnosis month median (range)	24 (1-180)	24 (6-180)	36 (5-120)		0.8786[a]	
Atopic history						
Asthma (%)	8 (10.39)	4 (25.00)	1 (7.14)	0.0767[b]	0.5647[b]	0.1010[b]
Allergic rhinitis (%)	33 (42.85)	9 (56.25)	5 (35.71)	0.2025[b]	0.3991[b]	0.1008[b]
Atopic dermatites (%)	8 (10.39)	4 (25.00)	0 (0.00)	0.0767[b]	0.2153[b]	0.0178[b]*
Food allergy (%)	8 (10.39)	6 (37.50)	1 (7.14)	0.0040[b]*	0.5647[b]	0.0130[b]*

[a] Kruskal Wallis Test, [b] binomial test proportions
*considered statistically significant at a level < 0.05;
EoE, Eosinophilic esophagitis; GERD, Gastroesophageal reflux disease.

Table 3 – Features of the symptom, endoscopic and histopathological scores of the cohort groups

	EoE (N = 16)	GERD (N = 14)	Control (N = 77)
Symptom score			
Heartburn / regurgitation (%)	14 (87.5)	10 (71.4)	54 (70.1)
Abdominal pain (%)	14 (87.5)	12 (85.7)	66 (85.7)
Nausea / vomiting (%)	15 (93.8)	12 (85.7)	61 (79.2)
Anorexia / early satiety (%)	11 (68.8)	12 (85.7)	52 (94.5)
Dysphagia (%)	8 (50.0)	7 (50.0)	31 (41.6)
Symptom induced nocturnal wakening (%)	8 (50.0)	4 (28.6)	31 (40.3)
Gastrointestinal bleeding (%)	0 (0.0)	0 (0.0)	0 (0.0)
Endoscopic score – EREFS			
Edema (%)	13 (81.2)	7 (50.0)	23 (30.0)
Fixed rings (%)	2 (12.5)	0 (0.0)	1 (1.3)
Exsudates (%)	8 (50.0)	2 (14.3)	3 (3.9)
Furrows (%)	9 (56.3)	1 (7.1)	1 (1.3)
Stictures (%)	1 (6.3)	1 (7.1)	0 (0.0)
Epitelial histophatological score			
Peak eosinophil count/HPF (mean +/- SD)	47.8 +/- 31.2	2.4 +/- 3.9	0.5 +/- 1.0
Basal zone hyperplasia			
mild (21% - 50%)	7 (43.8)	1 (7.1)	7 (9.0)
moderate (51% - 75%)	5 (31.3)	0 (0.0)	1 (1.3)
severe (> 75%)	2 (12.5)	0 (0.0)	0 (0.0)
Dilated intercelular spaces (%)	16 (100.0)	14 (100.0)	73 (94.8)
Epithelial desquamation (%)	5 (31.3)	0 (0.0)	0 (0.0)
Eosinophil clusters / abscesses (%)	0 (0.0)	8 (57.1)	0 (0.0)
Degranulated eosinophils (%)	12 (75.0)	4 (28.6)	6 (7.8)

EoE, Eosinophilic esophagitis; GERD, Gastroesophageal reflux disease; HPF, high powerful field; SD, standard deviation.

Table 4 – Clinical and laboratory characterization of the subjects whose samples were used for proteomic analyses

	Demographics				Symptoms		Endoscopy		Histopathology	
Subject	Diagnoses	Age (y)	Gender	Atopy	SS (features)	SS (max. 14 pts)	EREFS (findings)	EREFS (max. 13 pts)	PEC (HPF)	Epithelial score (max. 12 pts)
3 (E1)	EoE	4	Female	AS, AR, EC	AP, NV, ANE, NA	7	EXU, EDE	2	16	4.5
8 (E2)	EoE	8	Male	AS, AR, EC	HR, AP, NV, ANE, DYS	9	FUR, EDE	2	18	4
16 (E3)	EoE	4	Male	AR, FA	HR, NV, ANE, DYS, NA	7	EXU, EDE	3	72	7
6 (G1)	GERD	11	Female	AR	AP	2	EXU, FUR, EDE, T/RG	5	7	2.5
9 (G2)	GERD	9	Female	-	AP, NV, ANE	4	-	0	1	0.5
24 (G3)	GERD	9	Male	-	HR, NV, ANE	3	EDE	2	2	1.5
1 (C1)	Control	9	Male	-	AP, NV, ANE	5	-	0	0	1
2 (C2)	Control	6	Female	-	ANE	1	-	0	0	1
4 (C3)	Control	1	Male	-	DYS	1	-	0	0	0.5
13 (C4)	Control	2	Male	-	AP, NV, ANE, NA	4	-	0	0	0
15 (C5)	Control	11	Male	-	AP, NV, ANE, NA	8	EDE	1	0	1
17 (C6)	Control	10	Male	FA	HR, AP, NV, ANE, DYS	8	-	0	0	0.5
21 (C7)	Control	10	Female	-	HR, AP, NV, ANE	6	EDE	2	0	1
29 (C8)	Control	13	Male	AR	AP, ANE	4	EDE	2	0	1

NOTE: Atopic patients had asthma (AS), eczema (EC), allergic rhinitis (AR), and food allergy (FA). SS, Symptom scoring tool: ANE indicates anorexia/early satiety; AP, abdominal pain; HR, heartburn/regurgitation; NA, nocturnal awakening; NV, nausea/vomiting; DYS, dysphagia. EREFS, Endoscopic Reference Score: EXU indicates exudate; EDE, edema; FUR, furrows; T/RG, transit rings. PEC, peak eosinophil count; HPF, high powerful field.

