



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE CIÊNCIAS BIOMÉDICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA
E PARASITOLOGIA APLICADAS

Perfil de anticorpos específicos ao extrato de pólen de *Lolium multiflorum* (azevém italiano) e suas frações em indivíduos alérgicos e não-alérgicos

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Uberlândia

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Perfil de anticorpos específicos ao extrato de pólen de *Lolium multiflorum* (azevém italiano) e suas frações em indivíduos alérgicos e não-alérgicos

Tese de doutorado apresentada ao Colegiado do Programa de Pós-Graduação em Imunologia e Parasitologia Aplicadas como parte do processo para obtenção do título de Doutor.

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Em 13 de agosto de 2024, às 14:00 horas, reuniu-se por vídeo conferência, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, assim composta pelos membros titulares: Presidente: Carlos Henrique Gomes Martins - ICBIM- UFU; Titulares: Ernesto Akio Taketomi - ICBIM - UFU; Prof. Dra. Celene Maria de Oliveira Simões Alves - ICBIM / UFU; Prof. Dra. Letícia de Souza Castro Filice - FAMED/ UFU; Dr. Adriano Gomes da Silva - Fiocruz / RJ; Prof. Dra. Cinthya Covessi Thom de Souza - UEM.

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



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**pois a sabedoria é mais preciosa
do que rubis;
nada do que vocês possam desejar
compara-se a ela. "Eu, a sabedoria,
moro com a prudência,
e tenho o conhecimento
que vem do bom senso.**

Provérbios 8:11-12

Resumo

As doenças alérgicas são usualmente provenientes de uma forte resposta imunológica mediada por anticorpos da classe IgE a diferentes proteínas e outros componentes exógenos. Esses alérgenos podem ser provenientes de diversas fontes como polens, ácaros, fungos, epitélios de animais, elementos corpóreos e peçonhas de insetos, alimentos e medicamentos. Os polens de *Lolium multiflorum* (Lm), uma gramínea amplamente cultivada no sul do Brasil, são reconhecidamente uma importante fonte alergênica, e a cada dia cresce o número de indivíduos sensibilizados ao pólen dessa planta. Apesar de bastante conhecida no ramo da engenharia agrônômica como uma planta forrageira, há pouquíssimos estudos que avaliam o papel dessa gramínea na sensibilização alergênica. Estudos que isolem e caracterizem os alérgenos do polen de Lm podem ser úteis no tratamento e no diagnóstico das doenças alérgicas, revelando quais proteínas tem maior capacidade de disparar sintomas. Por isso é tão necessário a execução de estudos nesse ramo, a fim de melhorar a qualidade de vida de pacientes alérgicos a Lm. A presente tese é composta por três capítulos referentes à capacidade alergênica do extrato bruto de polens de Lm e de suas frações isoladas por meio de técnicas de cromatografia. No capítulo I, a cromatografia de troca iônica isolou eficazmente a fração S2, contendo componentes clinicamente relevantes para pacientes sensibilizados ao pólen de Lm. Tanto o extrato S1 quanto a fração S2 foram eficazes na separação de indivíduos atópicos e não atópicos, utilizando técnicas de ELISA e Immunoblotting. A sensibilidade e especificidade dos testes ELISA foram semelhantes para S1 e S2. A banda de 32 kDa foi o componente imunodominante mais importante, reagindo com anticorpos IgE e IgG4 específicos para *L. multiflorum*. A espectrometria de massas revelou alérgenos de grupo 1 e 5 na banda de 32 kDa e uma proteína hipotética de *Oryza sativa* na banda de 58 kDa. A fração S2 é uma alternativa potencial ao extrato total para diagnóstico e imunoterapia específica. No capítulo II, duas frações (não ligada e ligada à coluna de ConA), com componentes clinicamente relevantes para pacientes sensibilizados ao pólen de *L. multiflorum* foram isoladas. Pela observação dos perfis eletroforéticos, componentes proteicos distintos foram isolados em cada fração. As frações e o extrato bruto foram eficazes na separação de indivíduos atópicos e não atópicos, utilizando ELISA e Immunoblotting. A reatividade da IgE foi evidenciada por meio de bandas específicas no soro de indivíduos atópicos, o que não ocorreu em não atópicos. Bandas reativas específicas foram observadas tanto no extrato bruto quanto frações ligadas e não ligada à ConA, demonstrando uma possível abordagem para diagnóstico e tratamento de alergias respiratórias causadas por *L. multiflorum*. Por fim, no capítulo III, foram obtidas as frações S2 e S3,

importantes para pacientes sensibilizados ao pólen de *Lm*. Foram observadas diferenças na reatividade de anticorpos IgG específicos entre os grupos, especialmente nas frações S2 e S3. O nível de IgG de alta avidéz superou 50% em todos os indivíduos testados. Diferenças significativas no índice ELISA foram encontradas entre diferentes subclasses de IgG nas frações S2 e S3. Correlações entre subclasses de IgG variaram entre frações, sugerindo S2 e S3 como promissoras para o tratamento de condições alérgicas induzidas pelo pólen de *L. multiflorum*.

Palavras-chave: alergia, atopia, *Lolium multiflorum*, cromatografia de troca iônica, cromatografia de afinidade, ConA, frações alergênicas, IgE, IgGs.

Abstract

Allergic diseases are usually derived from a strong immune response mediated by IgE antibodies to different exogenous proteins and other components. These allergens can come from various sources such as pollens, mites, fungi, animal epithelia, body elements and insect pellets, food and medicines. The pollens of *Lolium multiflorum* (Lm), a grass widely cultivated in southern Brazil, are recognized as an important allergenic source, and every day grows the number of individuals sensitized to the pollen of this plant. Although well known in the field of agronomic engineering as a forage plant, there are very few studies evaluating the role of this grass in allergenic sensitization. Studies that isolate and characterize Lm pollen allergens may be useful in the treatment and diagnosis of allergic diseases, revealing which proteins have greater ability to trigger symptoms. That is why it is so necessary to carry out studies in this field in order to improve the quality of life of patients allergic to Lm. The present thesis consists of three chapters referring to the allergenic capacity of the crude extract of Lm pollens and their fractions isolated by chromatography techniques. In Chapter I, ion exchange chromatography effectively isolated the S2 fraction, containing clinically relevant components for patients sensitized to Lm pollen. Both extract S1 and fraction S2 were effective in separating atopic and non-atopic individuals using ELISA and Immunoblotting techniques. The sensitivity and specificity of the ELISA tests were similar for S1 and S2. The 32 kDa band was the most important immunodominant component, reacting with *L. multiflorum*-specific IgE and IgG4 antibodies. Mass spectrometry revealed group 1 and 5 allergens in the 32 kDa band and a hypothetical *Oryza sativa* protein in the 58 kDa band. The S2 fraction is a potential alternative to the total extract for diagnosis and specific immunotherapy. In Chapter II, two fractions (unbound and linked to the ConA spine) with clinically relevant components for patients sensitized to *L. multiflorum* pollen were isolated. By observing the electrophoretic profiles, distinct protein components were isolated in each fraction. The fractions and the crude extract were effective in separating atopic and non-atopic individuals using ELISA and Immunoblotting. The reactivity of IgE was evidenced by specific bands in the serum of atopic individuals, which did not occur in non-atopic individuals. Specific reactive bands were observed in the crude extract as well as fractions linked and not linked to the ConA, demonstrating a possible approach for diagnosis and treatment of respiratory allergies caused by *L. multiflorum*. Finally, in chapter III, the fractions S2 and S3 were obtained, important for patients sensitized to Lm pollen. Differences were observed in the reactivity of specific IgG antibodies between the groups, especially in the fractions S2 and S3. The level of high avidity IgG exceeded 50% in all subjects tested. Significant differences in the ELISA index were found

between different subclasses of IgG in the fractions S2 and S3. Correlations between IgG subclasses varied between fractions, suggesting that S2 and S3 are promising for the treatment of allergic conditions induced by *L. multiflorum* pollen.

Keywords: allergy, atopy, *Lolium multiflorum*, ion-exchange chromatography, affinity chromatography, ConA, allergenic fractions, IgE, IgGs.

Lista de Abreviaturas

ABTS: 2,2'-diazino do ácido etil-benzotiazolina sulfônico

AI: índice de avidéz (avidity index)

AIT: allergen immunotherapy

APCs: células apresentadoras de antígeno profissionais (Antigen-presenting cells)

AT: atópicos

BSA: soroalbumina bovina (bovine serum albumin)

CD: Grupamento de diferenciação (cluster of differentiation)

CM: carboximetil

ConA: Concanavalina A

DAB: 3'3-tetrahidrocloro de diaminobenzidina

E.I.: índice Elisa (Elisa index)

ELISA: *enzyme linked immunosorbent assay*

GATA 3: GATA binding protein 3

HPLC: cromatografia líquida de alta performance (high performance liquid chromatography)

IC50: half-maximal inhibitory concentration

IgA: imunoglobulina A

IgE: imunoglobulina E

IgG: imunoglobulina G

IL-4: interleucina 4

IL-5: interleucina 5

IL-6: interleucina 6

IL-9: interleucina 9

IL-10: interleucina 10

IL-13: interleucina 13

IL-25: interleucina 25

IL-33: interleucina 33

ILC: célula linfoide inata

IFN- γ : interferon gama

ITA: imunoterapia específica com alérgenos

kDa: kilodaltons

Lm: *Lolium multiflorum*

M: molar

mM: mili molar

MHC-II: complexo de histocompatibilidade principal de classe II

MS-MS: espectrometria de massas

NaCl: Cloreto de sódio

NAT: não-atópicos

PARs: receptores ativados por proteases

PBS: salina tamponada com fosfatos (phosphate buffered saline)

PBS-T: salina tamponada com fosfatos acrescido de Tween a 0,05%

PRRs: receptores de reconhecimento padrão (pattern recognition receptor)

ROS: espécies reativas de oxigênio (reactive oxygen species)

S1: sobrenadante 1

S2: sobrenadante 2

S3: sobrenadante 3

SDS-PAGE: dodecil sulfato de sódio-eletroforese em Gel de Poliacrilamida

STAT: transdutor de sinal e ativador de transcrição (signal transducer and activator of transcription)

Th: células T helper

TCP: teste cutâneo de puntura

TCR: receptor de célula T (T cell receptor)

Tg: *Toxoplasma gondii*

TLRs: receptores semelhantes ao Toll (Toll like receptor)

Treg: células T regulatória

TSLP: linfopoietina estromal tímica (thymic stromal lymphopoietin)

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1. Fundamentação Teórica

1.1 Atopia, alergia e doenças alérgicas

O termo atopia tem sido utilizado há muitos anos, especificamente desde a década de 1920, para definir a predisposição genética que alguns indivíduos ou uma população têm para produzir imunoglobulina E (IgE) específica frente ao contato com doses extremamente baixas de alérgenos, geralmente proteínas inofensivas. Portanto, indivíduos atópicos respondem com altos níveis de IgE frente a diferentes alérgenos (Johansson et al., 2004; Baldaçara et al., 2013; Kirchhof; De Gannes, 2018).

Apesar de bastante confundida com atopia, a alergia é um sinônimo para designar a hipersensibilidade do tipo I (classificação de Gell e Coombs), que é uma reação imediata mediada por anticorpos da classe IgE frente a antígenos não próprios, que ocorre em indivíduos sensibilizados aos alérgenos, que ainda pode envolver citocinas, quimiocinas células, entre outros. É importante ressaltar que nem todas as reações alérgicas ocorrem apenas em indivíduos atópicos (Johansson et al., 2001; Baldaçara et al., 2013).

As doenças alérgicas como as conhecidas, rinite alérgica perene ou sazonal, dermatite atópica e asma são desencadeadas através de uma resposta exacerbada do sistema imunológico frente a antígenos normalmente inócuos. Esses alérgenos podem ser provenientes das mais diversas fontes como polens de árvores, como da família Betulaceae e de gramíneas como a família Poaceae, ácaros da poeira domiciliar e epitélio de animais domésticos (cães e gatos), mas podem também ser derivados de alimentos como leite de vaca, nozes, soja, ovos, frutas, além de frutos do mar, entre outros. Ainda, pode haver outras fontes de alérgenos como peçonhas de himenópteros e fármacos como os antibióticos (De Monchy et al., 2013; Pomiecinski et al., 2017; Blumenthal et al., 2019).

Nos últimos 50 anos têm se observado um aumento da prevalência das doenças alérgicas ao redor do mundo, em que os indivíduos têm sido expostos a grandes quantidades de alérgenos e produtos químicos com capacidade de sensibilização, isso tem ocorrido com mais frequência, provavelmente devido a mudanças ambientais locais. Nas últimas décadas, assustadoramente, têm se observado um aumento do número de indivíduos alérgicos, sendo que cerca de um quarto da população europeia está acometida com alguma patologia alérgica, e ao redor do mundo, até um terço dos adultos e 40% das crianças serão de forma similar acometidos (Manduzio et al., 2012; Pandey, 2016).

As doenças alérgicas respiratórias como a asma e a rinite tornaram-se um grave problema de saúde na sociedade atual. Pesquisas indicam que essas doenças são mais prevalentes em crianças de primeira infância e em idosos, devido a um sistema imune imaturo ou debilitado. Além disso, a prevalência é maior em adultos de países em desenvolvimento em comparação aos de países desenvolvidos. Em países com estilo de vida ocidentalizado, a prevalência das doenças alérgicas pode ser até 20 vezes maior do que em de baixa renda (Hatzler; Hofmaier; Papadopoulos, 2012; Lambrecht; Hammad, 2017; Arduzzo et al., 2019). Ainda sobre a prevalência das doenças alérgicas, pesquisas têm relatado haver uma maior ocorrência de doenças alérgicas em jovens que residem em áreas urbanas quando comparados aos de zona rural (Shpakou et al., 2012). A hipótese da higiene parece ser fundamental na maturação do sistema imune (Hatzler; Hofmaier; Papadopoulos, 2012; Perkin; Strachan, 2022; Choi, 2023), pois há muito tempo pesquisadores já relatam uma maior taxa de doenças alérgicas em populações com menor exposição a vírus, bactérias e helmintíases (Gerrard et al., 1976).

Além disso, devido a mudanças de hábitos, cada vez mais, as crianças na primeira infância, fase importante na maturação do sistema imune, passam por uma menor exposição aos antígenos microbianos e infecções parasitárias, o que pode levar a respostas mais exacerbadas e/ou desviadas frente a antígenos (Alkotob et al., 2020; Lunjani et al., 2022). Dessa maneira, a hipótese da biodiversidade/disbiose da microbiota, que fora proposta por Haahtela *et al.* (2013), descreve que a perda da biodiversidade da microbiota está relacionada ao desenvolvimento de doenças alérgicas e comprometimento da tolerância do sistema imunológico. O equilíbrio da microbiota pode ser afetado por fatores como dieta, uso indiscriminado de antibióticos e por fatores externos como a poluição do ar. Recentemente, um estudo demonstrou que crianças com risco de desenvolvimento de alergias têm diminuição de cepas dos gêneros *Lachnospira*, *Veillonella*, *Fecalibacterium* e *Rothia* enquanto outras, como as espécies da família Clostridiaceae, são aumentadas (Haahtela et al., 2013; Arrieta et al., 2015; Lambrecht; Hammad, 2017; Dharmage; Perret; Custovic, 2019).

Rook et al. (2003) propuseram mais recentemente, além da teoria da higiene, a hipótese da microbiota. Essa hipótese propõe que ambientes ricos em diversidade microbiana no trato gastrointestinal e respiratório desempenham um papel importante na ativação e regulação do sistema imunológico. O termo "exposoma" refere-se a todas as exposições ambientais e não genéticas que uma pessoa experimenta ao longo de sua vida. O exposoma da polinose inclui não apenas a exposição ao pólen, mas também fatores como poluição do ar, mudanças climáticas, dieta, estresse, hábitos de vida (como o tabagismo), infecções e até o microbioma

do trato respiratório (Rook; Martinelli; Brunet, 2003; D'amato et al., 2020; D'amato et al., 2020).

O conhecimento do exposoma em relação à polinose permite uma abordagem mais abrangente para a prevenção e tratamento da doença. Ao identificar e alterar as exposições ambientais que contribuem para a polinose, é possível criar métodos mais eficazes para diminuir a prevalência e a intensidade da doença. As mudanças climáticas, como um aumento nas temperaturas e nos níveis de CO₂, podem prolongar as temporadas de polinização, aumentar a produção de pólen e mudar a distribuição das espécies vegetais, tudo isso fazendo com que o exposoma tenha um impacto na polinose neste contexto. Isso pode resultar em uma exposição ao pólen maior, agravando os sintomas da polinose. Além disso, o pólen pode se misturar com poluentes como dióxido de enxofre, óxidos de nitrogênio e material particulado, tornando-o mais alergênico. A poluição também pode danificar a mucosa respiratória, aumentando a susceptibilidade aos alérgenos. Ainda, as áreas urbanas podem aumentar a exposição a poluentes e reduzir a exposição à biodiversidade natural, o que pode aumentar a prevalência de alergias, como a polinose. O exposoma também interage com microrganismos, que podem ser úteis ou nocivos. Fatores ambientais podem alterar o microbioma respiratório ou intestinal, afetando a resposta imunológica e a suscetibilidade a alergias (D'amato et al., 2020; D'amato et al., 2020; Celebi Sozener et al., 2022).