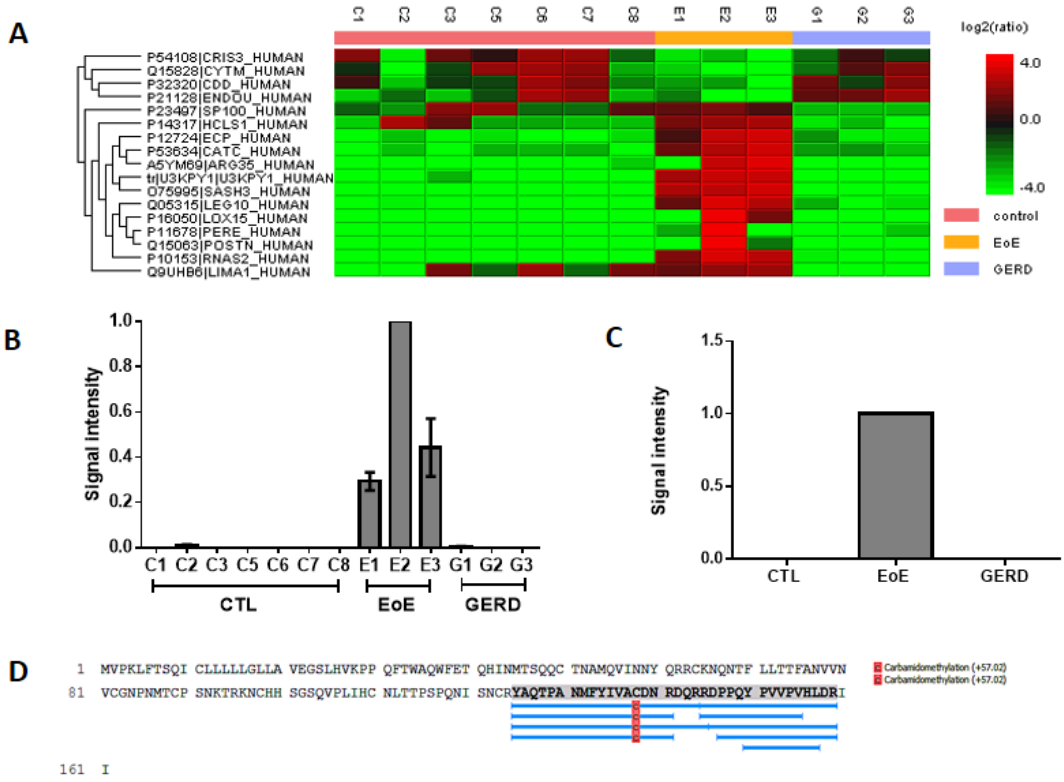


Figure 2: Mass spectrometry analyses of esophageal tissue samples from Eosinophilic Esophagitis (EoE), Gastroesophageal Reflux Disease (GERD) and control groups. Analysis of the Human RNase 2 protein target, also called Eosinophil-derived neurotoxin (EDN). (A) Heat map of the 17 best protein hits according to the peptide hits and coverage. (B) The data of the three methods of quantification of eosinophil-derived neurotoxin (EDN) are visualized as bar graphs (mean + SEM) for each sample of control (CTL), EoE and GERD groups. (C) the mean + SEM of all samples groups was calculated to compare control, EoE and GERD groups. (D) Native EDN identified and analyzed by mass spectrometry. The regions covered by identified peptides are shown in gray, each blue bar indicates a defined peptide sequence and the post-translational modification (PTM), and carbamidomethylation is shown in the red box.

Table 5 – Comparison of symptom, endoscopic, histopathological and EDN Immunohistochemistry scores among control, EoE and GERD groups

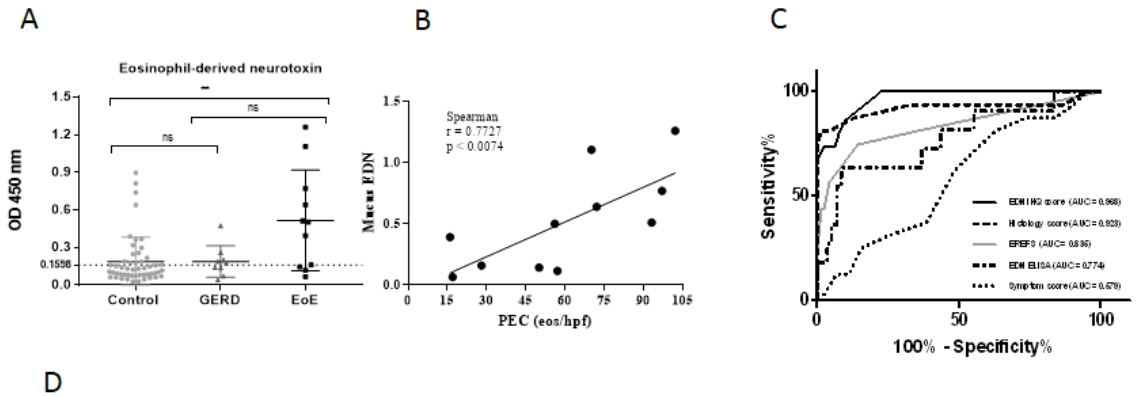
	Control	EoE	GERD	p-value		
				Control X EoE	Control X GERD	EoE X GERD
Symptom score (max 14 pts) **mean ± SD (max. 14 pts)**	6.39 ± 2.98	7.31 ± 2.82	7.14 ± 3.28		0.4235[a]	
EREFS (max 13 pts) **median [P25-P75]** **(range)**	0.0 [0-1] (0-4)	3.0 [1.25-5.75] (0-7)	0.5 [0-2] (0-5)	<0.0001*[b]	0.2562[b]	0.0519[b]
Epithelial histology score (max 12 pts) **median [P25-P75]** **(range)**	1.0 [1-1.5] (0-3.5)	6.0 [3.6-7.5] (0.5-11)	1.0 [1-1.75] (0.5-3)	<0,0001*[b]	>0,9999[b]	0,0007*[b]
EDN IHC score (max 12 pts) **median [P25-P75]** **(range)**	0.0 [1-2.0] (0-4)	9.0 [5-11] (3-12)	5.0 [3-6] (1-7)	<0,0001*[b]	<0,0001*[b]	0,7560[b]

[a]1 way ANOVA Test, [b]Kruskal Wallis Test with Dunn's multiple comparisons test
*considered statistically significant at a level < 0.05
EoE, Eosinophilic esophagitis; GERD, Gastroesophageal reflux disease.

Table 6 – Spearman Rank Correlation Coefficients of symptom, endoscopic, histopathologic and EDN Immunohistochemistry scores

Scores	PEC	IHC score	Histology score	EREFS
IHC score	0.541 (*P*<0.001)			
Histology score	0.841 (*P*<0.001)	0.422 (*P*<0.001)		
EREFS	0.395 (*P*<0 .001)	0.405 (*P*<0.001)	0.453 (*P*<0.001)	
Symptom score	0.140 (*P*=0.176)	0.088 (*P*=0.394)	0.126 (*P*=0.222)	0.132 (*P*=0.203)

PEC, Peak eosinophil count; IHQ, Immunohistochemistry; EREFS, Endoscopic Reference Score.



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Diagnostic parameters with different cut-off values	EDN mucus ELISA			EDN IHC total score		
	> 0.1420	*> 0.1598*	*> 0.3905*	*> 2.5*	*> 4.5*	*> 6.5*
Sensitivity %	81.82	72.73	63.64	100.00	80.0	73.33
Specificity %	56.67	63.33	91.67	77.78	92.59	97.53
Positive predictive value %	7.47	7.82	24.62	16.14	31.58	55.93
Negative predictive value %	98.65	98.19	98.33	100.0	99.09	98.84

EDN, eosinophil-derived neurotoxin; IHC immunohistochemistry

Figure 3 (A) Biomarker levels in esophageal mucus of control, GERD, and EoE subjects at baseline. The scatter plot graph shows the distribution of every individual measure with central line drawn at the mean of the group, and whiskers showing the values lower and greater of the standard deviation of the mean. The dotted horizontal line marks a cutoff point. The horizontal brackets on the top show the significance level between the means of the groups; ns, not significant; ** *P = 0.009* (B) Spearman correlation between mucus EDN ELISA and PEC (eos/hpf) in EoE patients (C) Receiver operator characteristic curves of clinical scores (Symptom and EREFS), histology score, EDN IHC score, and EDN ELISA biomarker levels in esophageal mucus. All scoring tools and EDN ELISA in mucus were used to analyze the receiver operating characteristic to discriminate between patients with and without eosinophilic esophagitis (EoE), and for predicting the presence of indicates cases of EoE. AUC indicates area under the curve of each used diagnostic tool. (D) Operating Characteristics of EDN mucus ELISA from esophageal mucus and EDN IHC score at three different cutoff points, and their diagnostic parameters (sensitivity, specificity, positive predictive value and negative predictive value).

Table 7 – Clinical and laboratory characterization of outliers subjects according EDN ELISA in the esophageal mucus at 0.1598 cut-off value.

	Demographics				Symptoms		Endoscopy		Histopathology		EDN IHC		EDN ELISA
Subject ID	Diagnoses	Age (y)	Gender	Atopy	SST (features)	SST (max. 14 pts)	EREFS (findings)	EREFS (max. 13 pts)	PEC (hpf)	Epithelial score (max. 12 pts)	PEC (hpf)	EDN IHC score (max. 12 pts)	EDN mucus level
34	EoE	10	Female	-	HR, AP, NV, ANE, NA	8	-	0	17	1.5	2	4	0.0665
103	GERD	10	Female	AS, AR	HR, AP, NV, ANE, DYS	12	-	0	1	1.5	7	7	0.4730
33	Control	10	Male	-	HR, AP, NV	4	-	0	0	1	0	0	0.7390
52	Control	2	Male	-	AP	1	-	0	0	1	1	2	0.8120
68	Control	12	Male	AD	HR, AP, NV	10	EDE	1	2	1.5	1	0	0.6395
90	Control	10	Male	AR	HR, AP, NV, DYS	8	EDE	1	0	1	2	0	0.8965

Note: Atopic patients had asthma (AS), allergic rhinitis (AR), atopic dermatitis (AD), and food allergy (FA). SS, Symptom scoring tool: ANE indicates anorexia/early satiety; AP, abdominal pain; HR, heartburn/regurgitation; NA, nocturnal awakening; NV, nausea/vomiting; DYS, dysphagia. EREFS, Endoscopic Reference Score: EDE, edema; FUR, furrows; EXU, exudate. PEC, peak eosinophil count; HPF, high powerful field; EDN, eosinophil-derived neurotoxin; IHC, immunohistochemistry.

4.2 Artigo 2 – Título: *“Endoscopic Reference Score is a Good Predictive Tool Differing Patients with Eosinophilic Esophagitis and Gastroesophageal Reflux Disease in a Pediatric Cohort.”*

Parecer do CEP consubstanciado (ANEXO A)

Normas de submissão para autores – Gastrointestinal Endoscopy (ANEXO C)

Title: Endoscopic Reference Score is a Good Predictive Tool Distinguishing Patients with Eosinophilic Esophagitis and Gastroesophageal Reflux Disease in a Pediatric Cohort.

Short running title: EREFS predicts pediatric EoE diagnosis

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Declaration of interest: None

Conflict of interest

All authors declare no conflict of interests

ABSTRACT

Background and aims: Endoscopic Reference Score (EREFS) is an endoscopic metric tool used to predict the diagnosis and evaluate the treatment of eosinophilic esophagitis (EoE). Clinical and endoscopic overlaps between EoE and gastroesophageal reflux disease (GERD) are challenging during diagnostic investigation. Little is known on the interference of GERD findings in the ability of EREFS to distinguish EoE patients. We aimed to evaluate the usefulness of EREFS to predict EoE diagnosis, differing from GERD in pediatric patients.

Methods: This is a prospective study in a pediatric cohort (1-18y) undergoing diagnostic endoscopy to investigate gastrointestinal complaints. 110 consecutive endoscopies were scored by EREFS in real time. EoE and GERD cases were defined upon consensus guidelines, and control group for unremarkable endoscopies and biopsies. Total EREFS (T-EREFS), inflammatory, and fibrostenotic scores were described. Predictive values and receiver operating characteristic curve were calculated. (ClinicalTrials.gov NCT03069573)

Results: 107 diagnostic endoscopic procedures were included. The follow up identified 16 (14.5%) EoE, 14 (12.7%) GERD and 77 (70.0%) control patients. All

inflammatory characteristics were able to identify EoE patients, except transited rings. Edema was the unique that showed balanced sensitivity (81.3%) and specificity (67.0%) with moderate accuracy (69.1%). Fibrostenotic features were rare. T-EREFS medians groups distinguished EoE from Control (2.5 vs. 0.0, *P*<0.0001) and GERD (2.5 vs. 0.5, *P*=0.0375). Predictive values for T-EREFS at cut off point 2 were 75% of sensitivity, 85.7% of specificity, 84.1% of accuracy, and AUC=0.80.

Conclusion: EREFS was a good metric tool to predict the diagnosis of EoE in a pediatric endoscopic cohort, without interference of GERD findings. The modified EREFS seems to be suitable for diagnostic purposes in children.

Key words: Eosinophilic esophagitis, Gastroesophageal reflux disease, EREFS, diagnostic metric tool.

INTRODUCTION

Eosinophilic esophagitis (EoE) is an inflammatory disease clinically defined by symptoms of esophageal dysfunction, and histologically by predominant eosinophilic inflammation in esophageal tissue.[1] The current consensus guideline recommends the presence of at least 15 eosinophils per high-power field (eos/hpf), obtained by routine biopsies through esophagogastroduodenoscopy (EGD), as a diagnostic criterion for adults and children. [2]

Pediatric EoE symptoms are often non-specific. In infants and toddlers, the disease presents with failure to thrive, feeding difficulties, vomits, and abdominal pain. In older children, frequent vomiting and abdominal pain are also reported, as well specific symptoms such as dysphagia and food impaction. Even though symptoms tend to vary with age, any manifestation of gastroesophageal dysfunction may occur.[3, 4] Once EoE is suspected, its presence should be confirmed by EGD with appropriate biopsies. Four or more biopsies of both proximal and distal esophagus need to be taken, as the disease often has a patching distribution[1, 5]. EoE endoscopic findings are also non-specific and may be seen in other esophageal conditions as GERD, infection, connective tissue diseases, and achalasia.[3] Although the presence of edema, furrows, white mucosal plaques, rings, stricture, and mucosal tearing after endoscope tube passage are suggestive of EoE, subtle and initial abnormalities may be confused with those other conditions. In addition, pediatric EoE has been reported as a normal endoscopic appearance in up to 32% of patients, [6-8] and children with GERD showed only 12.4% of erosive esophagitis as a typical endoscopic finding in a large population-based study.[9]