A hipótese da barreira epitelial (proposta por Pothoven e Schleimer, 2017) sugere que danos a essa barreira estão ligados a doenças inflamatórias crônicas, como asma e rinite. Por exemplo, a exposição a detergentes comuns pode levar à ruptura da barreira epitelial, favorecendo o surgimento e agravamento de doenças alérgicas. Essa quebra aumenta a permeabilidade do tecido e pode promover um microbioma disbiótico, frequentemente associado à inflamação do tipo 2, que exacerba condições crônicas como asma e rinite alérgica. Patógenos oportunistas, como o *Staphylococcus aureus*, podem comprometer ainda mais as barreiras epiteliais, agravando a doença. As células epiteliais são, portanto, essenciais para manter a integridade da barreira e regular as respostas imunes, prevenindo a inflamação crônica. A interrupção das junções estreitas no epitélio pode permitir a entrada de alérgenos e patógenos nos tecidos, desencadeando respostas imunes (Schleimer; Berdnikovs, 2017; Akdis, 2021; Lu et al., 2024).

Fatores genéticos também são importantes no desenvolvimento das doenças alérgicas. Pesquisas tem revelado que crianças e adolescentes com um ou dois pais alérgicos possuem uma maior prevalência de rinite alérgica, em comparação a indivíduos de mesma idade que não

possui progenitores com histórico de alergia (Keil et al., 2010). Uma outra pesquisa com adultos jovens, identificou que o gênero está associado a desenvolvimento de alguns tipos de doenças alérgicas em detrimento de outras. Como por exemplo, foi observado que a asma é mais comum em indivíduos do sexo masculino, e principalmente os que apresentam histórico de exposição a cães e gatos no ambiente domiciliar, tabagismo, e apresentem histórico de parentes alérgicos. Todavia, patologias tais como eczema foram mais frequentes em indivíduos do sexo feminino (Ziyab, 2017). Além disso, nos casos de multimorbidade alérgica (onde existe duas ou mais condições alérgicas), foi demonstrado que além da presença da alergia familiar, sintomas de alergia no início da vida e parto cesáreo, configuram-se como fatores de risco para as doenças alérgicas (Sigurdardottir et al., 2021).

1.2 Mecanismos imunológicos no desenvolvimento das doenças alérgicas

Dentre as capacidades dos antígenos, uma bastante importante é ultrapassar a barreira de células epiteliais dos tecidos, como no trato respiratório, ou serem capturados por células apresentadoras de antígenos profissionais (APCs), como macrófagos e células dendríticas. Isso pode desencadear um efeito em cadeia, promovendo o desenvolvimento e a subsequente ativação de células T *helper* do tipo 2 (Th2), além de células linfoides inatas (ILCs) do grupo 2 (Fujita et al., 2012; Melum et al., 2014; Eifan; Durham, 2016).

As APCs, após o contato com o alérgeno, irão capturá-lo, processá-lo, apresentando os seus peptídeos através das moléculas do complexo de histocompatibilidade principal de classe II (MHC-II) para células T CD4⁺ *naïves*. As moléculas MHC-II ligadas ao peptídeo antigênico interagem com o receptor de célula T (TCR) e com o *cluster of differentiation* (CD) 4 que presentes nas células T *helper naïve*, iniciando o primeiro sinal de ativação da resposta imune. Subsequentemente, para estabelecer um firme contato célula-a-célula entre APCs e células T CD4⁺ ocorre a ligação entre as moléculas coestimulatórias, como B7-1/2 com CD28, constituindo o segundo sinal de ativação. Caso neste momento essas células forem ativadas, as APCs liberam citocinas como a Interleucina 4 (IL-4), caracterizando o terceiro sinal da resposta imune. A IL-4 liberada pelas APCs ativa STAT 6 na célula T CD4⁺ *naïves*, levando à ativação do fator de transcrição GATA 3 (*GATA binding protein 3*). Em cooperação com a ligação MHC-II e TCR (primeiro sinal da resposta imune) e a ligação de moléculas coestimulatórias (segundo sinal de ativação da resposta imune), esses eventos ativam genes de perfil Th2 (Ho; Pai, 2007; Barnes, 2008; Holgate; Polosa, 2008). Esses genes, por sua vez, expressam diversas citocinas típicas do perfil Th2, como IL-4, IL-5, IL-9 e IL-13 que são abundantes no processo

inflamatório alérgico (Larché; Akdis; Valenta, 2006; Chaplin, 2010; Wynn, 2015; Palomares et al., 2017).

Há ainda outro mecanismo paralelo e independente das APCs que contribui para o desenvolvimento das doenças alérgicas, relacionado à natureza proteolítica de determinados antígenos e à capacidade de reconhecimento do lipopolissacarídeo (LPS). Alérgenos proteolíticos podem ser reconhecidos por receptores de reconhecimento padrão (PRRs), como os receptores ativados por proteases (PARs), e o LPS pode ser reconhecido por receptores semelhantes ao *Toll* (TLRs) 4, presentes nas superfícies de células epiteliais. Quando esses alérgenos se ligam aos PRRs, junto com espécies reativas de oxigênio (ROS), ocorre a ativação das células epiteliais, que então produzem diversas citocinas conhecidas como alarminas, como interleucina 25 (IL-25), interleucina 33 (IL-33) e linfopoietina estromal tímica (TSLP). A IL-25, e em conjunto com a IL-33 regula positivamente a ativação de NF- κ B, e junto com a TSLP, induz a ativação de STAT-5. Isso resulta no aumento da expressão de genes com perfil Th2, promovendo a produção de citocinas e o desenvolvimento das doenças alérgicas (Bulek et al., 2010; Georas; Rezaee, 2014; Lambrecht; Hammad, 2014; Divekar; Kita, 2015; Palomares et al., 2017; Taketomi et al., 2017).

Em adição, a produção de alarminas, especialmente IL-33 e TSLP, pelas células epiteliais ativadas por proteases, particularmente do trato respiratório, também pode levar à ativação de células ILC2 produtoras de IL-5 e IL-13, responsáveis pela indução de um processo inflamatório eosinofílico. Esse processo não envolve a produção de anticorpos IgE (endotipo não alérgico), devido à falta de produção de IL-4 pelas ILC2. A IL-13 é amplamente responsável por induzir hipersecreção por meio da ativação de células caliciformes e também pelo processo de remodelamento (Brusselle; Bracke, 2014). Após a ativação das células de perfil Th2 alérgeno-específicas, essas irão se multiplicar e produzir citocinas de perfil Th2, dentre as quais, a IL-4 e IL-13, são fundamentais, pois são elas que levam ao *switch* de classes de imunoglobulinas (Ig) produzidas pelas células B, que por sua vez passa a produzir e secretar IgE específica. A IgE específica aos alérgenos, então se ligará a receptores de alta afinidade (FC ϵ RI) em mastócitos e basófilos (Larché; Akdis; Valenta, 2006; Min, 2010). A IgE ainda pode se associar no sangue com alérgenos, formando um complexo IgE-alérgeno, que por sua vez, poderá se ligar a receptores de baixa afinidade (FC ϵ RII), presentes nas superfícies de células B, que então funcionará como uma APC, reiniciando o processo de produção de citocinas de perfil Th2, como já descrito (Xu et al., 2015; Shamji et al., 2019). Após uma nova

exposição aos alérgenos em indivíduos previamente sensibilizados, podem apresentar o desencadeamento de reações alérgicas (Goss et al., 2013).

A resposta alérgica em geral se divide em duas fases, na primeira a reação é do tipo imediata (na primeira hora), os granulócitos principalmente basófilos e mastócitos, sensibilizados com IgE-específica ao alérgeno, em contato com o mesmo, induzem a uma reação clássica de hipersensibilidade do tipo I. Os granulócitos após excitação, levam a diversos sintomas de fase imediata através da degranulação de mediadores pró-inflamatórios pré-formados que se encontram no interior das células, como histamina, leucotrienos, heparina, triptase, cininas, entre outros. Logo após isso, uma segunda fase ocorre, mais tardia, que se inicia cerca de 6 a 10 horas após o contato com o alérgeno, que pode levar ao aparecimento devido a síntese e liberação dos mediadores neoformados como prostaglandinas, leucotrienos e citocinas, esses sintomas podem ter uma duração média de 3 a 48 horas, com redução lenta e gradual. Um recente estudo ainda sugere que, neutrófilos podem funcionar como APCs e apresentar antígenos durante a fase tardia (James; Durham, 2008; Polak et al., 2019).

Ao contrário do que é observado na reação imediata, a quimiotaxia de eosinófilos é o mecanismo principal na reação tardia, devida a liberação dos mediadores inflamatórios os quais provém da reação imediata que recrutam diversas células inflamatórias, tais como: neutrófilos, eosinófilos, linfócitos T e mastócitos, os quais têm a capacidade de migrar para o local da lesão (diapedese), levando a inflamação, produção de muco, a remodelação do tecido (Min, 2010; Stone; Prussin; Metcalfe, 2010; Polak et al., 2019).

1.3 Rinite alérgica sazonal

A rinite alérgica sazonal, também denominada de polinose, hipersensibilidade nasal e alergia nasal, é uma doença mediada por anticorpos da classe IgE, desencadeada pela inalação de alérgenos derivados de polens por indivíduos sensibilizados a eles. É caracterizada por levar a inflamação e/ou disfunção da mucosa de revestimento nasal em que é caracterizada por diversos sintomas, tais como: obstrução nasal, rinorreia, espirros, prurido nasal e hiposmia. Recentes estudos demonstram que a prevalência da rinite alérgica sazonal tem aumentado. Devido à forte presença da doença nas zonas urbanas, têm sido denominadas de doença da civilização moderna. Há estudos ainda que relatam até um terço dos indivíduos que sofrem de polinose também apresentam asma (Ibiapina et al., 2008; Giavina-Bianchi, 2015; Okubo et al., 2017; Cocco et al., 2018; Sakano et al., 2018; Ridolo et al., 2022).

Um estudo revelou que a presença de rinite alérgica pode chegar até 30% da população quando contabilizadas todas as faixas etárias, entretanto nos Estados Unidos da América a

confirmação clínica só atinge apenas 14%, enquanto na Ásia, apenas 9% são diagnosticados e na América Latina apenas 7% recebem a confirmação do diagnóstico (Meltzer et al., 2012; Wallace; Dykewicz, 2017).

No Brasil, os estudos sobre a rinite alérgica sazonal derivam desde a década de 1940, devido ao início da contagem de aeroalérgenos, em regiões como Sul e Sudeste do país, com a ocorrência dos primeiros casos relatados. A frequência da polinose, está intimamente relacionada com regiões que possuem estações climáticas bem definidas que possuem uma localização meridional elevada e que sofrem ação continental. Além disso, as taxas de sensibilização aos grãos de polens sofrem variação devido a abundância e a distribuição de plantas com potencial alergênico pelas localidades (Moreira et al., 2015; Araujo; Rosario; Mari, 2016).

Uma pesquisa realizada recentemente na região sul do Brasil examinou a correlação entre os picos locais de pólen e o início dos sintomas da polinose, enfatizando a importância de compreender sobre a exposição ao pólen para melhorar as respostas de saúde pública e fornecer informações sobre métodos de gerenciamento de alergias. Os resultados indicaram que os sintomas da polinose geralmente aparecem em um período específico após a exposição aos picos de pólen, com variações significativas conforme a proximidade à fonte. Os indivíduos que residem próximo de fontes de pólen apresentaram sintomas mais rapidamente do que as pessoas que habitam em lugares mais distantes, mostrando como a proximidade afeta as reações alérgicas. Esses resultados ressaltam que as previsões de pólen personalizadas por localização são necessárias para ajudar as pessoas a controlar sua exposição e reduzir os sintomas de alergias mais rapidamente (Camargo et al., 2021).

1.4 Diagnóstico das doenças alérgicas

A produção de anticorpos da classe IgE específicos pode ser diagnosticada tanto por testes *in vitro* (determinação dos níveis de anticorpos da classe IgE sérica e local específicos aos alérgenos) quanto através de métodos *in vivo* (intradérmicos ou teste cutâneo de puntura). Entretanto, a presença ou ausência de IgE sérica específica, por si só, não é suficiente para determinar se um indivíduo é ou não alérgico, afinal um indivíduo IgE⁺ pode ser assintomático, porém são sensibilizados. Para determinar a alergia é necessária uma correlação com o diagnóstico clínico dado por um profissional especialista no assunto. Os testes cutâneos são de extrema valia, afinal são bastante sensíveis e específicos, rápidos, econômicos e fáceis de se realizar, quando comparados a testes *in vitro* que dependem de profissionais treinados. Contudo, os testes cutâneos podem muitas vezes apresentar resultados falso-positivos devido a

reatividade cruzada, ou falsos negativos devido as baixas concentrações do alérgenos inoculadas no indivíduo, que sofrem alterações de acordo com o método de extração utilizado. Além disso, fatores como hora do dia, sexo e tratamento medicamentoso podem afetar sua eficácia (Ownby, 1988; Ballmer-Weber, 2014; Wagner; Rudert, 2019; Zimmer et al., 2022).

Por outro lado, testes *in vitro* são mais utilizados, quando pacientes não podem ser submetidos ao teste cutâneo devido a história de anafilaxia, presença de lesões cutâneas, ou ainda ter consumido medicamentos que podem interagir ou prejudicar a detecção da interação alérgeno-IgE, porém sua desvantagem é clara em relação ao tempo de execução, maior custo em comparação ao teste *in vivo* e a necessidade de ser realizado por um profissional treinado. Da mesma forma que nos testes cutâneos a positividade não determina presença ou ausência de doença, antes sendo necessário a associação com a clínica do paciente. Entretanto, vários estudos demonstram que a determinação dos níveis de IgE sérica específica a alérgenos inaláveis pode ser uma ferramenta valiosa no diagnóstico de pacientes alérgicos. Destacam-se hoje como testes *in vitro*, a técnica de ELISA e Immuncap (Bernstein; Storms, 1995; Mastrandrea et al., 1997; Daher et al., 2009; Berings et al., 2017).

1.5 Tratamento das doenças alérgicas através da imunoterapia específica com alérgenos (ITA)

A fim de tratar as doenças alérgicas e reverter o quadro sintomático dos pacientes, em 1911 pesquisadores ingleses, Leonard Noon e John Freeman, introduziram uma abordagem não medicamentosa para tratar pacientes que possuíam polinose, consequente à inalação de polens, denominada de imunoterapia específica com alérgenos (ITA). Os pacientes recebiam doses crescentes de alérgenos ao longo do tempo, e apresentavam uma melhora no quadro clínico. Na atualidade, o uso da ITA é indicado para indivíduos que possuam alguma doença respiratória mediada por anticorpos da classe IgE tais como asma, rinite alérgica, ou ainda alergia a peçonha de insetos como os himenópteros e com alérgenos alimentares, seguindo o mesmo padrão de tratamento, com aumento de doses dos alérgenos ao longo do tempo, sendo a única opção de tratamento que modifica o quadro da doença (Aarestrup, 2017; Gutermuth et al., 2022; Fiala; Fleit, 2023).

Em relação a modelos de ITA, um recente estudo, utilizando camundongos BALB/c em um modelo de indução de asma e tratamento por imunoterapia específica com polens de Bétula demonstrou que após a terapia os animais apresentaram intensa redução na eosinofilia e produção de muco nos pulmões, redução de citocinas IL-4, IL-5, IL6, IL-10, IL-17 e TGFβ-1

no lavado broncoalveolar, bem como diminuição nos níveis séricos de IgE, IgG1 (correspondente à IgG4 humana) e IgG2a (correspondente à IgG1 humana) (Xie; Yin, 2019). Um estudo imunoterápico realizado no Brasil demonstrou ainda que a associação de extratos alergênicos de ácaros da espécie *Dermatophagoides pteronyssinus* com derivados de paredes celulares de bactérias, leva uma redução dos sintomas, do uso de medicamentos, e diminuição da reatividade no teste cutâneo de puntura (TCP) e dos níveis de IgE, bem como aumento de IgG1 frente ao extrato total e aumento de IgG4 para o alérgeno principal da espécie, após 1 ano de tratamento (Guimarães et al., 2008).

Os mecanismos imunológicos durante a ITA, são opostos aos observados no desenvolvimento das doenças alérgicas, uma característica importante é o reequilíbrio da resposta imune. A resposta imunológica durante a ITA envolve a produção precoce de anticorpos específicos da classe IgE nos primeiros meses. Contudo, ao longo do tempo aumentam os níveis de anticorpos com função bloqueadora e protetora como IgG, IgG4, IgA1 e IgA2, além de que a primeira passa a ser a principal classe de anticorpos produzida na ITA prolongada. Isso está relacionado à participação de fatores de transcrição FOXP3, que têm sua expressão aumentada, bem como o aumento de citocinas de perfil regulatório como TGF- β e IL-10 produzidas por células T reguladoras (T-reg), as quais induzem supressão de células inflamatórias e citocinas de perfil Th2 (Akdis; Akdis, 2014; Shamji et al., 2021a; Shamji et al., 2021b; Gunaydin et al., 2022).

À medida que o tratamento da imunoterapia com alérgenos é continuado, os níveis de IgG específicos ao alérgeno, sobem até atingirem um platô. Os anticorpos IgG, principalmente da subclasse IgG4, induzidos pela ITA podem atuar como anticorpos bloqueadores do alérgeno, competindo com os anticorpos IgE na ligação com o alérgeno, a fim de bloquear a ativação de granulócitos sensibilizados com IgE. Adicionalmente, estudos sugerem que a IgG4 compete com o complexo IgE-alérgeno pela ligação aos receptores de baixa afinidade a IgE, denominado de CD23, impedindo assim que a célula B atue como uma APC. Além disso, a imunoterapia específica pode levar a diminuição dos números totais de mastócitos, basófilos e eosinófilos e diminuição na reatividade na pele (Kowalski; Jutel, 1998; Fujita et al., 2012; Akdis; Akdis, 2014).