The correlation between GERD and EoE is complex, not only by symptoms and endoscopic overlaps, but also due to the response to proton pump inhibitors (PPIs)

therapy. Around 50% of patients with EoE are PPI-responsive esophageal eosinophilia (PPI-REE), reaching clinical and histologic remission with this exclusive therapy.[10] In addition to the ability of PPIs healing damaged mucosa affected by acid secretion, they also have an anti-inflammatory action that may decreased the esophageal eosinophilia blocking the production of cytokines responsible for eosinophil recruitment.[11] For this reason, PPI therapy is no longer a criterion used to make the differential diagnosis between EoE and GERD.[1] Evidence in pediatric EoE patients demonstrates that the duration of the disease progression, without effectiveness therapeutic intervention, may guide the esophageal tissue for remodeling and fibrosis.[12] Therefore, in order to achieve an assertive diagnosis avoiding esophageal dysfunction and nutritional deficits, it is important to establish screening methods for early diagnosis. The supremacy of the histological evaluation to establish EoE diagnosis cannot yet be questioned, so clinical metric tools should have the role to direct timely and accurate diagnostic investigation.

The Endoscopic Reference Score (EREFS), proposed by Hirano et al[13], standardized the endoscopic findings of EoE, providing grades of severity. It has been used for diagnostic prediction, assessment of the disease's activity and remission after therapeutic intervention, and indication of phenotypic profile for prognostic prediction. Some studies in adult and children showed the accuracy improvement for predicting EoE diagnosis before histopathological analysis [14, 15]. Validated studies demonstrated that EREFS system has good inter-observer agreement and high feasibility in real-time procedures.[13, 16] However, there are few prospective studies showing the effectiveness of EREFS used to predict the diagnosis of EoE in a pediatric population. In this prospective pediatric cohort, we aimed to evaluate the usefulness of EREFS in

predicting EoE diagnosis in children, and whether the score is influenced by GERD endoscopic features.

METHODS

Study design and Population

Patients were prospectively enrolled from consecutive EGDs in a pediatric cohort from January 2015 to August 2016, at Clinical Hospital of Federal University of Uberlândia, Minas Gerais, Brazil. No patients who were taking PPI, antihistamine medications, and steroids (topical or systemic) within the last 4 weeks prior to the endoscopy. The included patients were followed up in an outpatient clinic after the endoscopic procedure for diagnostic investigation. The exclusion criteria were a prior diagnosis of some eosinophilic gastrointestinal disorder (eosinophilic gastroenteritis/colitis, celiac disease, inflammatory bowel disease, or Barrett's esophagus, Connective disease, or hypereosinophilic syndrome) and congenital or acquired esophageal stenosis. Written informed consent was provided from all parents. Teenagers older than 12 years of age signed a statement of assent. The institutional review board approved this study under the protocol number CAAE 36787714.0.0000.5152

Diagnostic definitions

The diagnoses of EoE and GERD were based on current clinical diagnostic guidelines.[1, 17] EoE patients were defined as having the presence of any symptom related to esophageal dysfunction with at least one esophageal biopsy with 15 or more eos/hpf, and their outcome did not reveal any evidence of other eosinophilic disorder.

The number of eosinophils on the stomach and duodenum mucosa was not increased. GERD patients (infants and children under the age of 8) showed the presence of some symptoms related to reflux disease, associated to erosion esophagitis or, in its absence, abnormal esophageal pH monitoring study (reflux index - RI ≥ 7%). For older children and adolescents the same criterion was applied adding heartburn improvement upon PPI therapy. Control patients consisted of those whose esophageal epithelium was unremarkable, without esophageal eosinophilia, and their outcome did not reveal the possibility of GERD.

Endoscopic procedure, histological biopsies and EREFS analysis

Two pediatric endoscopists performed all endoscopies following the standard procedure with general anesthesia, using pediatric gastroscope for children under 10 kg and standard adult gastroscope for others.[18]. Four biopsies were collected from each distal esophagus, mid-proximal esophagus, stomach, and duodenum tissues. All samples were analyzed by a senior pathologist, using a high powerful field (hpf) area of 0.225 mm² for analyses (40 objective; 400 total magnification). The endoscopists fulfilled real-time Endoscopic Reference Score (EREFS) based on the endoscopic esophageal features. The reference for both endoscopists was the published classification slides[13]. The original EREFS consist of the severity graduation of some signs – fixed esophageal rings (trachealization) (0–3), exudates (0–2), furrows (0–2), edema (0–2), and the detection of presence of others – strictures (0–1), transient esophageal rings (felinization) (0-1), narrow caliber esophagus (0–1), and crepe paper esophagus (0–1). The total EREFS (T-EREFS) ranges from 0 to 13 points.[13] The composed scores consider the inflammatory or fibroestenotic profile of the endoscopic features. The inflammatory EREFS (I-EREFS) was calculated by the sum of exudates,

furrows, transient esophageal rings, and edema, ranging from 0 to 7. The fibrostenotic EREFS (F-EREFS) was performed by the sum of fixed rings and any other type of strictures feature (strictures, narrow caliber, and crepe paper esophagus), ranging from 0 to 6.

Statistical Analysis

Comparative statistical analyses were performed using non-parametric Kruskal-Wallis tests followed by Student-Newman-Keuls post-test and Binomial Proportions Test for Independent Data. The baseline characteristics of the study population are described by means of percentages, median, interquartile range (IQR) and body mass index (BMI) Z-score. The control and case groups (EoE and GERD) were described by means, minimum value, first quartile, median, third quartile and maximum value besides interquartile range (IQR), amplitude and n-sample. Predictive epidemiological parameters of diagnostic efficiency were calculated by measuring sensitivity, specificity, positive and negative likelihood ratio, positive and negative predictive value, and accuracy. The cutoff point was also estimated. All statistical analyses were performed using PASW Statistics 17 and BioEstat 5.3 software. The *significance level was considered* to be less than 0.05.

RESULTS

Demographic characteristics and diagnostic classification

A hundred and ten patients were enrolled over the study period. Three patients were lost to the follow up because they did not return to outpatient clinic to complete the diagnostic investigation. The final cohort included 107 pediatric patients 16 (14.5%) EoE, 14 (12.7%) GERD and 77 (70%) Controls. The baseline characteristics of the study population with demographics and the description of the first symptom are described in **Table 1**.

EREFS findings

The EREFS findings in this cohort are showed in pictures of **Figure 1**, except for feline esophagus, which is a dynamic feature. The frequency of all findings was higher in EoE than GERD or Control patients. Transient rings and edema at grade 1, were the only inflammatory characteristics that were not statistic significantly higher in EoE patients. Fibrostenotic features (fixed rings and strictures) were not able to differentiate EoE x GERD groups. The features of inflammatory score were present in 13 (81.2%) EoE patients, and most features could be noticed in a frequency of 50% or higher (exudate 50.0%, furrows 56.3%, edema 81.3%, and transient rings 6.3%). In this cohort, signs of fibrostenotic score were infrequent in all groups. In EoE patients, fibrostenotic findings were fixed rings (12.5%) and strictures (6.3%) only, both at grade1. 75% of the EoE group subjects revel I-EREFS and T-EREFS greater than 2 points, and it only occurs in less than 50% GERD and 10% of Control groups. In none of the analyzed groups there was more than 1 point in F-EREFS. **(Table2)**

Description of total EREFS Total and composite scores

The descriptive analysis of I-EREFS, F-EREFS, and T-EREFS with medians, percentiles, ranges, and *P* values, according to the groups is shown in **Table 3**. The comparison of EREFS medians across groups showed statistically significant difference only in I-EREFS and T-EREFS, differing EoE group from Control and GERD.

Accuracy of EREFS to predict EoE diagnosis

The predictive values from all EREFS features, I-EREFS, F-EREFS, and T-EREFS, considering 16 EoE patients as positive cases and 91 Control/GERD patients as negative cases were analyzed **(Table 4)**. The features of I-EREFS and F-EREFS generally had more specificity than sensitivity, except for edema. Fibrostenotic score and its features (fixed rings and strictures) showed accuracy more than 80%, although sensitivity and specificity were unbalanced, with very low sensitivity. Edema at cut off point 1 was the only isolated feature that showed balanced sensitivity (81.3%) and specificity (67%), but accuracy lower than 70%. In this cohort study T-EREFS at cut off point 2 seems to be the best score for predicting EoE diagnosis with an acceptable sensitivity (75%), good specificity (85.7%) and 84.1% of accuracy. The Receiver-Operating Characteristic curve was built for T-EREFS at cut off point 2, and the area under the curve (AUC) was 0.80 **(Figure 2).**

DISCUSSION

This is the first prospective study using EREFS for EoE diagnostic investigation in an unselected pediatric population. The characteristics of EoE symptoms and endoscopic features, similar to GERD manifestations, request special approach in the diagnostic process. This process requires an endoscopy procedure with biopsies, carefully

indicated by the presence of the symptoms. Our cohort did not demonstrate in the baseline characteristics any special clinical condition that allows the prediction of EoE among GERD or other diagnoses. Only the male gender was more frequent in EoE in comparison with analyzed groups (Table 1). In pediatrics, EoE affects mainly school-aged patients, with a peak incidence around 10 years of age, and with a greater predominance in males.[20]

Due to the presence of non-specific clinical data, the EREFS could be considered as a useful metric tool that allows increasing the diagnostic accuracy of the disease before the histological analysis, as an additional biomarker of EoE.[21] The first meta-analysis including EoE and non-EoE control patients demonstrated modest sensitivity and predictive values for endoscopic features to predict esophageal inflammation, and the prevalence of endoscopic features was very heterogeneous.[6] However, in our study, we could observe that the presence of endoscopic findings from EREFS was frequent in EoE patients (>81%). This may occur by the fact that this study has prospective design, using the EREFS metric immediately after the procedure without any information about the patient diagnosis, only their symptoms.

Endoscopic features of children and adults with EoE usually show different presentation. Children typically present inflammatory features, and fibrostenotic features are more commonly seen in adolescents and adults.[6, 22] The analysis of phenotype EoE in inflammatory or fibrostenotic profile was based on a publication, which carried out a multicenter prospective cohort with 25% of pediatric population.[22] The physiopathology mechanism has not been totally known, but maybe it depends on the patients' age and the untreated time of the disease.[12] The fibrostenotic features are thought to be the result of esophageal remodeling by fibrotic repair in an evolution of eosinophilic inflammation. However, this natural evolution of the inflammatory

disease to fibrostenotic condition has not been proved yet. [23] The risk for a fibrostenotic phenotype seems to be twice as much for every 10-year increase in age.[24] A recent published pediatric cohort has demonstrated that there is some relationship between the period of esophagus exposure to a chronic inflammation, and signs of decreased esophageal distensibility, as the result of esophageal remodeling.[12] We found plenty of inflammatory endoscopic profiles, unlike the fibrostenotic profiles. This result seems to be in agreement with the epidemiological data from this study, which detected EoE diagnosis in children with school-age presenting nonspecific symptoms, as abdominal pain, vomiting, regurgitation, and nausea.

The distribution of the inflammatory features showed a significant predominance in EoE children in comparison with both GERD and Control patients (Table 2). The prevalence of these findings was considerable, and the power of the test was acceptable to reduce the type II error (β) and consequently the risk of false negative, which is an important condition for a diagnostic test. The analyses of predictive values have suggested good accuracy for EoE diagnosis only for the feature edema and T-EREFS. This favorable capacity from edema to distinguish the EoE patient may occurred because in this study the analyses were conducted by pediatric endoscopists. This is a subtle endoscopic sign usually evaluated in pediatric GERD, in initial levels of esophagitis without erosion, as described by Hetzel & Dent Classification.[25] The T-EREFS showed the best accuracy (>80%) for the EoE diagnoses with acceptable sensitivity and good specificity, and AUC>0.7 (Table 4 and Figure 2). Although, the original EREFS publication suggest the suppression of some analyzed features which, despite the good specificity, showed low prevalence and a small role in the accuracy of the score.[13, 26] Our data have not demonstrated some EREFS features, as crepe paper esophagus and narrow caliber of esophagus; and very low prevalence of feline

esophagus. Maybe, these features would be also suppressed for the diagnostic EoE pediatric analysis, as in the modified EREFS classification used in adults.

One of the strong points of this study was the validation of EREFS in an unselected pediatric Brazilian population. Most EoE studies are related to the northern hemisphere, and in Caucasian population. This is one of the few Brazilian studies in pediatric EoE and the first prospective endoscopic cohort analyzing the EREFS characteristics at the moment of the diagnoses. A new publication raises the need to promote a better understanding about the demographic influence on EoE behavior, because some underdiagnosed population, such as the Afro-Americans, may present the disease with atypical characteristics.[27] The second point is the suggestion of EREFS as a tool to differentiate EoE from GERD (Table 3), which can be shown by the significant difference between T-EREFS and I-EREFS medians from EoE x GERD. This result has demonstrated that they are actually different diseases, despite the similarity and the possibility of concomitant manifestation.[10]

The major limitation of this study is the low prevalence of EoE patients, and the possibility of a low variance of the data. Other drawback of the manuscript is the absence of inter-observer analysis which may compromise the reliability of the assigned scores and the reproduction of the results. Therefore, we suggest a validated multicenter prospective study using the original EREFS in pediatric population with inter-observer analysis to better validate the use of this endoscopic metric tool for diagnostic purpose.