A ITA é atualmente a principal forma específica de tratamento das doenças alérgicas, sendo demonstrado que esse recurso terapêutico leva a melhora do quadro clínico dos pacientes alérgicos a polens mesmo após 2 anos de descontinuação da terapia, ainda que o nível de anticorpos IgG decresça (James et al., 2011). O grande problema hoje da ITA é a busca por

biomarcadores efetivos, isso seria de grande valia a fim de avaliar se o tratamento está sendo efetivo nos indivíduos tratados, quando parar o tratamento e impedir recaídas após finalizar a terapia (Shamji et al., 2017). Pesquisadores recentemente sugeriram a dosagem de anticorpos IgG séricos, a fim de monitorar a eficácia da ITA juntamente com a avaliação clínica, com análise do grau e/ou da frequência dos sintomas clínicos (Taketomi et al., 2017), enquanto ainda outros sugerem que os níveis de IgG4 nasal, seriam os melhores aliados no monitoramento da imunoterapia (Taketomi et al., 2017; Shamji et al., 2019).

Por muito tempo, foram utilizados extratos proteicos totais de alérgenos como de ácaros da poeira domiciliar e polens de árvores e gramíneas, no diagnóstico bem como na ITA. Devido ao fato de que a imunoterapia é um tipo de tratamento que envolve administração por anos, os extratos alergênicos oferecem risco de ocasionar reações adversas. Diante disso, avanços na pesquisa têm relatado as vantagens de se utilizar em substituição ao extrato total, peptídeos naturais e sintéticos, bem como proteínas purificadas, que promovem de forma eficaz o aumento de IL-10 por células T-reg FOXP3⁺, que poderá levar a uma maior produção de anticorpos da subclasse IgG4, com função bloqueadora (Cox, 2017; Mosges et al., 2018; Boonpiyathad et al., 2019). Além disso, um estudo demonstrou que a utilização de peptídeos purificados é mais vantajosa, pois se ligam fracamente a IgE e apresentam menor reatividade a granulócitos, porém induzem da mesma forma os benefícios da ITA (Ellis et al., 2017; Shamji et al., 2018). Adicionalmente, tem sido mostrado que os indivíduos sensibilizados apresentam heterogeneidade de resposta frente a diversos alérgenos principais do extrato proteico, apresentando reatividade a algumas proteínas e sendo indiferentes a outras, por isso uma resposta direcionada pode ser mais eficaz do que a utilização do extrato total de proteínas (Taketomi et al., 2006a).

1.6 Composição e principais alérgenos provenientes de polens

A composição dos grãos de polens pode variar, apresentando de a 4-12 % de proteínas como as albuminas, prolaminas, gluteminas, globulinas, entre outras (Stanley; Linskens, 1974). Devido a essa ampla gama de proteínas, algumas apresentam homologia estrutural entre componentes de diversas fontes alergênicas, principalmente entre plantas. Isso leva muitas vezes à reatividade cruzada, onde os anticorpos reconhecem os epítomos compartilhados. Essa reatividade pode ser entre outras plantas, alérgenos alimentares e até mesmo entre helmintos devido à existência de panalérgenos. Estes são componentes bem preservados na maioria dos organismos vivos, com capacidade de desencadear uma resposta dependente de anticorpos da classe IgE. Devido a isso, a taxonomia revela que quanto mais estreito for o relacionamento

entre as plantas, maior a reatividade cruzada devido ao maior compartilhamento de antígenos. Os anticorpos das classes IgE, apesar de altamente específicos contra um alérgeno reconhecem peptídeos similares em outras fontes alergênicas, o que acaba dificultando o diagnóstico da rinite alérgica sazonal (Taketomi et al., 2006b; Canonica et al., 2013; Aleksic et al., 2014; Miguères et al., 2014; Popescu, 2015; Igetei et al., 2018; Yagami; Ebisawa, 2019).

A polinose, em todo o planeta, acomete até 50% dos pacientes alérgicos. Diversos polens de plantas podem causar esse problema, dentre os principais, pode-se listar, polens de vegetais lenhosos de ordens como Fabales, Fagales, Lamiales, Proteales e Pinales e palmeiras (Arecales), além é claro de gramíneas (Poales), em especial a família Poaceae. Esses táxons, são as principais fontes alergênicas, que levam além dos sintomas e diminuição na qualidade de vida, a um forte impacto econômico, cujo absenteísmo ao trabalho pode ser diretamente associado a rinite alérgica. Muitos alérgenos de polens têm sido estudados e caracterizados, conforme demonstrado na Tabela 1 (Corti et al., 2005; Taketomi et al., 2006b; Neckel et al., 2013; Asam et al., 2015).

Há uma gama de alérgenos proteicos em polens de gramíneas. Dentre as proteínas principais de polens, os alérgenos do grupo 1, presentes em toda a família Poaceae, apresentam bastante destaque. Os principais alérgenos desse grupo são denominados de β -expansinas, e são bastante abundantes nessas plantas. Há diversas β -expansinas, detectadas e caracterizadas até o momento, e estudos recentes têm mostrado que esses são os principais alérgenos envolvidos no desencadeamento das respostas alérgicas na polinose (Pacharn et al., 2019; Sterner et al., 2019). Além disso, outro grupo de alérgeno importante na sensibilização de pacientes alérgicos é o grupo 5, sendo encontrados até o momento apenas em membros da subfamília Pooideae. Pode-se citar ainda outros alérgenos que desempenham relevante papel em polens de gramíneas como os do grupo 13. Alguns que possuem menor reconhecimento por anticorpos IgE específicos são os dos grupos 2, 4, 23 e 24, além de como já mencionado, os panalérgenos dos grupos 7 e 12, que são denominados bioquimicamente de polcacasinas e profilinas respectivamente, os quais são alérgenos importantes na reatividade cruzada. Todos esses alérgenos, bem como alguns dos organismos da ordem Poales, que são comuns de se encontrar, estão exemplificados, conforme a Tabela 1 (Davies et al., 2008; Timbrell et al., 2014; García-Mozo, 2017; Kailaivasan; Davies, 2018; Yagami; Ebisawa, 2019).

Tabela 1. Caracterização dos principais grupos de alérgenos presentes em gramíneas da ordem Poales.

Fontes alergênicas	kDa	Alérgenos	
		Grupo	Nome bioquímico
<i>Anthoxanthum odoratum</i>	27	Grupo 1	Beta expansina
<i>Urochloa mutica</i>	30	Grupo 1	Beta expansina
<i>Dactylis glomerata</i>	32	Grupo 1	Beta expansina
	11	Grupo 2	-
	14	Grupo 3	-
	60	Grupo 4	-
	25/28	Grupo 5	-
<i>Festuca pratensis</i>	60	Grupo 4	-
<i>Oryza sativa</i>	35	Grupo 1	Beta expansina
	14	Grupo 12	Profilina
<i>Poa pratensis</i>	33	Grupo 1	Beta expansina
	34	Grupo 5	-
<i>Lolium perenne</i>	27	Grupo 1	Beta expansina
	11	Grupo 2	-
	11	Grupo 3	-
	57	Grupo 4	-
	31	Grupo 5	-
	16	Grupo 11	Proteína relacionada à Ole e 1
<i>Phleum pratense</i>	27-28	Grupo 1	Beta expansina
	10-12	Grupo 2	Grupo gramínea II/III
	55	Grupo 4	Enzima ponte berberina
	32	Grupo 5	-
	11	Grupo 6	-
	6	Grupo 7	Polcacina

	20	Grupo 11	Proteína relacionada à Ole e 1
	14	Grupo 12	Profilina
	55	Grupo 13	Poligalacturonase
<i>Paspalum notatum</i>	29-30	Grupo 1	Beta expansina
<i>Zea mays</i>	25-35	Grupo 1	Beta expansina
	28	Grupo 8	Quitinase de classe IV
	14	Grupo 12	Profilina
	9	Grupo 14	Proteínas não específicas de transferência lipídica
	-	Grupo 25	Tiorredoxina
<i>Cynodon dactylon</i>	32	Grupo 1	Beta expansina
	12	Grupo 7	Polcacina
	14,5	Grupo 12	Profilina
	9	Grupo 15	-
	-	Grupo 22w	Enolase
	9	Grupo 23	-
	21	Grupo 24	PR-1 (proteína relacionada à patogênese)
<i>Sorghum halepense</i>	30-35	Grupo 1	Beta expansina
	12	Grupo 2	Beta expansina
	54-55	Grupo 13	Exopoligalacturonase (Glicosil hidrolase 28)
<i>Lolium multiflorum</i>	-	-	-

Fonte: (ALLERGEN, 2024)

1.7 *Lolium multiflorum* (Lm)

Lolium multiflorum (Lm) é uma gramínea que filogeneticamente pertence a ordem Poales, família Poaceae, subfamília Pooideae e tribo Poeae, seu gênero é *Lolium*. É também

conhecida como azevém anual, ou azevém italiano, na língua inglesa *Italian ryegrass*. Essa gramínea foi trazida ao Brasil por imigrantes europeus, e se adaptou muito bem a estações climáticas bem definidas no país, desde então têm sido bastante utilizada na produção de forragem no sul (Vieira; Ferreira; Matter, 2005; Bernardes et al., 2010a; Timm et al., 2019; Correa et al., 2023).

Adicionalmente, Lm têm ampla capacidade de produção polínica e devido a sua fácil adaptação e forte utilização na pecuária, têm levado a sensibilização de muitos indivíduos no sul do Brasil. Um estudo, na capital do estado do Paraná, relatou que 15% dentre os indivíduos alérgicos observados no estudo eram atópicos a *L. multiflorum*. Outro estudo, envolvendo cidades do estado do Rio Grande do Sul e do Uruguai, revelou aumento dos sintomas de polinose no período de estação de florescimento de Lm, demonstrando que o aumento dos sintomas da rinite alérgica sazonal está intimamente ligado a presença do pólen da gramínea no ar. Além disso, um outro estudo revelou que *Lolium multiflorum* pode apresentar forte reatividade cruzada com alérgenos de outras gramíneas como *Phleum pratense* e *Cynodon dactylon* (Vieira; Ferreira; Matter, 2005; Moreira et al., 2015; Araujo; Rosario; Mari, 2016; Bonissoni; Neto, 2016).

Apesar de sua importância na rinite alérgica sazonal, não há ainda caracterização dos principais alérgenos de *L. multiflorum*. Um trabalho na literatura cita Lol m 5, como um alérgeno de Lm, entretanto não foi realizada nenhuma caracterização deste. Uma recente pesquisa de nossa equipe, indica forte reatividade com componentes de peso molecular de 32 kDa, com a presença de componentes dos grupos 1 e 5 (Mohapatra; Lockey; Shirley, 2005; Correa et al., 2023). Dessa forma, existem poucos estudos sobre Lm, essa falta de informação dificulta o diagnóstico bem como o tratamento dos indivíduos atópicos.

1.8 Purificação de alérgenos principais de gramíneas no estudo das doenças alérgicas a partir de extratos naturais

Como demonstrado na Tabela 1, há uma variedade de grupos de alérgenos importantes no tocante as gramíneas com capacidade de sensibilização, em que se destacam os alérgenos do grupo 1, as β -expansinas. Esses componentes alergênicos desempenham funções de extensão e relaxamento das paredes celulares, contribuindo, portanto, para processos de crescimento e amadurecimento de órgãos, extensão do tubo polínico, facilitando a entrada do grão de pólen no estigma da flor, dentre outros. Ainda uma característica especial das gramíneas é que nesses vegetais, as β -expansinas se acumulam no gameta masculino (pólen). Esses

alérgenos foram primeiramente isolados em 1993 em estudos de mecanismo da parede celular. Essas proteínas atuam em pH baixo (4,5), dessa forma, a atividade dessas é influenciada por fatores ambientais e hormonais. Posterior a identificação dos alérgenos, a nomeação dos mesmos segue a ordem com as três letras iniciais do gênero, seguido da primeira letra do epíteto específico e o número do grupo de alérgenos no qual se insere, como por exemplo as β -expansinas de *Phleum pratense* chamada de Phl p 1 (Cosgrove; Li, 1993; Cosgrove et al., 2002; Li et al., 2003; Jin et al., 2006; Valdivia et al., 2007; Geilfus et al., 2015; Marowa; Ding; Kong, 2016; Wang et al., 2016).

As β -expansinas são os principais alérgenos das gramíneas e há muito tempo se investiga o papel que desempenham nas doenças respiratórias mediadas por IgE, como a asma sazonal e a rinite alérgica sazonal. Já existem experimentos com o isolamento desses alérgenos com métodos como a cromatografia de troca iônica em coluna de carboximetil (CM) Sepharose juntamente com HPLC em gramíneas. Outros estudos, demonstraram que *Paspalum notatum*, conhecida popularmente por grama da Bahia apresenta no mínimo três isoformas de β -expansinas de 29 kDa isoladas do pólen com forte reatividade com anticorpos da classe IgE de soros de indivíduos alérgicos, após isolamento dos alérgenos através da técnica de cromatografia por exclusão por tamanho. É importante o isolamento desses componentes pois estudos recentes também detectaram esses alérgenos são os mais importantes agentes polínicos na sensibilização de indivíduos alérgicos e que muitas dessas proteínas apresentam semelhanças intraespécies de 64% a 85%, e surpreendentemente similitude de 97% até 100% entre diferentes espécies de gramíneas. Dessa forma, esses alérgenos do grupo 1, se destacam na sensibilização a alérgenos por polens de gramíneas e demandam cada vez mais de maior atenção para compreender os caminhos no diagnóstico e também no tratamento da rinite alérgica sazonal e de outras doenças alérgicas (Malley; Reed; Lietze, 1962; Li et al., 2003; Hrabina et al., 2008; Drew et al., 2011; Aud-In; Somkid; Songnuan, 2019).

1.9 Utilização de frações alergênicas para aplicação na detecção e tratamento da alergia

Em 1988, foi realizada a primeira clonagem da sequência completa de alérgenos em todo o mundo, a proteína de grupo 1 do ácaro da poeira domiciliar *Dermatophagoides pteronyssinus*, Der p 1, que acabou sendo publicada apenas mais tarde naquele ano, o que levou ao antígeno do grupo 5 de *Dolichovespula maculata* denominada como Dol m 5 a ser o primeiro clone publicado na história. No ano seguinte, houve a clonagem e expressão do primeiro alérgeno clonado de plantas, o alérgeno do grupo 1 da espécie *Betula verrucosa*, designado Bet v 1. Em comum, esses alérgenos compartilham que sua expressão foi realizada em bactérias da

espécie *Escherichia coli* após serem infectados por fagos λ gt11 (Chua et al., 1988; Fang et al., 1988; Breiteneder et al., 1989; Tscheppe; Breiteneder, 2017).

Os estudos em alérgenos têm avançado e a utilização de clones de proteínas têm sido cada vez mais frequentes para detecção e tratamento de indivíduos alérgicos. É de extrema importância o uso de alérgenos recombinantes no diagnóstico das doenças alérgicas, devido ao fato de se usar apenas extratos alergênicos naturais ou comerciais influenciar negativamente nos testes devido a presença de alérgenos insignificantes e que podem reagir de forma cruzada com anticorpos específicos, além do fato de alguns estudos demonstrarem que alérgenos importantes podem estar em baixa concentração nos extratos proteicos. Por isso, pesquisas demonstram que a adição de alérgenos recombinantes em meio a esses, para fins de diagnóstico, como por exemplo no SPT. Um processo denominado, *spiking*, que se baseia na adição de alérgenos recombinantes em meio a extratos alergênicos potencializa o diagnóstico *in vivo* em pacientes atópicos. Na atualidade, já existem plataformas *singleplex* (um ensaio por amostra) e *multiplex* (vários ensaios por amostra), que indicam a presença de anticorpos específicos frente a alérgenos recombinantes (Casset et al., 2012; Kespohl et al., 2013; Yoshida et al., 2015; Tscheppe; Breiteneder, 2017).

Da mesma forma que no diagnóstico, os alérgenos recombinantes também são utilizados no tratamento das doenças alérgicas. Há muito tempo se utiliza na ITA extratos alergênicos, porém esses podem conter em si contaminantes, alérgenos insignificantes do ponto de vista atópico que possam levar a um aumento na sensibilização a novas moléculas, além de estarem susceptíveis a degradação por proteases, temperatura e pH, levando a uma menor eficácia na terapia. Por outro lado, as proteínas recombinantes, derivadas da engenharia genética são capazes de gerar uma menor resposta a IgE (hipoalergênicos) e driblar todos os problemas listados, bem como fundir alérgenos principais de diversas fontes (Casset; Valenta; Vrtala, 2013; Douladiris et al., 2015; Zuidmeer-Jongejan et al., 2015; Freidl et al., 2017; Tscheppe; Breiteneder, 2017; Najafi et al., 2019). Estudos têm mostrado a efetividade das proteínas recombinantes ou purificadas no tratamento das doenças alérgicas como a rinite alérgica, levando a longo prazo a uma redução dos níveis de IgE específica, bem como ao aumento de anticorpos IgG, IgG1, IgG2, IgG3 e IgG4 específicos, demonstrando a eficácia desses componentes (Jutel et al., 2005; Pauli et al., 2008; Goldblum et al., 2018; Sánchez Acosta et al., 2020).

1.10. Cromatografia como método de separação de frações alergênicas

A cromatografia tem sido utilizada como método na separação dos principais alérgenos de diversas fontes, como alimentos, ácaros da poeira domiciliar e grãos de pólen. Técnicas cromatográficas, como troca iônica (IEC) e cromatografia de afinidade, são empregadas para purificar ou auxiliar na purificação de alérgenos principais, além de frações ricas em componentes glicosilados (Cases; Pastor-Vargas; Perez-Gordo, 2017; Resende et al., 2019; Correa et al., 2023).