In conclusion, this study suggests that total EREFS is a useful diagnostic tool for pediatric EoE with acceptable sensitivity, good specificity, and accuracy. The inflammatory features are more frequently found than fibrostenotic features in the assessment of pediatric endoscopic EoE diagnosis, which is in agreement with the

clinical presentation of the disease in the pediatric age group. Some features present in the original EREFS (transient rings – transient rings, narrow caliber of esophagus, and “crepe paper” esophagus) may be likely suppressed for the diagnostic EoE pediatric analysis, as in the modified EREFS classification used in adults.

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Table 1 - Baseline characteristics of the study population

Demographics / Clinical Features	**Control**	**EoE**	**GERD**	***P*-value**		
				Control x EoE	Control x GERD	EoE X GERD
n (%)	77(70%)	16 (14.5%)	14(12.7%)			
Median age (range)	9 (0-16)	8.5 (1-17)	10 (5-12)		0.5120[a]	
Male n (%)	36 (46.8)	12 (75)	7 (50)	0.0415[b*]	>0.9999[b]	0.0768[b]
Median BMI z-score (P25,P75)	0.03 (-0.32,1.14)	0.51 (-0.77,0.51)	0.09 (-1.06,1.47)		0.9350[a]	
First symptom n (%)						
Abdominal pain	39 (50.6)	6 (37.5)	3 (21.4)	0.1691	0.0218*	0.1690
Vomiting, regurgitation, nausea	24 (31.2)	8 (50.0)	8 (57.1)	0.0745	0.0306*	0.3478
Heartburn	3 (3.9)	1 (6.3)	2 (14.3)	0.3364	0.2928	0.2321
Dysphagia	3 (3.9)	1 (6.3)	0 (0.0)	0.3364	0.6129	0.5484
Food impactation	2 (2.6)	0 (0.0)	0 (0.0)	0.6900	0.7202	1

[a] Kruskal Wallis Test, [b] Binomial test proportions

*considered statistically significant at a level < 0.05;

EoE=Eosinophilic esophagitis, GERD=Gastroesophageal reflux disease, EREFS=Endoscopy Reference Score

Table 2 - Comparison between EREFS findings from EoE, GERD and Control groups

		Control		EoE		GERD		Control x EoE		Control x GERD		EoE x GERD	
		n	**%**	**n**	**%**	**n**	**%**	***P*-value**	power	***P*-value**	power	***P*-value**	power
Inflammatory EREFS	Furrows (Grade 0)	76	98.7%	7	43.8%	13	92.9%	<0.0001*	99.7%	0.0850**	29.5%	0.0022*	93.2%
	Furrows (Grade 1)	0	0.0%	4	25.0%	0	0.0%						
	Furrows (Grade 2)	1	1.3%	5	31.3%	1	7.1%	<0.0001*	87.2%	0.0850**	29.5%	0.0498*	51.2%
	Exsudates (Grade 0)	74	96.1%	8	50.0%	12	85.7%	<0.0001*	98.0%	0.0583**	36.4%	0.0192*	68.7%
	Exsudates (Grade 1)	3	3.9%	4	25.0%	2	14.3%	0.0018*	68.2%	0.0583**	36.4%	0.2321	17.7%
	Exsudates (Grade 2)	0	0.0%	4	25.0%	0	0.0%						
	Edema (Grade 0)	54	70.1%	3	18.8%	7	50.0%	<0.0001*	99.6%	0.0703**	41.0%	0.0350*	56.4%
	Edema (Grade 1)	19	24.7%	3	18.8%	4	28.6%	0.3059	12.0%	0.3788	8.9%	0.2629	15.4%
	Edema (Grade 2)	4	5.2%	10	62.5%	3	21.4%	<0.0001*	99.8%	0.0180*	50.0%	0.0118*	75.7%
	Transient rings (Grade 0)	75	97.4%	15	93.8%	12	85.7%	0.2259	18.5%	0.0249*	43.3%	0.2321	17.8%
	Transient rings (Grade 1)	2	2.6%	1	6.3%	2	14.3%	0.2259	18.5%	0.0249*	43.3%	0.2321	17.8%
	Inflammatory EREFS (score 0)	52	67.5%	3	18.8%	7	50.0%	0.0002*	99.1%	0.1032	33.9%	0.0350*	56.4%
	Inflammatory EREFS (score 1)	19	24.7%	1	6.3%	1	7.1%	0.0513**	60.5%	0.0725**	50.2%	0.4610	5.3%
	Inflammatory EREFS (score 2)	4	5.2%	3	18.8%	5	35.7%	0.0307*	45.6%	0.0002*	80.5%	0.1473	27.1%
	Inflammatory EREFS (score 3)	1	1.3%	2	12.5%	0	0.0%	0.0105*	48.8%				
	Inflammatory EREFS (score 4)	1	1.3%	1	6.3%	0	0.0%	0.1071	27.7%				
	Inflammatory EREFS (score 5)	0	0.0%	3	18.8%	1	7.1%					0.1754	23.5%
	Inflammatory EREFS (score 6)	0	0.0%	3	18.8%	0	0.0%						
Fibrostenotic EREFS	Fixed rings (Grade 0)	76	98.7%	14	87.5%	14	100%	0.0105*	48.8%	0.3340	0.0%	0.0855**	40.1%
	Fixed rings (Grade 1)	1	1.3%	2	12.5%	0	0.0%	0.0105*	48.8%				
	Stricture (Grade 0)	77	100%	15	93.8%	13	92.9%	0.0137*	39.5%	0.0092*	40.1%	0.4610	5.3%
	Stricture (Grade 1)	0	0.0%	1	6.3%	1	7.1%					0.4610	5.3%
	Fibrostenotic EREFS (score 0)	76	98.7%	13	81.3%	13	92.9%	0.0009*	65.3%	0.0850**	29.5%	0.1754	23.5%
	Fibrostenotic EREFS (score 1)	1	1.3%	3	18.8%	1	7.1%	0.0009*	65.3%	0.0850**	29.5%	0.1754	23.5%
Total EREFS	EREFS (score 0)	52	67.5%	3	18.8%	7	50.0%	0.0002*	99.1%	0.1032	33.9%	0.0350*	56.4%
	EREFS (score 1)	18	23.4%	1	6.3%	1	7.1%	0.0610**	55.5%	0.0846**	45.5%	0.4610	5.3%
	EREFS (score 2)	5	6.5%	4	25.0%	4	28.6%	0.0114*	57.3%	0.0055*	62.4%	0.4127	7.2%
	EREFS (score 3)	1	1.3%	1	6.3%	1	7.1%	0.1071	27.7%	0.0850**	29.5%	0.4610	5.3%
	EREFS (score 4)	1	1.3%	0	0.0%	0	0.0%						
	EREFS (score 5)	0	0.0%	3	18.8%	1	7.1%					0.1754	23.5%
	EREFS (score 6)	0	0.0%	3	18.8%	0	0.0%						
	EREFS (score 7)	0	0.0%	1	6.3%	0	0.0%						