A IEC é um processo amplamente utilizado na indústria biotecnológica pois é eficaz na separação de moléculas ionizáveis e na preparação para uso analítico. Essa abordagem, depende de interações iônicas ou eletrostáticas entre íons analitos ou moléculas altamente polares e uma fase estacionária com carga oposta, as trocas catiônicas e aniônicas são as duas categorias fundamentais da IEC (Grönberg, 2018; Ngere et al., 2023). Dessa forma, se faz fundamental na separação de moléculas carregadas, tais como peptídeos, proteínas, DNA e RNA (ÖZlem Bahadır, 2013). Essa técnica, tem sido empregada na separação de alérgenos principais de plantas, como as β -expansinas (Li et al., 2003). Em nosso recente trabalho, conseguimos isolar uma fração por IEC com alérgenos principais de Lm, que apresentou capacidade de tratamento e diagnóstico de indivíduos atópicos (Correa et al., 2023), demonstrando o potencial de tal método de isolamento.

A cromatografia de afinidade é uma forma de cromatografia líquida extremamente versátil e seletiva, usa como fase estacionária, um agente biologicamente relacionado, para se ligar seletivamente a um alvo em uma amostra (Tozzi; Anfossi; Giraudi, 2003; Hage; Matsuda, 2015; Arora; Saxena; Ayyar, 2017).). A imobilização de peptídeos com atividade de ligação específica permite o uso de cromatografia de afinidade e isola compostos com alta pureza (Tozzi, 2003). Além disso, o uso de materiais de embalagem baseados em sílica com partículas pequenas aumentou o desempenho da cromatografia de afinidade, isso permite separações mais rápidas e maiores capacidades dinâmicas (Hollis et al., 1987). Nosso grupo, avaliou a reatividade de IgE, IgG1 e IgG4 a frações antigênicas derivadas do extrato bruto de ácaros da espécie *Dermatophagoides pteronyssinus* ligadas e não ligadas à Concanavalina A (ConA) separadas por meio de cromatografia de afinidade. O trabalho demonstrou que ambas as frações eram capazes de se ligar aos anticorpos, com níveis mais altos de IgG4 específicos para frações ligadas à ConA em comparação com frações não ligadas à ConA, enquanto os níveis específicos de IgG1 foram semelhantes para ambas as frações, sugerindo potencial uso das frações glicosiladas na imunoterapia (Resende et al., 2019). Um outro trabalho de nossa equipe mostrou que os principais alérgenos isolados na cromatografia de afinidade com ConA são cruciais na

sensibilização ao alérgeno resposta de IgE e na manutenção da homeostase (respostas de IgG1 e IgG4) (Almeida et al., 2006).

2. Objetivos

A presente tese foi dividida em manuscritos, apresentados nos capítulos I, II e III, cujos objetivos são descritos a seguir.

CAPÍTULO I

Objetivo geral

Obter e analisar as frações imunogênicas e alergênicas do extrato proteico de pólen de *Lolium multiflorum* e sua fração eluída por meio de cromatografia de troca iônica, bem como avaliar a reatividade destas contra anticorpos das classes IgE e IgG4 específicos a Lm.

Objetivos específicos

- Separar por meio de cromatografia de troca iônica (IEC) os componentes com potencial alergênico de *L. multiflorum*.
- Analisar o perfil eletroforético de S1 e de sua fração S2, obtida por meio da IEC.
- Quantificar os níveis de IgE e IgG4 específicos a Lm por ELISA (Enzyme Linked ImmunoSorbent Assay) no soro de pacientes alérgicos e indivíduos controles;
- Identificar os principais componentes alergênicos/antigênicos do extrato de *L. multiflorum* reconhecidos por anticorpos IgE e IgG4 por Immunoblotting no soro de pacientes alérgicos e indivíduos controles;
- Obter a sequência proteica dos principais componentes de S2 através de espectrometria de massas.

CAPÍTULO II

Objetivo geral

Obter e analisar as frações imunogênicas e alergênicas do extrato proteico de pólen de *Lolium multiflorum* e suas frações eluídas por meio de cromatografia de afinidade, bem como avaliar a reatividade destas contra anticorpos da classe IgE específicos a Lm.

Objetivos específicos

- Separar por meio de cromatografia de afinidade os componentes com potencial alergênico de Lm.
- Analisar o perfil eletroforético do extrato total e de suas frações ligadas e não ligadas obtidas por meio da cromatografia de afinidade.
- Quantificar os níveis de IgE específicos a Lm por ELISA (Enzyme Linked ImmunoSorbent Assay) no soro de pacientes alérgicos e indivíduos controles.
- Identificar os principais componentes alergênicos/antigênicos do extrato total de polens de *L. multiflorum* e de suas frações não ligadas e ligadas à coluna de ConA, reconhecidos por anticorpos IgE através da técnica de Immunoblotting no soro de pacientes alérgicos e indivíduos controle.
- Avaliar a capacidade de inibição da ligação a IgE das frações não ligadas e ligadas à coluna de ConA no soro de pacientes atópicos frente ao extrato proteico de pólen de *Lolium multiflorum*.

CAPÍTULO III

Objetivo geral

Obter e analisar as frações imunogênicas e alergênicas do extrato proteico de pólen de *Lolium multiflorum* e suas frações eluídas por meio de cromatografia de afinidade, bem como avaliar a reatividade destas contra anticorpos da classe IgG e suas subclasses (IgG1, IgG2, IgG3 e IgG4) específicos a Lm.

Objetivos específicos

- Quantificar os níveis de IgG específicos a Lm no soro de pacientes alérgicos e indivíduos controles.
- Comparar a reatividade de IgG no soro de pacientes alérgicos e indivíduos controles contra S1 (extrato proteico de polens) S2 (fração não-ligada a ConA) e S3 (fração ligada a ConA).
- Quantificar os níveis de IgG1, IgG2, IgG3 e IgG4 específicos a Lm no soro de pacientes alérgicos e indivíduos controles.

- Comparar a reatividade de IgG1, IgG2, IgG3 e IgG4 no soro de pacientes alérgicos e indivíduos controles contra S1 (extrato proteico de polens) S2 (fração não-ligada a ConA) e S3 (fração ligada a ConA).
- Correlacionar os níveis de IgG1, IgG2, IgG3 e IgG4 no soro de pacientes alérgicos e indivíduos controles frente a S1, S2 e S3.

3. Hipóteses

H0: As frações obtidas através da IEC e cromatografia de afinidade não apresentam potencial no diagnóstico e tratamento de indivíduos atópicos a Lm.

H1: As frações obtidas através da IEC e cromatografia de afinidade apresentam potencial no diagnóstico e tratamento de indivíduos atópicos a Lm.

CAPÍTULO I – Artigo publicado

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Identification of carboxymethyl (CM)-binding proteins derived from *Lolium multiflorum* pollen extract and antibody reactivity in Brazilian allergic patients

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Abstract

Lolium multiflorum grass is the major pollen allergen source in the southern region of Brazil, but most of its allergens remain poorly characterized. The aim of this study was to investigate antibody reactivity to *L. multiflorum* crude and carboxymethyl-ligand extracts in allergic patients and healthy individuals. Ion exchange carboxymethyl (CM) chromatography (CM-Sepharose) was used to isolate proteins (S2) from *L. multiflorum* crude extract (S1), which were assessed by SDS-PAGE. S1- and S2-specific IgE and IgG4 levels were measured by ELISA using sera from 55 atopic and 16 non-atopic subjects. Reactive polypeptide bands in S1 and S2 were detected by immunoblotting, and the most prominent bands in S2 were analyzed by mass spectrometry (MS-MS). Similar IgE and IgG4 levels were observed to both S1 (IgE median absorbance: 1.22; IgG4 median absorbance: 0.68) and S2 (IgE median absorbance: 1.26; IgG4 median absorbance: 0.85) in atopic subjects. S1 and S2 had positive correlations for IgE and IgG4 (IgE: $r=0.9567$; IgG4: $r=0.9229$; $P<0.0001$) levels. Homology between S1 and S2 was confirmed by IgE (84%) and IgG4 (83%) inhibition. Immunoblotting revealed that the 29–32 kDa band was recognized by 100% of atopic subjects in both S1 and S2. MS-MS analysis identified similarity profile to groups 1 and 5 grass allergens. This study revealed that carboxymethyl-ligand fraction played an important role for pollen allergy diagnosis by containing clinically relevant allergens and constituted a promising candidate for allergen-specific immunotherapy.

Key words: Allergen; Ion-exchange chromatography; Beta-expansin; *Lolium multiflorum*; IgE; IgG4

Introduction

Seasonal allergic rhinitis, also known as hay fever, is an upper respiratory disease caused by the inhalation of airborne pollen, affecting allergic people particularly from spring to early summer, with well-defined symptoms, such as nasal itching, nasal congestion, sneezing, runny nose, and conjunctival hyperemia (1). In the past, it was thought that allergic rhinitis was an isolated and exclusive condition in airway allergy, but it has been currently shown that it is a systemic pathology with an intimate relationship with asthma and atopic dermatitis (2–5). Rhinitis affects approximately 50% of allergic patients around the world and both tree and grass pollens are among the most important sources of airborne allergens. Also, seasonal allergic rhinitis causes a great economic impact, in part because of the significant absenteeism rates directly associated with this disease (6–8).

L. multiflorum, Poaceae family, commonly known as Italian ryegrass, was introduced in Brazil by European immigrants, and it has been an important source for sensitizing individuals in the southern region of the country (9–11). Despite its importance, there is little evidence in the literature regarding the characterization of its major allergens, which is a reasonable concern for establishing diagnosis and treatment-specific strategies, particularly with immunodiagnostic tools and allergen-specific immunotherapy.

Pollen contains a wide range of proteins with distinct characteristics and functions, including β -expansins, which are also common in other grass pollen grains, as *Lolium perenne* and have been highlighted for more than half a century as the main components for inducing seasonal allergic rhinitis and seasonal asthma caused by

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grass pollen (12–14). Notably, β -expansins constitute up to 10% of the whole pollen grain, and Lol p 1, Poa p 1, Phl p 1 group 1 allergens derived from *Lolium perenne*, *Poa pratensis*, and *Phleum pratense*, respectively, are the most representative forms of this class of proteins in addition to group 5 allergens, which are exclusive to Pooideae subfamily. Many studies have demonstrated that these groups of allergens are immunodominant in grass pollen allergy by triggering robust IgE-mediated immune response. In addition, groups 2, 4, 7, 12, 13, 23, and 24 have also been described as mid-tier and minor allergens (15–18). Notably, Cyn d 1 was recently identified as 30 kDa- β -expansin major allergen from *Cynodon dactylon* (Bermuda grass) with high reactivity in patients with allergic rhinitis in subtropical regions, even though it has been responsible for the cross-reactivity with other grasses of the same region (19,20). In another study, groups 1 and 5 pollen isoforms were identified by 2D electrophoresis, and specific IgE antibodies found in sera demonstrated that they are essential for sensitization (7). Despite this, few studies have elucidated the role of pollen allergens as a potential application in the diagnosis and specific treatment of allergies. In this context, this study aimed to characterize protein fractions isolated from the total extract of *L. multiflorum* grass pollen, assessing IgE and IgG4 reactivity using sera of patients with seasonal allergic rhinitis.

Material and Methods

Subjects

A total of 71 subjects were recruited from the southern region of Brazil and distributed in two groups: atopic patients (AT) (n=55; 26 men/29 women; 29 $\pm$ 10 years old) with a clinical history of pollinosis and positive skin prick test (SPT) to *L. multiflorum* total extract; and healthy non-atopic subjects (NAT) (n=16; 2 men/14 women; 21 $\pm$ 12 years old), with no clinical history of pollinosis and negative SPT to *L. multiflorum* total extract (Table 1). The NAT

group had a smaller sample size than the AT group because the immune response of those subjects showed a uniform negative SPT (no IgE reaction) to *L. multiflorum* extract. This study was approved by the Ethics Committee in Human Research of the Federal University of Uberlândia (protocol number 3.342.127), and subjects signed an informed consent form.

Skin prick test

Immediate hypersensitivity response was assessed by SPT, as previously described (21). House dust mite (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Blomia tropicalis*), dog (*Canis familiaris*), cat dander (*Felis domesticus*), fungus (*Alternaria alternata*), and grass pollen (*Cynodon dactylon*, *Phleum pratense*, *Paspalum notatum*, and *L. multiflorum*) allergen extracts were tested (FDA Allergenic, Brazil). Histamine hydrochloride 10 mg/mL and 0.9% sodium chloride were used as positive and negative controls, respectively. A wheal size 3 mm greater than the negative control was considered positive. Also, 10 mL of blood was collected from each subject and sera were obtained by centrifugation at 700 g for 10 min at room temperature.

L. multiflorum extract

L. multiflorum was identified and registered by the University of Caxias do Sul Herbarium under specimen voucher HUICS 56105 available at <https://specieslink.net/search/>. Grass pollen was collected in Caxias do Sul, Rio Grande do Sul State, Brazil, during the flowering period of Italian ryegrass (September to November). Pollen content was sieved (0.50 mm pore/Tyler 32, Granutest-Telastem Sieves, Brazil) to eliminate potential contaminants. After separation, 5 g of grass pollen were mixed in 50 mL of 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, USA) and submitted to maceration at 4°C and extraction for 18 h at 4°C under orbital stirring. Subsequently, the solution was centrifuged at 15,000 g at

Table 1. Demographic and clinical characteristics of the subjects distributed according to clinical history of pollinosis and skin prick test.

Characteristics	Atopic n=55	Non-atopic n=16	P value
Gender ^a			<0.0001
Male	26 (47.3%)	2 (12.5%)	
Female	29 (52.7%)	14 (87.5%)	
Age ^b			0.1469
Median	29.5 (18–57)	21 (20–57)	
Mean wheal size (standard deviation, mm) ^b			<0.0001
<i>Lolium multiflorum</i>	10.9 $\pm$ 3.465	0	
Skin prick test (%) ^a			<0.0001
Positive	55 (100%)	0 (0%)	
Negative	0 (0%)	16 (100%)	

^aChi-squared test or Fisher's exact test, when appropriate; ^bMann-Whitney test.

4°C for 30 min and the supernatant was concentrated (S1) and stored at -80°C. The protein content was determined by the Bradford method (22), using bovine serum albumin (BSA, Sigma) as standard.

Ion-exchange chromatography

The *L. multiflorum* total extract S1 was separated by ion-exchange chromatography as previously described (13). Briefly, total extract S1 was thawed and centrifuged at 15,000 *g* at 4°C for 10 min. Then, the supernatant was dialyzed in equilibration buffer (20 mmol/L sodium citrate, pH 4.5) (Sigma) for 18 h at 4°C. Carboxymethyl (CM)-Sephacrose resin (Cytiva Life Sciences, USA) was packed and equilibrated as recommended by the manufacturer, and extract S1 (2 mg/mL) was loaded onto the column. Unbound proteins were removed by washing with equilibration buffer and bound fractions were eluted with equilibration buffer supplemented with 200 mmol/L NaCl and UV absorbance was measured at 280 nm (BioTek Instruments, Inc., USA). The CM-binding fractions with the same electrophoretic profile were pooled (S2) and the protein content of S2 fraction was also determined as previously described.

Measurement of IgE and IgG4 antibodies to *L. multiflorum* total extract (S1) and fraction (S2)

Serum IgE and IgG4 antibodies specific to *L. multiflorum* were measured by indirect ELISA as previously described (11). High-affinity microplates were coated with 50 µL of total extract S1 or fraction S2 at 20 µg/mL overnight at 4°C and blocked with PBS-Tween (T) containing 1% BSA for 1 h at room temperature. Serum samples were diluted at 1:2 (IgE) or 1:5 (IgG4) and then incubated at 37°C for 2 h in a humid chamber. Subsequently, secondary human biotinylated anti-human IgE (1:500) or anti-human IgG4 (1:1000) (Sigma) antibodies were incubated for 1 h at 37°C, followed by streptavidin-peroxidase conjugate (Sigma) diluted at 1:500 for 30 min at room temperature. The reaction was developed by the addition of 0.01 M 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) (Life Technologies, USA) in 0.07 M citrate-phosphate buffer, pH 4.2, containing 0.03% hydrogen peroxide. Absorbance was monitored at 405 nm. The results are reported as absorbance. The cut-off was defined by the average absorbance value of the negative control sera plus 3 standard deviations.

Inhibition ELISA

To evaluate the inhibition of S2 against extract S1, competitive inhibition ELISA was conducted as previously described (23). High-affinity microtiter plates were loaded with extract S1 (1 µg/well) in carbonate-bicarbonate buffer, and plates were then blocked with PBS-T containing 1% BSA for 1 h at room temperature. Subsequently, pooled serum from five positive samples determined by ELISA of the AT group were diluted (IgE: 1:2; IgG4: 1:5) and

preabsorbed for 18 h at 4°C with 10-fold diluted S2 fraction. *Toxoplasma gondii* (Tg) extract was used as irrelevant antigen. Residual uninhibited antibody reactivity to extract S1 was measured by ELISA as described below and results are reported as the inhibition of S2 fraction in the absence of inhibitors (inhibition %).

SDS-PAGE and immunoblotting

Total extract S1 and fraction S2 were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) prepared with 6% polyacrylamide for stacking and 14% for separation, under non-reducing conditions (24). The gels were stained with Coomassie blue and subsequently scanned (ChemiDoc XRS+, Bio-Rad, USA) and analyzed by Image Lab software (Bio-Rad Laboratories). Polypeptides were transferred to a nitrocellulose membrane (0.45 mm Millipore, USA) in buffer solution (25 mmol/L Tris, 192 mmol/L glycine, methanol), using a semi-dry transfer system (Multiphor II Electrophoresis Unit, Pharmacia LKB, Sweden) as described elsewhere (25). Nitrocellulose strips were blocked with PBS-T containing 2% skim milk (PBS-T-M) for 2 h at room temperature, followed by incubation overnight at 4°C under slow stirring with serum samples diluted 1:2 (IgE) or 1:5 (IgG4) in PBS. Then, the membranes were incubated with secondary human biotinylated anti-IgE (1:500) or anti-IgG4 (1:1000) antibodies diluted in PBS-T for 2 h at room temperature, followed by incubation with the streptavidin-peroxidase conjugate (Dako Corporation, USA) diluted 1:500 in PBS-T for 1 h at room temperature. After the final washing, 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) was added and bands were revealed.

Mass spectrometry

Liquid chromatography tandem-mass spectrometry (LC-MS/MS) was carried out to identify polypeptide bands. Fraction S2 was subjected to SDS-PAGE as described above, and the bands of 12, 29–32, and 58–69 kDa were excised and stored in 10 mmol/L ammonium bicarbonate (AMBIC, Sigma). For sample preparation, the solution was discarded and a washing solution (50 mmol/L AMBIC supplemented with 40% acetonitrile – ACN, Sigma) was added; the sample was stirred on a Thermomixer (Eppendorf, USA) at 2,200 *g* for 30 min, the washing solution was discarded, and the steps were repeated 5 times. ACN was added to the gel, vortexed for 5 min, and discarded. Dithiothreitol 10 mmol/L (DTT, Bio-Rad) solution was added to the samples and mixed on a Thermomixer at 56°C at 2200 *g* for 45 min. Then, the DTT was discarded, followed by the addition of 55 mmol/L iodoacetamide solution (IAA, Thermo Fisher Scientific Inc., USA). After shaking for 30 min at room temperature in the dark, the IAA solution was discarded. Afterward, the samples were treated with AMBIC (50 mmol/L) and vortexed for 5 min, followed by disposal of the solution. Also, ACN was added to the sample, vortexed for 5 min,

and then discarded. The samples were dried in a speed vacuum dryer, added to digestion buffer (100 ng of trypsin in 20 mL of 25 mmol/L AMBIC), and incubated for 16 h at 37°C at 800 g.

Peptide extraction was performed with the addition of trifluoroacetic acid (TFA, Thermo Fisher Scientific) to stop the digestion. The supernatant was collected in a LoBind microtube, mixed with an extraction solution (40% ACN in 0.1% TFA), and stirred for 15 min at room temperature. The process was repeated 4 times. The sample was dried in a speed vacuum dryer and resuspended in 0.1% TFA for desalting. Analyses were performed on LTQ-Orbitrap Velos ETD/Easy nanoLC II (Thermo Fisher Scientific). The peptides were separated on a C18RP column on a 95 min gradient. The instrumental conditions were checked using 100 fmol of a tryptic digest of BSA as standard. The carryover sample was completely removed between runs. The proteins were then filtered by $\sim 10 \log p > 15$, selecting those with > 3 unique peptides (UP) or at least 10% equality in the FASTA sequence, according to the UNIPROT database to select highly reliable non-redundant proteins. Alignment sequence analyses were performed using the Basic Local Alignment Search Tool (BLAST), followed by multiple sequence alignment analyses by CLUSTALW.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 6.0 (GraphPad, USA). The frequencies of the antigenic bands recognized by serum antibodies in immunoblotting were subjected to comparative analyses between two proportions by the Z statistic.

Kolmogorov-Smirnov normality test was used to determine distribution data model. The Mann-Whitney test was used to compare age, average wheal size, and the ELISA results. Pearson's test was used to determine correlation among data. All results were considered significant at a significance level of $P < 0.05$.

Results

Protein profile of *L. multiflorum* total extract (S1) and fraction (S2)

Protein content of all samples was determined by the Bradford method using BSA as a standard curve with correlation coefficient (r^2) of 0.996, using cubic curve fit calculated by applying the following equation: $\text{Absorbance} = 1.575 + 2.499(\text{Conc}) - 0.003899[(\text{Conc})^2] + 2.025 \times 10^{-6}[(\text{Conc})^3] + [(\text{Conc})^3]$. Figure 1A shows the fractionation profile of *L. multiflorum* extract by ion exchange-chromatography. Peak 1 indicates proteins that were not bound to CM-sepharose resin and peak 2 represents resin-binding fractions that were eluted with 200 mmol/L NaCl.

The electrophoretic profiles of the total extract S1 and fraction S2 are shown in Figure 1B. The major components that were identified in the total extract S1 (protein content, 2,000 $\mu\text{g/mL}$) were the 12, 29–32, and 58 kDa bands. In addition, bands ranging from 17 to 145 kDa were also seen in the extract S1. The analysis of the fraction S2 revealed predominantly Coomassie-stained bands of 32 and 58 kDa and minor bands ranging from 12 to 69 kDa. The fraction S2 (protein content, 500 $\mu\text{g/mL}$) was particularly enriched with 29–32 kDa components.

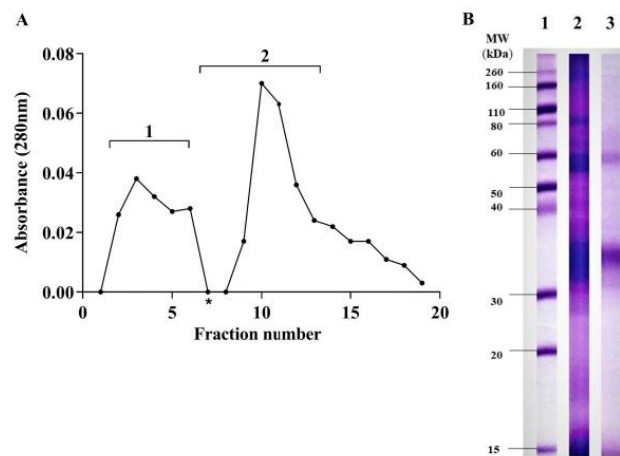


Figure 1. A, Fractionation profile of *Lolium multiflorum* total extract by ion-exchange carboxymethyl (CM) chromatography. Void volume (1) and fraction S2 (2) eluted with 20 mM NaCl. *Start of the elution. B, SDS-PAGE stained with Coomassie brilliant blue. Standard molecular weight values (lane 1), total extract S1 (lane 2), and fraction S2 (lane 3).

Specific IgE and IgG4 antibodies against total extract S1 and fraction S2 from *L. multiflorum*

The levels of serum IgE against *L. multiflorum* total extract (S1) were significantly higher in AT patients (median=1.22; 100%) compared to NAT subjects (median=0.15; 0%) ($P<0.0001$) as shown in Figure 2A. As expected, NAT subjects presented undetectable levels of IgE antibodies anti-S1. Likewise, levels of serum IgE against fraction S2 were significantly higher in AT patients (median=1.26; 96.4%) than in NAT subjects (median=0.09; 0%) ($P<0.0001$), as shown in Figure 2B. In addition, as shown in Figure 2E, a strong correlation ($r=0.9567$; $P<0.0001$) was found when comparing serum IgE antibody reactivity to extract S1 and fraction S2 from all AT and NAT individuals.

The levels of serum IgG4 against *L. multiflorum* total extract (S1) were significantly higher in AT patients

(median=0.68; 100%) compared to NAT subjects (median=0.27; 92.7%) ($P=0.0019$), as shown in Figure 2C. Also, levels of serum IgG4 against fraction S2 were significantly higher in AT patients (median=0.85; 96.7%) than in NAT subjects (median=0.32; 0%) ($P=0.0041$), as shown in Figure 2D. Additionally, a strong correlation ($r=0.9229$; $P<0.0001$) was found when comparing serum IgG4 antibody reactivity to the total extract S1 and fraction S2 from all AT and NAT individuals, as evidenced in Figure 2F.

Extract S1-specific IgE and IgG4 antibodies inhibited by fraction S2

IgE and IgG4 cross-reactivity to S1 extract was assessed by inhibition ELISA using S2 fraction. Tg was used as an irrelevant antigen. High homology was observed between extract S1 and fraction S2 for IgE (84%) of pooled sera from the AT group (Figure 3A) and

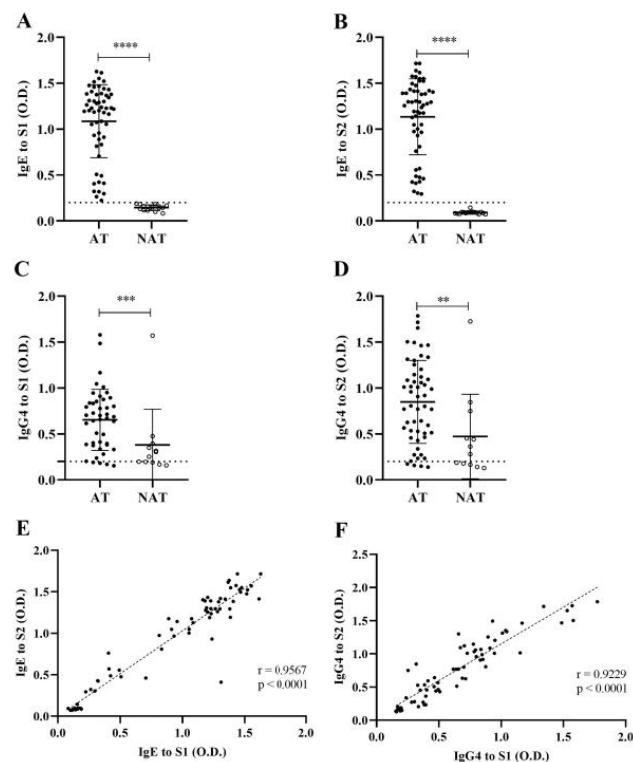


Figure 2. Levels of IgE and IgG4 specific to the *Lolium multiflorum* total extract (S1) (A and C) and carboxymethyl (CM)-binding fraction (S2) (B and D) in sera from 55 atopic patients (AT) with positive skin prick test to *L. multiflorum* and 16 non-atopic subjects (NAT), determined by ELISA. Differences between groups were compared by the Mann-Whitney test. Correlation coefficients (r) between levels of IgE (E) and IgG4 (F) specific to the total extract S1 and fraction S2 were calculated by Pearson's correlation coefficient test. Data are reported as absorbance (optical density (O.D.)). Median and positive threshold (0.2) are indicated by horizontal bars and dashed lines, respectively. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

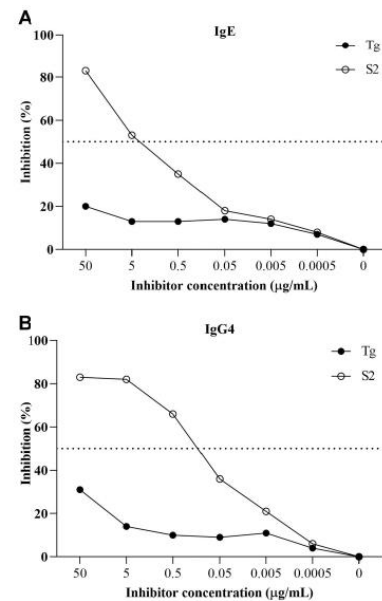


Figure 3. IgE (A) and IgG4 (B) inhibition curves using *Lolium multiflorum* total extract (S1) on the solid phase, by ELISA. Pooled sera from atopic patients were preabsorbed with different concentrations of CM-binding fraction derived from *Lolium multiflorum* (S2) or *Toxoplasma gondii* (Tg) as an irrelevant antigen (control). Dashed lines represent the half-maximal inhibitory concentration (IC50).

for IgG4 (83%) (Figure 3B) at the maximum concentration of 50 μg/mL of inhibitor. According to Figure 3A, 4.7 μg of the inhibitor was needed to inhibit 50% of IgE binding, while 0.37 μg of the inhibitor was necessary to inhibit IgG4 binding, as shown in Figure 3B. Tg exhibited low reactivity for IgE (20%) and IgG4 (31%) at the maximum concentration used.

Allergens in *L. multiflorum* total extract S1 and fraction S2 recognized in IgE and IgG4 immunoblotting

The reactivity analysis of *L. multiflorum* total extract S1 and fraction S2 components was evaluated in 35 serum samples of AT patients, which were divided into 7 pools (5 serum samples/pool), according to absorbance value obtained in ELISA for specific IgE antibody levels (maximum absorbance value: minimum absorbance value / number of pools). For negative control, the test was performed in 1 pool with 5 serum samples from NAT subjects. The representative images of immunoblotting for IgE antibody reactivity in AT patients (lanes 2–4) and NAT subjects (lane 5) are shown in Figure 4A (total extract S1) and Figure 4B (fraction S2).

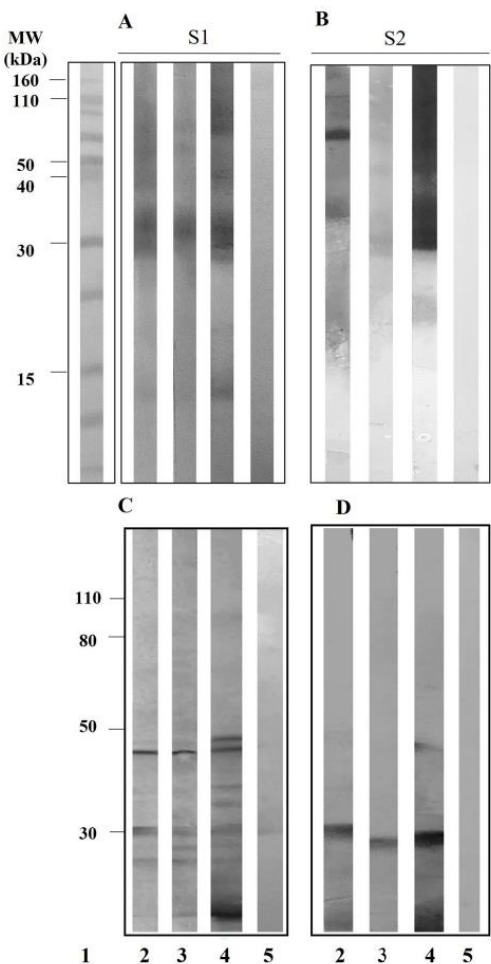


Figure 4. Representative immunoblotting of *Lolium multiflorum* total extract S1 and fraction S2 components recognized by IgE (A and B) and IgG4 (C and D) antibodies in three sera pools of atopic patients with pollinosis (lanes 2 to 4) and a sera pool of non-atopic subjects (lane 5). Protein standard molecular weight (MW) values are indicated in kDa (A, lane 1).

The frequency of serum-specific IgE reactivity to the total extract S1 and fraction S2 are reported in Figure 5A. The 29–32 kDa band was the most recognized by serum pools of all AT patients when using either extract S1 or fraction S2 from *L. multiflorum*, followed by the 69 kDa band, recognized in 85.7% of the samples. Interestingly, bands of 12, 38, and 58 kDa were recognized by IgE antibodies predominantly in the total extract S1 whereas IgE reactivity to 26 kDa band was predominantly seen in

the fraction S2. Other components with variable recognition were the bands ranging from 12 to 145 kDa as shown in Figure 5A. Serum samples of NAT subjects did not show any IgE reactivity to the extract S1 or fraction S2 components (Figure 4A and B, lane 5).

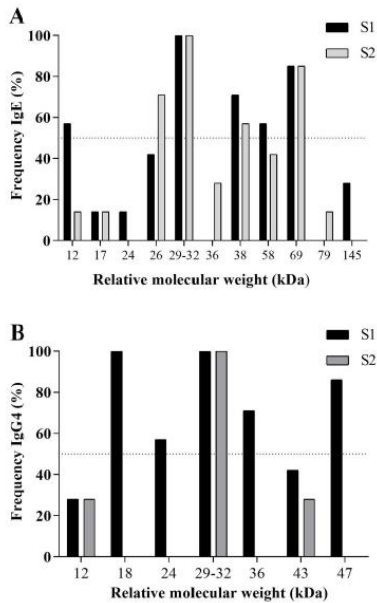


Figure 5. Frequency (%) of polypeptide bands of *Lolium multiflorum* total extract (S1) and carboxymethyl (CM)-binding fraction (S2) recognized by specific IgE (A) and IgG4 (B) antibodies in atopic patients with pollinosis, by immunoblotting. Dashed line indicates >50% of recognition.

Also, the presence of specific IgG4 antibodies in AT patients was classified according to the absorbance values obtained in ELISA for specific IgG4 antibodies (maximum absorbance value – minimum absorbance value / number of pools). For negative control, the test was performed in 1 pool with 5 serum samples from NAT subjects (Figure 4C and D, lane 5). The representative images of immunoblotting for the reactivity of IgG4 antibodies to *L. multiflorum* total extract S1 and fraction S2 components in patients AT (lanes 2–4) are illustrated in Figure 4C (extract S1) and 4D (fraction S2).

The frequency-specific IgG4 reactivity to the total extract S1 and fraction S2 components are reported in Figure 5B. The 29–32 kDa band was the most recognized by serum pools of the AT group when using either extract S1 or fraction S2 from *L. multiflorum*. Four bands in the extract S1 were exclusively recognized by IgG4 antibodies, namely 18 kDa (100%), 47 kDa (85.7%), 36 kDa (71.4%), and 24 kDa (57.1%). Interestingly, no IgG4 reactivity was seen exclusively to components of fraction S2. Other components with variable recognition were the bands of 43 kDa (42.9 % in S1; 28.6% in S2) and 12 kDa (28.6% in S1; 28.6% in S2).

LC-MS/MS characterization and properties of the main bands of fraction S2

Searching the protein database at the National Center for Biotechnology Information (NCBI) with the sequences obtained from an analysis of the 29–32 kDa band, at least 3 UP sequences were revealed for 2 proteins like those found in other grasses. The first protein found presented 5 UP compatible with Lol p 1, among others β -expansins, and the second protein presented 4 UP sequences compatible with Lol p 5, and 3 other proteins from group 5 (Table 2), with specific differences in some amino acids of the sequences (Figures 6 and 7). On the other hand, only a long peptide from the 58–69 kDa band was identified in MS/MS analysis. This peptide displays 10%

Table 2. Analysis by mass spectrometry of the main proteins recognized in the fraction S2 derived from *Lolium multiflorum* total extract.