*considered statistically significant at level < 0.05; ** significant at level of 0.05 <α <0.10

EoE=Eosinophilic esophagitis, GERD=Gastroesophageal reflux disease, EREFS=Endoscopy Reference Score

Table 3 - Comparison between F-EREFS, I-EREFS, and T- medians of Control, EoE and GERD groups

	Control	**EoE**	**GERD**	***P*-value**		
				Control x EoE	Control x GERD	EoE X GERD
Fibrostenotic EREFS – median (range) [P25-P75]	0 (0-1) [0-0]	0 (0-1) [0-0]	0 (0-1) [0-0]		0.5411[a]	
Inflammatory EREFS – median (range) [P25-P75]	0 (0-4) [0-1]	3 (0-6) [1.75-5]	0.5 (0-5) [0-2]	<0.0001[b]*	0.1269[b]	0.0314[b]*
Total EREFS – median (range) [P25-P75]	0 (0-4) [0-1]	2.5 (0-7) [1.75-5.25]	0.5 (0-5) [0-2]	<0.0001[b]*	0.1207[b]	0.0375[b]*

[a] Kruskal Wallis Test, [b]Kruskal Wallis Test followed by Student-Newman-Keuls
*considered statistically significant at a level < 0.05;
EoE=Eosinophilic esophagitis, GERD=Gastroesophageal reflux disease, EREFS=Endoscopy Reference Score

Table 4 – Description of predictive values from EREFS features considering 16 EoE patients as positive cases and 91 controls + GERD as negative cases

	Cut off point	Sensitivity (%)	Specificity (%)	Likelihood Positive Ratio	Positive predictive value (%)	Likelihood negative ratio	Negative predictive value (%)	Accuracy (%)
Fixed rings	1.0	12.50	98.90	11.36	66.73	0.88	86.50	85.94
Stricture	1.0	6.30	98.90	5.73	50.27	0.95	85.68	85.01
Narrow calibre	insufficient data							
Crepe paper esophagus	insufficient data							
F-EREFS	1.0	18.80	97.98	8.55	60.13	0.83	87.22	85.95
Exudates	insufficient data							
Furrows	insufficient data							
Edema	1.0	81.30	67.00	2.46	30.30	0.28	95.31	69.15
Transient rings	1.0	4.40	93.80	0.71	80.09	1.02	14.76	17.81
I-EREFS	insufficient data							
T-EREFS	2.0	75.00	85.70	5.24	48.07	0.29	95.10	84.10

F-EREFS = Fibrostenotic Endoscopy Reference Score, I-EREFS = Inflammatory Endoscopy Reference Score, T-EREFS = Total Endoscopy Reference Score

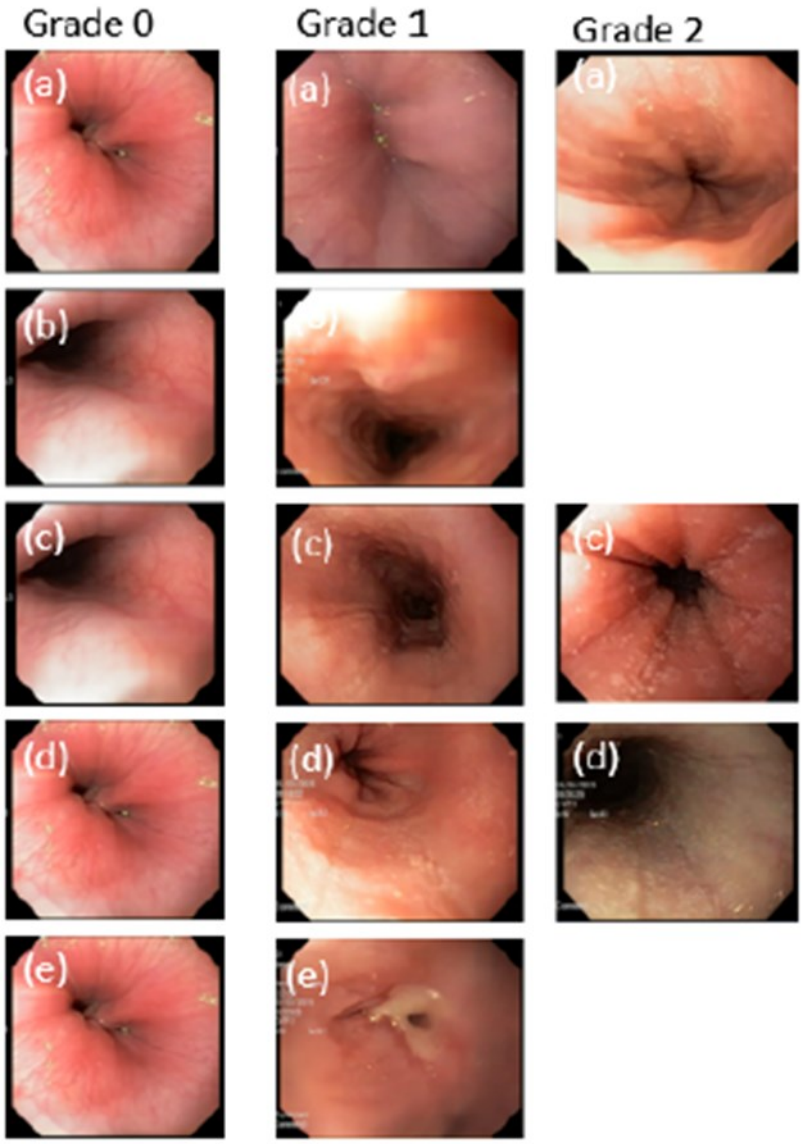

Figure 1: EREFS features found in study population. (a) Edema - loss vascular markings (Grade 0: Distinct vascularity, Grade 1: Decreased, Grade 2: Absent); (b) Fixed rings – trachealization (Grade 0: None, Grade 1: Mild-ridges); (c) Exudate - white plaques (Grade 0: None, Grade 1: Mild $\leq$ 10% surface area, Grade 2: Severe > 10% surface area); (d) Furrows - vertical lines (Grade 0: None, Grade 1: Mild, Grade 2: Severe-depth) (e) Strictures (Grade 0: absent, Grade 1: present)