Bands MW (kDa)	Protein	Organism	Similarity (%) / UP	Sequence ID (GenBank)
29–32	Lol p 1	<i>Lolium perenne</i>	5	CAB63699.1
–	Ph l p1	<i>Phleum pratense</i>	5	1N10_A
–	Dac g 1	<i>Dactylis glomerata</i>	5	AAP96760.1
–	Fes p 1	<i>Festuca pratense</i>	5	KAB8106316.1
29–32	Lol p 5	<i>Lolium perenne</i>	4	AAD20386.1
–	Ph lp 5	<i>Phleum pratense</i>	4	AAC25995
–	Dac g 5	<i>Dactylis glomerata</i>	4	AAK62278.1
–	Fes p 5	<i>Festuca pratense</i>	4	CCD28288.1
58–69	Hypothetical protein EE612_040618	<i>Oryza sativa</i>	(10%)	KAB8106316.1

MW: molecular weight; kDa: kilodaltons; UP: unique peptide.

CLUSTAL O (1.2.4) multiple sequence alignments

Band of 29-32 kDa. Group 1

Fes	-MASSSSVLLVVALFAVFLGSAHGIKVPFGPNITAEYGDKWLDKSTWYGKPTGAGPKD	59
Phl	-MASSSSVLLVVALFAVFLGSAHGIKVPFGPNITATYGDKWLDKSTWYGKPTGAGPKD	59
Dac	MASSSSVLLVVALFAVFLGSAHGIKVPFGPNITATYGDKWLDKSTWYGKPTGAGPKD	60
Lol	-MASSSSVLLVVALFAVFLGSAHGIKVPFGPNITAEYGDKWLDKSTWYGKPTGAGPKD	59
Seq1	-----STWYGKPTGAGPK-----	13
Seq2	-----D-----	1
Seq3	-----	0
Seq4	-----	0
Seq5	-----	0

Fes	NGGACGYKDVDPKAPFNGMTGCGNT?IFKDGRCGSCFELKCTKPESCSGEAVTVITDDN	119
Phl	NGGACGYKDVDPKPPFSGMTGCGNT?IFKSGRCGSCFELKCTKPACSGEPVVVHITDDN	119
Dac	NGGACGYKDVDPKAPFNGMTGCGNT?IFKDGRCGSCFELKCTKPESCSGEAVTVITDDN	120
Lol	NGGACGYKDVDPKAPFNGMTGCGNT?IFKDGRCGSCFELKCTKPESCSGEAVTVITDDN	119
Seq1	-----	13
Seq2	NGGACGYK-----	9
Seq3	-----GcGScFELK-----	9
Seq4	-----	0
Seq5	-----	0

Fes	EEPIAPYHFDLSGHAFGSMKKGEEQKLSAGELELQFRVKCKYPDGTKPTFHVEKGSN	179
Phl	EEPIAPYHFDLSGHAFGAMAKKGDEQKLSAGELELQFRVKCKYPGKTKVTFHVEKGSN	179
Dac	EEPIAPYHFDLSGHAFGSMKKGEEQKLSAGELELQFRVKCKYPGKTKVTFHVEKGSN	180
Lol	EEPIAPYHFDLSGHAFGSMKKGEEQKLSAGELELQFRVKCKYPDDTKPTFHVEKASN	179
Seq1	-----	13
Seq2	-----	9
Seq3	-----	9
Seq4	-----SAGELELQFR-----	10
Seq5	-----	0

Fes	PNYLAILVKYVDGDDVVAVDIKEKGKDKWIELKESWGAVNRIDTPDKLTGPFTVRYTTE	239
Phl	PNYLAILVKYVNGDDVVAVDIKEKGKDKWIELKESWGAIWRIDTPDKLTGPFTVRYTTE	239
Dac	PNYLAILVKYVDGDDVVAVDIKEKGKDKWIELKESWGAIWRVDTDPDKLTGPFTVRYTTE	240
Lol	PNYLAILVKYVDGDDVVAVDIKEKGKDKWIELKESWGAVNRIDTPDKLTGPFTVRYTTE	239
Seq1	-----	13
Seq2	-----	9
Seq3	-----	9
Seq4	-----	10
Seq5	-----	0

Fes	GGTKSEVEDVIPEGWKADTSYSAK	263
Phl	GGTKTEAEDVIPEGWKADTSYESK	263
Dac	GGTKSEVEDVIPEGWKADTSYSAK	264
Lol	GGTKSEFEDVIPEGWKADTSYSAK	263
Seq1	-----	13
Seq2	-----	9
Seq3	-----	9
Seq4	-----	10
Seq5	---SEVEDVIPEGWK-----	12
	:*:*****	

Figure 6. Sequence alignment of carboxymethyl (CM)-binding fractions derived from *Lolium multiflorum*. Samples compared with four FASTA sequences of several proteins obtained and aligned on the blastp (protein-protein BLAST) in the NCBI database. Fes: Fes p 1 (Sequence ID: CCD28289.1); Phl: Phl p 1 (Sequence ID: P43213.1); Dac: Dac g 1 (Sequence ID: AAP96760.1); Lol: Lol p 1 (Sequence ID: CAB63699.1). The equivalent amino acids in the analyzed proteins are marked with asterisks (*). A colon (:) indicates conservation between groups of strongly similar properties.

coverage with a hypothetical protein from *Oryza sativa* (Figure 8).

Discussion

Lolium multiflorum is one of the main allergen sources that elicit type 2 (T2) inflammatory response in subjects with IgE-mediated hypersensitivity. In the pathophysiological process involved in type I hypersensitivity, allergen proteases from *L. multiflorum* disrupt the epithelial barrier, thereafter captured by professional antigen presenting cells and presented to naive CD4⁺ T cells by

co-interaction with T cell receptor (TCR) and MHC-II. Those cells will differentiate into Th2 cells by producing interleukin (IL)-5, IL-4, and IL-13 cytokines, which also play an important role in the recruitment of eosinophils and IgE production, which characterize the T2-high endotype (allergic eosinophilic pattern of the airway inflammatory diseases) (26). The IgE interaction with its receptor FcεRI will trigger allergic inflammatory immune response mediated by vasoactive amines, such as histamine, prostaglandin, leukotrienes, and platelet-activating factor, released by activated mast cells and basophils. Although the allergic response to plant allergens is well described,

CLUSTAL O (1.2.4) multiple sequence alignments

Band of 29-32 kDa. Group 5

Dac	MAVQKRYTVALFLAVLVAGPVASYAADAGYTPAAAAAT-----PATAGGKAMTEEQTLI	53
Fes	MAVQKRYTVALFLAVLVAGPVASYAADAGYTPAAAAATPATFAATFAAGGKATTEQKLL	60
Phl	MAVQKRYTVALFLAVLVAGPVASYAADAGYTPAA-----AGAEAGKATTEQKLL	53
Lol	MAVQKRYTVALFLAVLVAGPVASYAADAGYTPAAAAATPATFAATFAAGGKATTEQKLL	60
Seq1	-----LL	2
Seq2	-----	0
Seq3	-----	0
Seq4	-----	0

Dac	EDVNAGFKAATAAASAPPADKFKTFEATTTAAACKANIAAAATKVPFLVAKLDAAYAVAY	113
Fes	EDVNAGFKAATAAANAPPADKFKIFEAAFTSESSKGLATSKAPGLVPKLDATYDIAY	120
Phl	EDINVGFKAATAAASVPADKFKTFEAAFTSSSKA-----ATAKAPGLVPKLDAAYSVAY	109
Lol	EDVNAGFKAATAAANAPPADKFKIFEAAFTSESSKGLLATSAKAPGLVPKLDATYDVAY	120
Seq1	EDVNAGFK	10
Seq2	-----AAVAAAANAPPADK-----	14
Seq3	-----	0
Seq4	-----	0
	:*:***:*:***	
Dac	KTATGPTPEAKYDAFVATLALRVIAAGLEVHAVKPAEEVPAKIPAGELQIVDKIDA	173
Fes	KAAEGATPEAKYDAFVTALTEALRVIAAGLEVHAVKPAEEVPAKIPAGELQIVDKIDA	180
Phl	KAAVATPEAKYDAFVATLALRVIAAGLEVHAVKPAEEVPAKIPAGELQIVDKIDA	169
Lol	KAAEGATPEAKYDAFVTALTEALRVIAAGLEVHAVKPAEEVPAKIPAGELQIVDKIDA	180
Seq1	-----	10
Seq2	-----	14
Seq3	-----	0
Seq4	-----	0
Dac	AYKIAATAANAAPANDKFTVFEGAFNKAKESTGGAYESYKFIPTLEAAVQQAAYATVAA	233
Fes	AFKIAATAANAAPANDKFTVFESSFNKALKECTGGAYETKFIPTLEAAVQQAAYATVAA	240
Phl	AFKVAATAAATAPADDKFTVFEEAFNKAKESTGGAYDYKCIPTLEAAVQQAAYATVAA	229
Lol	AFKIAATAANAAPTNDKFTVFESAFNKAKECTGGAYETKFIPTLEAAVQQAAYATVAA	240
Seq1	-----	10
Seq2	-----	14
Seq3	-----QAYAAATVAA	9
Seq4	-----	0

Dac	APEVKYAVFEAALTKAITAMSEAKQVATPAAVAT-----GAATAAASATGAATAAGGYK	289
Fes	APEVKYAVFEAALTKAITAMSEAKQAGKFAAAATGAATVATGAATAAG-----AAAGGYK	297
Phl	APQVKYAVFEAALTKAITAMSEVQKVSQPATGAATVAAGAAATTGAASGAATVAAGGYK	289
Lol	APEVKYAVFEAALTKAITAMTQAGKAGKFAAAATGAATVATGAATAAAGAAATAAGGYK	300
Seq1	-----	10
Seq2	-----	14
Seq3	APEVK-----	14
Seq4	-----YAVFEAALTK-----	
10		
	:*:***	
Dac	V	290
Fes	A	298
Phl	V	290
Lol	A	301
Seq1	-	10
Seq2	-	14
Seq3	-	14
Seq4	-	10

Figure 7. Sequence alignment of carboxymethyl (CM)-binding fractions derived from *Lolium multiflorum*. Samples compared with four FASTA sequences of several proteins obtained and aligned on the blastp (protein-protein BLAST) in the NCBI database. Fes: Fes p 5 (Sequence ID:CCD28288.1); Phl: Phl p 5 (Sequence ID:AAC25995.1); Dac: Dac g 5 (Sequence ID:AAK62278.1); Lol: Lol p 5 (Sequence ID: AAD20386.1).The equivalent amino acids are marked with asterisks (*). A colon (:) indicates conservation between groups of strongly similar properties.

few studies are focused on *L. multiflorum*, which is associated with seasonal allergic rhinitis and asthma in several regions, including Brazil (10,11,27,28). Therefore, a better understanding of the specific immune response induced by this source of allergens that leads to severe pathological conditions becomes relevant, as is the development of diagnostic tools and allergen-specific immunotherapy approaches using biological products derived from *L. multiflorum*.

This study analyzed patients from southern Brazil who had been exposed to a variety of allergens during their lifetime, especially *Lolium multiflorum*, and reacted to pollen allergens from this grass. This was confirmed by SPT, with the average size of wheals larger in the AT group than the NAT group, as expected. Also, IgE- and IgG4-specific antibodies against *L. multiflorum* were detected using ELISA and immunoblotting techniques only in the AT group. These data are consistent with our

CLUSTAL O (1.2.4) multiple sequence alignments

Band of 58 kDa.

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Seq 1 ----- 0
Oryza sativa MSHVRSAPAGKSGGGGSTPAKGRPFPGSTTSGGAAAAAAAGDAAAPALVGPQLQV 60

Seq 1 ----- 9
Oryza sativa -----VPGLLDALL 120
                *****

Seq 1 QVVVLLMPFVAAATCSRPGGSGNLQTRIVTLPPASLR----- 47
Oryza sativa QVVVLLMPFVAAATCSRPGGSGNLQTRIVTLPPASLRSTSVYLLSSCTMVFKRHGPGQLQ 180
                *****

Seq 1 ----- 47
Oryza sativa DLGFLPLAPIPLPPESCQYPOTDENIDDDRDIAMPKDHTKPPRVRTLGVNTLSGFGHE 240

Seq 1 ----- 47
Oryza sativa NVEKVYSDTTTPSDDQTKTADSTVTKRSAGFLFDEGLEFNVDDEGRTEKQCAVAASNI 300

Seq 1 ----- 47
Oryza sativa IRNFSFMPENETVMVQHRHCLSTVFQCLEDQNTDNEPFLPAIPQIYKRLVDLLSVPADV 360

Seq1 ----- 47
Oryza sativa AQAAAIISALYNVAEVMDFRLKLASERWAVDRLLKVVKTTPHPVPEVCRKASMIVESLVSE 420

Seq1 ----- 47
Oryza sativa PQNRMHLLVHENTFAEILTSEKYSOTFARILYELTARPSNKTAGQAIWGNIN 474

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Figure 8. Sequence alignment of carboxymethyl (CM)-binding fractions derived from *Lolium multiflorum*. Samples compared with four FASTA sequences of several proteins obtained and aligned on the blastp (protein-protein BLAST) in the NCBI database. *Oryza sativa*: hypothetical protein *Oryza sativa* (Sequence ID: KAB8106316.1). The equivalent amino acids are marked with asterisks (*).

previous studies (10,11). When evaluating the sensitization profile of the patients against *L. multiflorum*, serum IgE reactivity easily discriminated subjects from atopic and non-atopic groups, using either total extract S1 or fraction S2 derived from *L. multiflorum*, with no significant difference between them. By analyzing the electrophoretic profile of *L. multiflorum* total extract S1 and fraction S2, we observed that components with 12, 29–32, and 58 kDa were present in the highest intensity, particularly the band of 29–32 kDa in both fractions. The importance of these bands as major allergens was later confirmed by immunoblotting due to their high reactivity. Previous investigations have demonstrated that major components around 30 kDa are glycoproteins like β -expansins, which are related to papain-like proteases, corresponding to group 1 allergens (20,29–31).

Several studies have demonstrated that glycoproteins with molecular weights ranging from 25 to 37 kDa belonging to groups 1 and 5 allergens are the most important molecules involved in the induction of IgE antibodies to grass pollen, such as *L. perenne* and *P. pratensis* (11,29,32). Recently, Sterner and colleagues identified two isoforms of Cyn d 1, a β -expansin with about 29–30 kDa of *Cynodon dactylon* (Bermuda grass), by mass spectrometry as the major allergen in patients with allergic rhinitis in subtropical regions and responsible for cross-reactivity with other grasses in the same region (20,29,33). An additional Swedish study also revealed two β -expansins as the major sensitizing allergens in Phl p 1

and Cyn d 1 (33). These data indicate that β -expansins are the major group 1 grass pollen allergens. Our results, using mass spectrometry, revealed exactly the presence of group 1 proteins, β -expansins, and group 5 proteins, another important protein group. Therefore, we can sustain for the first time that ion exchange chromatography was able to enrich the major allergens derived from *L. multiflorum* and that the proteins likely responsible for cross-reactivity or other minor allergens did not link to the resin. As a result, these less relevant components were absent in fraction S2. This feature of the enriched fraction S2 may be considered more appropriate than the total extract S1 for allergy diagnosis since fraction S2 was able to keep sensitivity and specificity in ELISA comparable to the total extract S1. Therefore, we demonstrated the relevance of fraction S2 by inhibition ELISA. S2 revealed a high inhibition capacity similar to S1 and high-capacity binding to IgE and IgG4 specific for S1, which means that S2 kept with the main components of extract S1.

Furthermore, we can speculate that fraction S2 has a better indication than the *L. multiflorum* total extract for use in allergen-specific immunotherapy (AIT) since fraction S2 lacks many components that do not have clinical relevance (34,35). Although a previous study has shown that antibodies against *L. multiflorum* can highly cross-react with several other kinds of grass, including *P. pratense* and *C. dactylon* (11), several studies on AIT have demonstrated that it is always better to use a genuine allergen that contains clinical important

allergens and lacks components that are not relevant to atopic patients, because immunotherapy might induce new allergen sensitization. For this reason, it is more appropriate to use purified allergens, peptides, or fractions than crude allergenic extracts, particularly in AIT (36,37).

L. multiflorum total extract S1 and fraction S2 containing major allergens of 29–32 kDa, groups 1 and 5, showed strong specific IgG4 reactivity in both indirect ELISA and immunoblotting. A protective function against allergy has been greatly attributed to the generation of specific IgG4 subclass, which are antibodies that compete with IgE for binding to allergens and consequently decreasing mast cells and basophils activation by Fc_ε receptors, preventing histamine degranulation and allergic reactions (29,38,39). Currently, a total allergenic extract derived from mites, pets, and pollen is commonly used for both *in vivo* and *in vitro* diagnostic tests, as well as in AIT. In the last decade, studies have demonstrated that the use of purified peptides or proteins is also highly effective in AIT and promote an increase in IL-10-producing T-reg FOXP3+ cells, which are fundamental for the success of the treatment, because IL-10 is a crucial cytokine for inducing the production of specific IgG4 subclass in B cells, acting as a blocking antibody (39).

Altogether, we demonstrated that fraction S2 derived from total allergenic extract of *L. multiflorum* was able to bind to serum-specific IgE and IgG4 antibodies of atopic patients comparable to the total extract S1, and we observed that the 29–32 kDa band contained the main components of group 1 and group 5 allergens recognized by specific serum IgE and IgG4 antibodies of seasonal allergic patients.

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CAPÍTULO II – Artigo a ser submetido

IgE reactivity to *Lolium multiflorum* and concanavalin A binding fractions in atopic and non-atopic Brazilian subjects

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Abstract

In southern Brazil, *Lolium multiflorum* (Lm) pollen is one of the main causes of allergic rhinitis due to it is spread widely. The major pollen allergens are group 1 glycoproteins (β -expansins) and group 5 components. However, few studies have developed therapy and diagnostic methods. This study assessed the serum reactivity of atopic (AT) and non-atopic (NAT) patients to crude Lm extract and its fractions. Concanavalin A (ConA) column was used for affinity chromatography of Lm crude extract. Lm crude extract, and the unbound and bound fractions were dosed using the BCA method. The electrophoretic profiles of the extracts were examined by SDS-PAGE. IgE reactivity was assessed in the sera of 56 subjects 18-60 years (AT = 43; NAT = 13). Sera were examined using ELISA and immunoblotting against Lm crude extract, unbound and bound fractions. The electrophoretic profile of Lm crude extract varied with bands from 153 to 12 kDa, while unbound fraction contained clear bands between 87 and 12 kDa, and bound fraction contained bands between 87 and 12 kDa. AT patients had larger wheals and higher IgE levels than non-atopic patients against Lm extract. Significant variations were observed in EI values for serum specific IgE to Lm crude extract, unbound fraction, and bound fraction between the AT and NAT groups. Additionally, unbound fraction (84%) and bound fraction (86%) showed inhibitory effects on Lm crude extract. Specific IgE antibody reactivity was detected in AT serum by immunoblotting, with bands of 50, 33, and 26 kDa against Lm crude extract; 33 kDa against unbound fraction; and 58, 33, and 31 kDa against bound fraction, while NAT serum did not react. Unbound and bound fractions are potential candidates for the treatment and diagnosis of respiratory allergies caused by Lm, and more research is needed to compare their effects in therapy.

Keywords: atopy, *Lolium multiflorum*, IgE, concanavalin A.

Introduction

Atopy is defined as individual genetic predisposition to produce IgE antibodies in response to environmental allergens and diagnosis is determined by positive Skin Prick Test (SPT) and/or detection of allergen-specific IgE by *in vitro* assays (Johansson et al., 2001). Allergy is not defined by total IgE in individuals' serum but is mediated by antibodies and cells, particularly by the T2 profile, and symptomatic allergic patients usually have increased IgE antibodies specific to the allergen, thus this condition is classified as an IgE-mediated allergy (Johansson et al., 2004; Mohammadi Farsani; Yaghubi-Tabar; Mohammadi Farsani, 2020).

Asthma and rhinitis are the main respiratory allergies have been growing as public health concerns, mainly caused by exposure to mite, animal dander, and plant allergens (Dramburg et al., 2023). Recently, there has been a significant increase in allergies to grass pollen, mainly caused by members of the Poaceae family (Gruzieva et al., 2015; González-Fernández et al., 2022).

The major allergens in Poaceae pollen include glycoproteins of group 1 (β -expansins) and proteins of group 5, which are exclusive to plants of the Pooideae subfamily (Correa et al., 2023). Other components are panallergens of groups 7 and 12, polcacins, and profilins, as well as components of groups 2, 3, and 4 (oxidoreductase), 6 (isoflavone reductase), 10 (cytochrome C), 11 (proteins related to Ole e 1), and 13 (polygalacturonase) (García-Mozo, 2017; Visez et al., 2021). The pollen of *Lolium multiflorum* (Lm), a grass belonging to the Poaceae family found in southern Brazil, brought to the region by European immigrants, has become an important source of allergenic sensitization, however, few studies have been conducted to establish treatment and diagnostic tools (Moreira et al., 2015; Araujo; Rosario; Mari, 2016).

Chromatography is a fundamental technique for separating and identifying allergens present in pollen. Precise separation of pollen components can be achieved by chromatography, which utilizes different physicochemical properties of molecules, such as size, charge, and affinity for stationary and mobile phases. In allergology, this technique, along with mass spectrometry, is particularly useful for identifying allergens that trigger allergic reactions in patients (Landa-Pineda et al., 2016; Wang et al., 2023). Therefore, to better understand Lm allergens, affinity chromatography using a Concanavalin A (ConA) column can assist in separating the main components of crude extract Lm pollen, because of their ability to bind to glycoproteins (Resende et al., 2019), to verify whether these proteins are clinically relevant in

allergic sensitization, aiding in the development of diagnosis and treatment of respiratory diseases caused by the pollen of this grass. Thus, the objective of this study was to evaluate the serum reactivity of atopic and non-atopic subjects to the crude extract of Lm and its fractions derived through ConA chromatography.

Methodology

Lm protein extraction

Lm pollens from Caxias do Sul, RS, Brazil (Figure 1) were collected and identified by the Herbarium of the University of Caxias do Sul (HUCS), with identification number 56105, and can be accessed at <https://specieslink.search/>. Antigens were extracted from Lm pollen according to a previously described protocol (Correa et al., 2023). In summary, pollen was homogenized 1:10 in phosphate-buffered saline (PBS) 0.01 M, (pH 7.2) containing the complete inhibitor cocktail. The mixture was macerated on ice followed by overnight orbital shaking at 4°C. Subsequently, the mixture was centrifuged at $15,000 \times g$ and 4°C for 30 min, and the supernatant was collected and dialyzed against PBS overnight. Finally, the dialyzed extract was centrifuged, and the supernatant was collected, aliquoted, and stored at -80°C. The total protein concentration in the Lm extract was determined using the bicinchoninic acid (BCA) protein quantification method, as described by Smith et al. (Smith et al., 1985). After storage, the extract was designated as Lm crude extract.

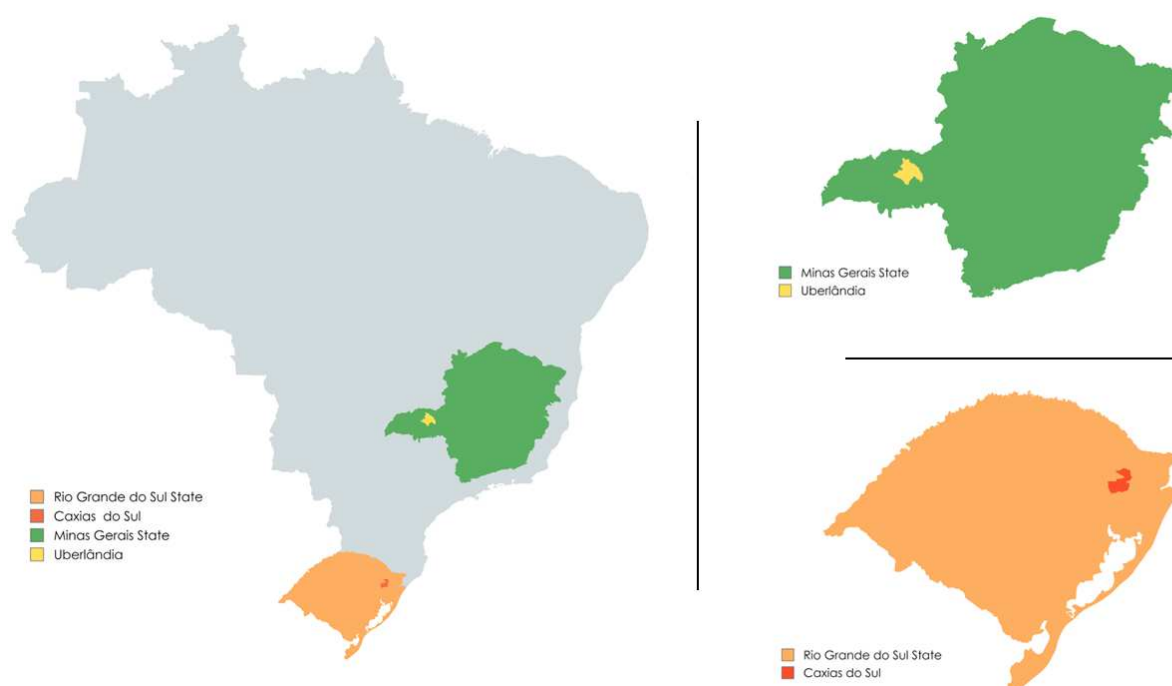


Figure 1. Demonstrative map of cities in Brazil. In orange, the map of the boundaries of the state of Rio Grande do Sul; in red, the boundaries of the municipality of Caxias do Sul. Green: map of the boundaries of the state of Minas Gerais; yellow: the boundary of the municipality of Uberlândia.

Affinity Chromatography with Concanavalin A-Sepharose

Affinity chromatography was performed as previously described (Resende et al., 2019). In summary, ConA-Sepharose 4B matrix (5 mL; GE Healthcare, Uppsala, Sweden) was packed and equilibrated according to the manufacturer's instructions. The Lm extract (2 mg/mL) was loaded onto the column for 2 h and washed with binding buffer (1 mmol MgCl_2 and 1 mmol CaCl_2 in PBS) until the entire volume of the unbound fraction was completely eluted. An elution buffer (binding buffer with 0.5 M glucose) was then added to the column. The eluate was collected until the entire volume of the bound fraction was completely removed. Unbound and bound fractions were monitored by ultraviolet absorbance (UV) at 280 nm (BioTek Instruments, Inc., Winooski, VT, USA).

Subjects, Blood Samples and Skin Prick Test (SPT)

A total of 56 subjects, male and female, aged 18 to 60 years with typical symptoms of allergic rhinitis and asthma, and control individuals, were recruited for the study in Caxias do Sul, RS, and Uberlândia, MG. The Ethics Committee for Research of the Federal University of Uberlândia approved the study under protocol number 3.342.127 (CAAE:

09978918.6.0000.5152). To participate in the study, participants signed the informed consent form (ICF).

Blood samples were collected from all subjects, and sera were stored at -20°C until serological tests, simultaneously with skin prick test (SPT). For analysis of immediate hypersensitivity, SPT was conducted as previously described (Moreira et al., 2015), using Lm pollen extract, physiological saline solution containing 50% glycerol as negative control, and histamine hydrochloride (10 mg/mL) as positive control. Punctures were performed with appropriate lancets on the inner forearm over drops (10 µL) of each allergenic extract (FDA Allergenic/Immunatech, Rio de Janeiro, RJ, Brazil), keeping 3 cm between the extracts. The test was read after 15 min, and an average wheal size equal to or greater than 3 mm compared with the wheal obtained to the negative control was considered positive.

Lm-Specific IgE Levels

Specific IgE antibodies to Lm were quantified in sera of atopic patients and non-atopic individuals using conventional ELISA (cELISA), as previously described (Correa et al., 2023). High-affinity plates (Corning, São Paulo, SP, Brazil) were coated with Lm crude extract, unbound fraction, or bound fraction at a concentration of 20 µg/mL in carbonate-bicarbonate buffer for 18 h at 4°C. The plates were washed with 0.05% Tween phosphate-buffered saline (PBS-T) and blocked with PBS-T containing 1% bovine serum albumin (BSA) for 1 h at room temperature. The plates were washed in a similar manner after each reaction step. Next, 1% PBS-T-BSA was used as the diluent. Serum samples were diluted 1:2 and incubated at 37°C for two hours in a humid chamber. Subsequently, biotinylated human anti-IgE secondary antibody (1:1000; Sigma-Aldrich) was added for 1 h at 37°C. Finally, streptavidin-peroxidase conjugate was added at a dilution of 1:500 for 30 min at room temperature. The reaction was developed by the addition of 2,2'-diazine of ethylbenzothiazole sulfonic acid (ABTS; Sigma-Aldrich) to 0.01M in 0.07M citrate-phosphate buffer, pH 4.2, containing 0.03% hydrogen peroxide. Optical density (OD) was measured at 405 nm using an Epoch Microplate Spectrophotometer (Biotek Instruments, Inc.). OD values were used to calculate the ELISA Index (EI) = OD of test sample/cut-off point (PC). The cut-off point was determined by average absorbance value of negative control sera plus 3 standard deviations.

Lm Specific IgE Inhibition ELISA

A competitive inhibition ELISA was performed to assess the inhibition of unbound fraction and bound fraction in relation to the Lm crude extract, as previously described (Resende et al., 2019). High-affinity plates were coated with Lm crude extract (20 µg/mL) in a carbonate-bicarbonate buffer overnight at 4°C. After each reaction step, the plates were washed with PBS-T in a similar manner. Next, blocking was performed with PBS-T containing 1% BSA for 1 h at room temperature. Subsequently, a pool of five samples positive for Lm-specific IgE antibodies was added, diluted in PBS-T-BSA 1% (IgE: 1:2), and pre-absorbed overnight at 4°C with unbound fraction or bound fraction at different concentrations (10^2 to 10^{-4} µg). In the same proportions as previously described, ovalbumin (OVA) extract was used as an irrelevant antigen. The residual reactivity of uninhibited antibodies to the crude extract of *Lolium multiflorum* (Lm) was quantified by ELISA. The results were expressed as the optical density (OD) of the sample at the indicated concentration, divided by the OD in the absence of the sample and multiplied by 100 to calculate the percentage of inhibition.

SDS-PAGE and Immunoblotting

The main bands of Lm crude extract, unbound fraction, and bound fraction were obtained by performing sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 14% under denaturation conditions, as previously described (Laemmli, 1970). The polyacrylamide gel was stained with Coomassie blue and digitized for image analysis using ImageLab software version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA). After vertical electrophoresis in SDS-PAGE, Lm crude extract, unbound fraction, and bound fraction were transferred to a nitrocellulose membrane (0.45 µm, Millipore, Bedford, MA, USA) using system Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories). The strips of nitrocellulose membrane were blocked with 1% skimmed milk in PBS-T for 1 h at 4°C, and then incubated with a pool of serum samples diluted in 1% skim milk containing PBS-T (PBS-T-M) at a dilution of 1:2 for IgE antibody detection overnight at 4°C with slow shaking. After each reaction step, the strips were washed five times with PBS-T for 5 min each. Subsequently strips were incubated with biotinylated human anti-IgE secondary antibodies diluted in PBS-T-M (1:500) for 2 h at 4°C. Then, the nitrocellulose strips were incubated with streptavidin-peroxidase complex (HRP, Dako Corporation, Carpinteria, CA, USA) diluted in PBS-T-M (1:500) for 1 h at 4°C. After the final wash step, the bands were visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma), digitized with the ChemiDoc™ XRS+ system, and analyzed using Image Lab™ software (Bio-Rad Laboratories).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.4 software (GraphPad Software, Inc. San Diego, CA, USA). The differences between the gender and wheal size were analyzed by Fisher's exact test. Student's t-test, Mann-Whitney test, ANOVA or Kruskal-Wallis's test were used for age, mean wheal size, and ELISA index (EI). All results were considered statistically significant when $p < 0.05$.

Results

Protein profile: Lm extract and fractions

The protein concentration determined by BCA method in Lm crude extract was 7,500 $\mu\text{g/mL}$. Then, Lm crude extract was subjected to affinity chromatography using a Con A-Sepharose 4B matrix. Chromatographic analysis showed two peaks of intensity at a wavelength of 280 nm. Peak 1 indicates the elution of unbound fraction, whereas peak 2 indicates the elution of bound fraction after the addition of the elution buffer, as shown in Figure 2A. The eluate was then collected and concentrated. The protein concentrations in unbound and bound fractions were determined using the same method as that for Lm crude extract. The concentrations for unbound fraction and bound fraction were 6,200 and 5,350 $\mu\text{g/mL}$, respectively.

The electrophoretic profiles of Lm crude extract, unbound fraction, and bound fraction were evaluated using SDS-PAGE. The bands in SI with the highest visibility were 55, 32 and 12 kDa. Bands with 55, 32, 29, and 12 kDa had the highest intensity observed in unbound fraction. The most prominent bands in bound fraction were 54, 36, 32, 29, 19, and 12 kDa (Figure 2B).