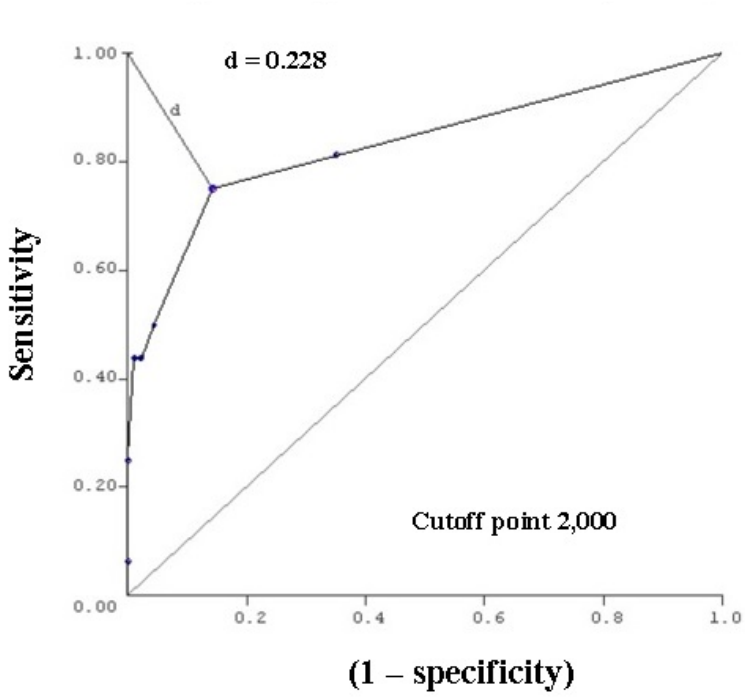

Figure 2: The Receiver-Operating Characteristic curve for T-EREFS at cutoff point 2.

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ANEXO A – PARECER CONSUBSTANCIADO DO CEP

UFU Comitê de Ética em Pesquisa — UNIVERSIDADE FEDERAL DE UBERLÂNDIA/MG — PlataForma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Caracterização Fenotípica de Pacientes Pediátricos Portadores de Eosinofilia Esofágica e Investigação de Biomarcadores Aplicados ao Diagnóstico da Esofagite Eosinofílica

Pesquisador: Luiz Ricardo Goulart Filho

Área Temática:

Versão: 2

CAAE: 36787714.0.0000.5152

Instituição Proponente: Universidade Federal de Uberlândia/ UFU/ MG

Patrocinador Principal: Universidade Federal de Uberlândia/ UFU/ MG
Universidade Federal de Uberlândia/ UFU/ MG
Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 930.413

Data da Relatoria: 04/12/2014

Apresentação do Projeto:

Conforme apresenta o protocolo: A esofagite eosinofílica (EEo) é uma doença inflamatória que tem como característica importante a dificuldade de passagem do alimento do esôfago para o estômago. Sua incidência parece aumentar, e ocorre em proporção relevante de pacientes com doença de refluxo gastroesofágico (DRGE). As duas doenças mostram muitas similaridades, e a distinção é atualmente feita com teste terapêutico pelo uso de inibidores de bombas de prótons. Contudo, persistem problemas nessa distinção.

Objetivo da Pesquisa:

Caracterizar fenotipicamente pacientes pediátricos portadores de eosinofilia esofágica através da descrição de suas características clínicas, histológicas, endoscópicas e bioquímicas. Desenvolver e avaliar o papel de biomarcadores na Esofagite Eosinofílica e seus espectros fenotípicos como forma de aprimorar o diagnóstico e a classificação da doença.

Avaliação dos Riscos e Benefícios:

Segundo os pesquisadores: Os procedimentos médicos que serão realizados são inerentes à investigação e tratamento das doenças de interesse. Portanto, não se identifica aumento

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significativo de risco para os participantes. Medidas satisfatórias para resguardo de sigilo de informações pessoais foram apresentadas. Há potencial de benefício indireto, mediante a identificação de marcadores moleculares da EEo.

Comentários e Considerações sobre a Pesquisa:

Deverão ser avaliados indivíduos menores de idade (0 a 18 anos), com indicação de endoscopia digestiva alta para investigação diagnóstica proveniente de médico assistente. Serão excluídos casos que apresentarem complicações durante o exame, com outras co-morbidades relacionadas à EEo, que fizerem uso de medicação corticosteroide, ou que não seguirem o protocolo do estudo. Estima-se a investigação de 2.000 participantes, divididos em condições clínicas de interesse específico. A investigação se divide em duas fases. Na primeira fase, os participantes serão submetidos a endoscopia (digestiva alta) e biópsia; as amostras serão avaliadas em exame anátomo-patológico para investigação de esofagite eosinofílica e em seguida os pacientes serão mantidos em acompanhamento ambulatorial – pacientes com diagnóstico de esofagite eosinofílica serão mantidos em tratamento com inibidor de bomba de prótons; em seguida, tais pacientes serão submetidos a nova endoscopia e finalmente os casos serão divididos segundo a condição do paciente. Na segunda fase, as amostras biológicas obtidas (tecido lesionado, líquido esofágico, saliva, e sangue periférico) serão utilizadas em ensaios laboratoriais de biologia molecular para identificar e caracterizar potenciais marcadores da EEo. Registros médicos serão utilizados para caracterização da amostra e obtenção de dados de interesse específico.

Considerações sobre os Termos de apresentação obrigatória:

Apresentados de forma satisfatória.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

As pendências apontadas no parecer 878.265 foram atendidas.

De acordo com as atribuições definidas na Resolução CNS 466/12, o CEP manifesta-se pela aprovação do protocolo de pesquisa proposto.

O protocolo não apresenta problemas de ética nas condutas de pesquisa com seres humanos, nos limites da redação e da metodologia apresentadas.

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Considerações Finais a critério do CEP:
Data para entrega de Relatório Parcial ao CEP/UFU: Maio de 2016.
Data para entrega de Relatório Parcial ao CEP/UFU: Outubro de 2018.

OBS.: O CEP/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEP PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

O CEP/UFU lembra que:
a- segundo a Resolução 466/12, o pesquisador deverá arquivar por 5 anos o relatório da pesquisa e os Termos de Consentimento Livre e Esclarecido, assinados pelo sujeito de pesquisa.
b- poderá, por escolha aleatória, visitar o pesquisador para conferência do relatório e documentação pertinente ao projeto.
c- a aprovação do protocolo de pesquisa pelo CEP/UFU dá-se em decorrência do atendimento a Resolução CNS 466/12, não implicando na qualidade científica do mesmo.

Orientações ao pesquisador :
• O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 466/12) e deve receber uma via original do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado.
• O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS 466/12), aguardando seu parecer, exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de regime oferecido a um dos grupos da pesquisa que requeiram ação imediata.
• O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS 466/12). É papel de o pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu

posicionamento.

• Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projetos do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma, junto com o parecer aprobatório do CEP, para serem juntadas ao protocolo inicial (Res.251/97, item III.2.e).

UBERLANDIA, 09 de Janeiro de 2015

Assinado por:
Sandra Terezinha de Farias Furtado
(Coordenador)

ANEXO B – NORMAS DE SUBMISSÃO PARA AUTORES

(American Journal of Gastroenterology)

https://edmgr.ovid.com/ajg/accounts/ifauth.htm

ANEXO C – NORMAS DE SUBMISSÃO PARA AUTORES

(Gastrointestinal Endoscopy)

https://www.elsevier.com/journals/gastrointestinal-endoscopy/0016-5107/guide-for-authors