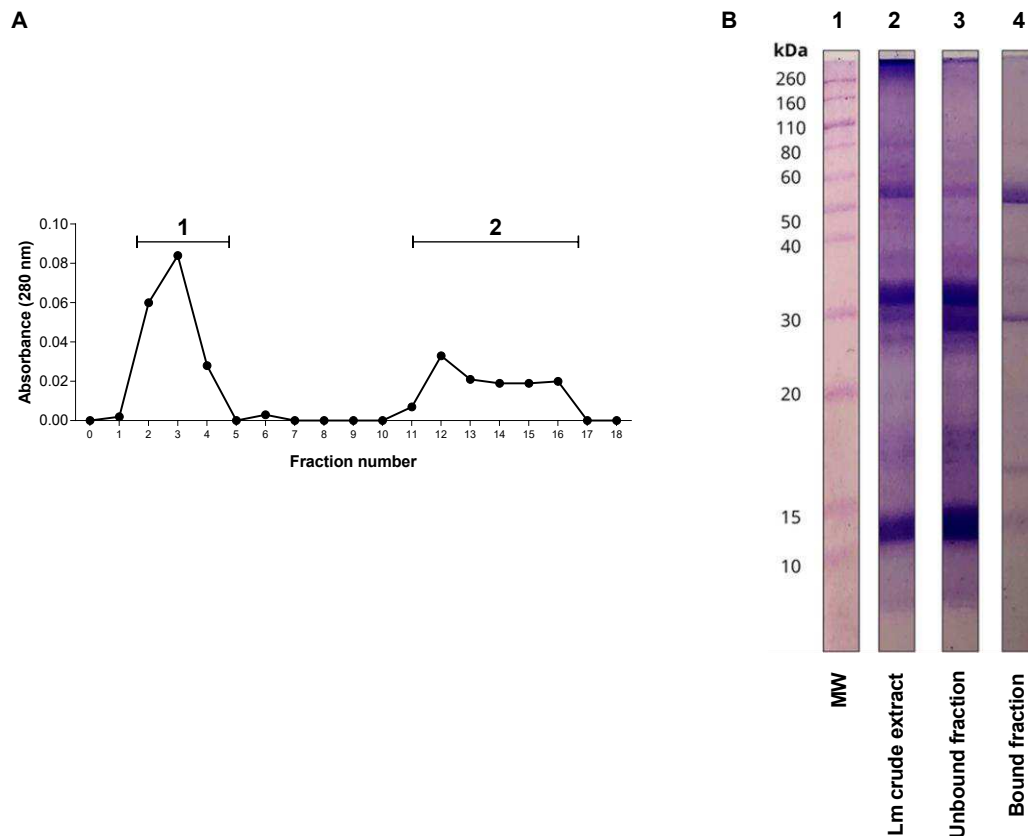


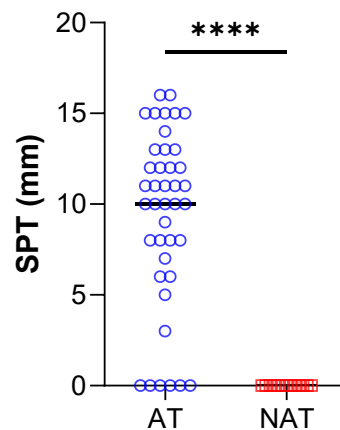
Figure 2. Fractionation profile of *Lolium multiflorum* extract on ConA column A. Unbound and bound fractions in ConA column, indicated by 1 and 2, respectively. B. SDS-PAGE stained with Coomassie brilliant blue. MW: Standard molecular weights (lane 1), Lm crude e extract (lane 2), unbound fraction (lane 3) and bound fraction (lane 4).

Subjects

Table 1 shows the demographic and clinical data of the patients. The AT group exhibited a higher median of wheal size than the NAT group when exposed to Lm extract (AT = 10.00 vs NAT = 0.00, $p < 0.0001$), where most individuals in the AT group showed positivity in SPT compared to the NAT group (TA = 86.1% vs. NAT = 0.0%; $p < 0.0001$), as shown in Table 1 and Figure 3. Similarly, when evaluating IgE levels against Lm crude extract, a statistically significant difference was observed between the AT and NAT groups (AT = 5.61 vs NAT = 0.67, $p < 0.0001$), where the majority of individuals in the AT group were as shown in Table 1.

Table 1. Demographic and clinical characteristics of the subjects according to pollinosis history and SPT and EI results.

Characteristics	Atopic (AT) n=43	Non-atopic (NAT) n=13	p value
Genre			
Male	20 (46.5%)	5 (38.4%)	0.7536
Female	23 (53.5%)	8 (61.6%)	
Age			
Median	26 (18-60)	29 (21-52)	0.2937
Lm median wheal size (mm)			
Lm	10.00	0	< 0.0001****
SPT to Lm			
Positive	37 (86.1%)	0 (0%)	< 0.0001****
Negative	6 (13.9%)	13 (100%)	
Lm specific IgE levels			
EI median	5.61	0.67	< 0.0001****
IgE to Lm			
Positive	40 (93.0%)	0 (0%)	< 0.0001****
Negative	3 (7.0%)	13 (100%)	

**Figure 3.** Skin Prick test between individuals in the AT and NAT groups against Lm. Bold lines indicate the median. The difference between the groups was evaluated using the Mann-Whitney test. The p value was considered significant when < 0.05.

We also investigated whether sera from AT and NAT groups were able to distinguish between specific IgE production against Lm crude extract, unbound and bound fractions. The EI values for specific IgE against Lm crude extract in the sera of AT and NAT individuals

differed, since 93% of the subjects positive in SPT had positive EI levels for IgE-specific antibodies, while no serum of NAT group was positive for Lm crude extract (Figure 4A). Differences were also observed in the EI values for specific IgE against unbound fraction when compared to subjects with AT and NAT, although 90.7% of AT individuals presented a positive result, only 38.3% of individuals with NAT were reactive to the same fraction tested (Figure 4B). Similarly, serum from AT individuals showed significant differences in EI values for specific IgE for bound fraction compared to NAT group ($p < 0.0001$). Thus, 81.4% of individuals with AT presented a positive result for specific IgE against bound fraction, while only 7.7% of the NAT group presented a positive result for this fraction (Figure 4C).

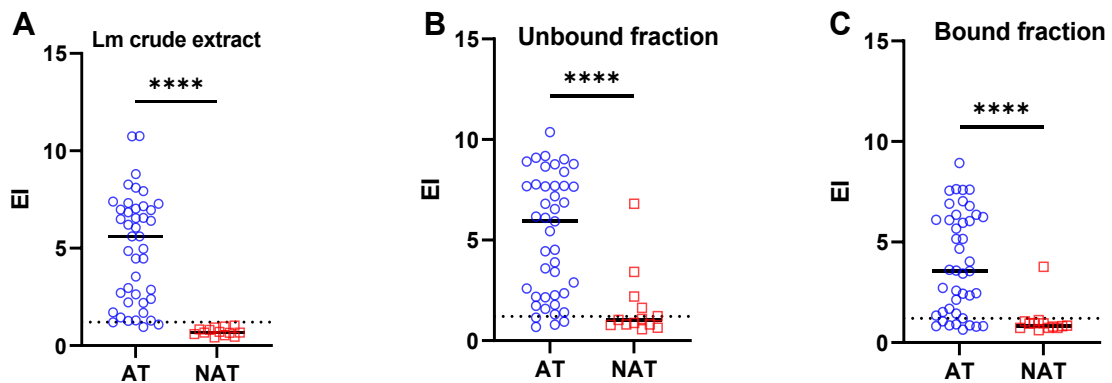


Figure 4. Specific IgE levels in AT and NAT against Lm crude extract, unbound and bound fraction. A. EI levels for IgE-specific antibodies against Lm crude extract in AT and NAT. B. EI levels for IgE-specific antibodies against unbound fraction in AT and NAT. C. EI levels for IgE-specific antibodies against bound fraction in AT and NAT. The dashed line indicates the positivity threshold. The difference between the groups was assessed by the Mann-Whitney test. The p-value was considered significant when < 0.05 .

We conducted a comparison of IgE-specific EI levels in order to assess any variations in reactivity to Lm crude extract, unbound and bound fractions in the individuals' serum. We noticed that, in all participants (Figure 5A), and solely in AT subjects (Figure 5B), there was no difference between IgE-specific EI levels between the extracts tested. However, higher IgE-specific EI levels were identified in NAT against unbound fraction individuals when compared to NAT against Lm crude extract, but no difference was seen in respect to individuals tested against bound fraction (Figure 5C).

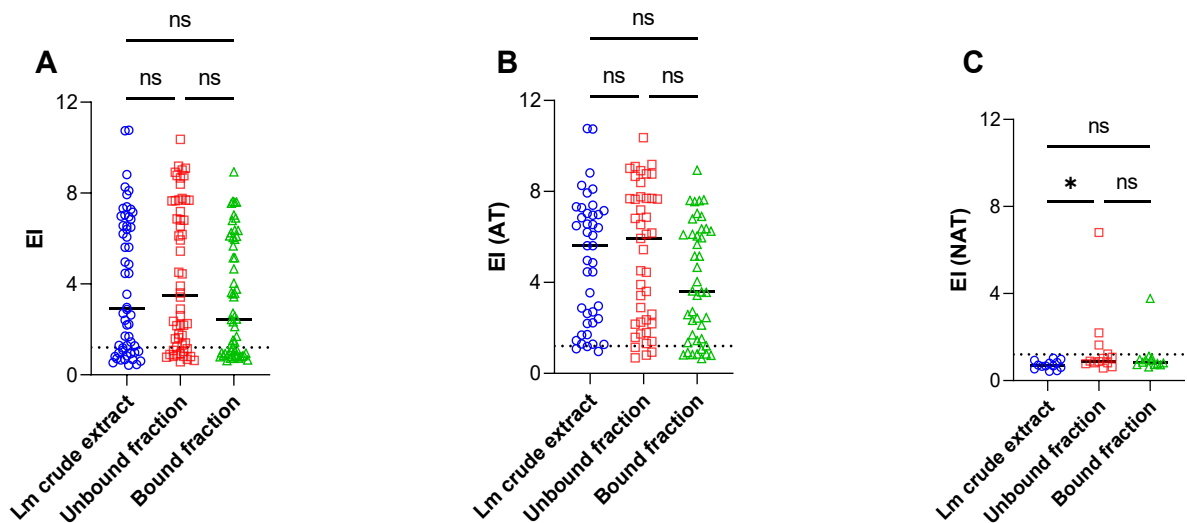


Figure 5. Specific IgE levels in all subjects against Lm crude extract, unbound and bound fractions. A. EI levels for IgE-specific antibodies against Lm crude extract, unbound and bound fractions. B. EI levels for IgE specific antibodies in AT against Lm crude extract, unbound and bound fractions. C. EI levels for IgE specific antibodies in NAT against Lm crude extract, unbound and bound fractions. The dashed line indicates the positivity threshold. The difference between the groups was assessed using the Kruskal-Wallis test. Bold lines indicate the mean. The p-value was considered significant when < 0.05 .

Inhibition ELISA was performed to assess the IgE cross-reactivity to Lm crude extract using unbound and bound fractions. Unbound fraction showed an inhibitory effect of more than 50% against Lm crude extract in the serum of AT subjects at concentrations of 10^2 – 10^{-4} , with a maximum inhibition capacity of 84%. Bound fraction, however, showed a maximum inhibition of IgE binding to Lm crude extract of 86%. Additionally, it demonstrated inhibition of more than 50% in the serum of AT subjects at concentrations of 10^2 – 10^0 . No substantial inhibition was observed for the irrelevant antigen at any concentration used (Figure 6).

Using immunoblotting, we investigated the sensitivity of specific IgE antibodies against Lm in the serum of AT and NAT subjects. As can be noted, the sera showed reactivity to the bands of 50, 33 and 26 kDa against Lm crude extract in the serum pool of the AT subjects, whereas no reactivity was detected in the serum pool of the NAT group. The serum pool of the AT group showed reactivity in the 33 kDa band against unbound fraction, while the NAT subjects showed no reactivity in any band. In the serum of AT subjects, antibody reactivity to bound fraction was observed against bands of 58, 33, and 31 kDa. No activity was detected in the serum pool of the NAT subjects (Figure 7).

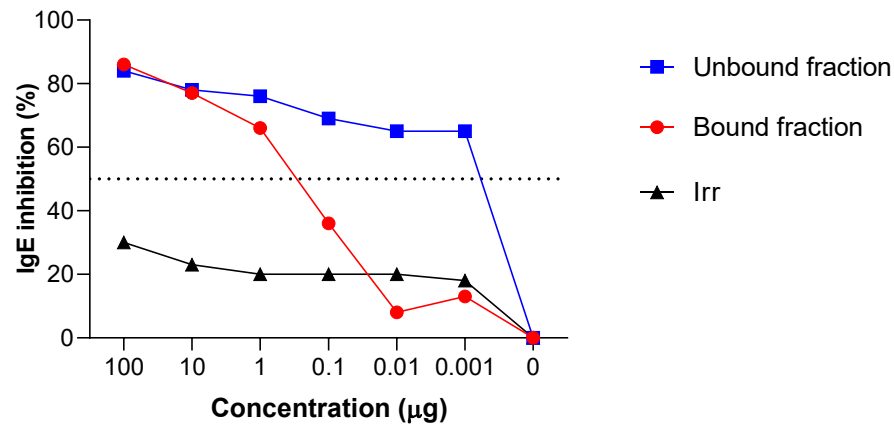


Figure 6. Inhibition curves of specific IgE antibodies using the Lm crude extract in the solid phase were determined by ELISA. Pooled sera from atopic patients were pre-adsorbed with different concentrations of fraction unbound fraction (blue square), bound fraction (red circle) or OVA as irrelevant antigen (Irr; black triangle). The dashed lines represent the half-maximal inhibitory concentration (IC₅₀).

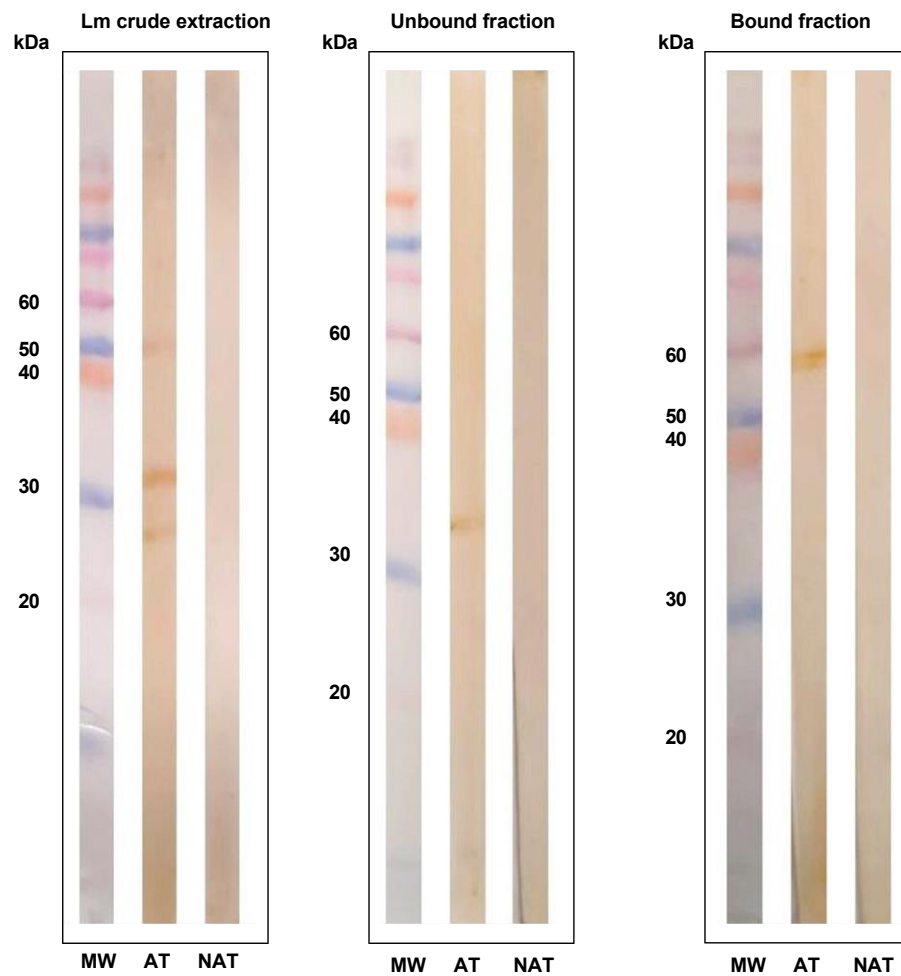


Figure 7. Immunoblotting representative of the Lm crude extract, and fractions unbound and bound fractions with demonstration of the main components recognized by IgE antibodies specific to Lm in serum pools of AT patients and serum pool of NAT individuals. The molecular weight (MW) of the bands is displayed next to each band in kDa.

Discussion

The impact of climate change and air pollutants on pollen allergens underscores the need for multi-sectoral strategies to prevent and control pollen allergies, particularly in low-income and middle-income countries. Pollen production, distribution, and composition are affected by these factors (Ravindra; Goyal; Mor, 2022). Therefore, pollen allergy has become a common and growing problem caused by the release of pollen from various grasses (Vusthepalli et al., 2022). In southern Brazil, Lm has become one of the main causes of this issue due to its widespread use in agriculture, favoring the adaptability of this specie (Matzrafi; Preston; Brunharo, 2021; Correa et al., 2023).

The main allergens that cause sensitization to Lm and other grasses include glycoproteins, which are usually classified into groups 1 and 5 and range in size from 25 to 37 kDa. Glycoproteins can contain carbohydrate-specific epitopes known as cross-reactive carbohydrate determinants (CCDs), which induce the production of specific IgE antibodies (Altmann, 2006; Manduzio et al., 2012; Shamji et al., 2021b). These allergens have been used as markers of genuine sensitization to tree and grass pollen, which has been beneficial for the treatment of polysensitized patients through immunotherapy (Gangl et al., 2015).

To isolate the glycoproteins, we used an affinity chromatography technique with a ConA column, which has capacity to bind to α -D-glucosyl and α -D-mannosyl residues of polysaccharides and glycoproteins (Sparbier; Wenzel; Kostrzewa, 2006; Dong et al., 2015). Our chromatographic analysis showed two absorption peaks at a wavelength of 280 nm, the first corresponding to the unbound volume and the second to the Con A-bound fraction. Electrophoretic profile analysis revealed that unbound fraction had a profile that was relatively similar to Lm crude extract, but retained most of the polypeptide bands, whereas bound fraction had an electrophoretic profile that was significantly different. Both unbound fraction and bound fraction showed bands in the 30 kDa range, which are typical of group 1 (β -expansins) and 5, the main pollen allergens, as well in the range of 58 kDa, typically associated with hypothetical proteins of *Oryza sativa*, as recently demonstrated (Pacharn et al., 2019; Correa et al., 2023). Thus, we sought to evaluate the reactivity of IgE antibodies specific to Lm in the serum of atopic (AT) and non-atopic (NAT) subjects against Lm crude extract, unbound and bound fractions.

Based on the literature, individuals were classified as AT and NAT when they showed positive results in SPT or in the test for IgE specific for Lm (16, 17). As observed, a low percentage of individuals positive in the SPT were negative in the specific IgE ELISA; just as

in some cases, positivity in the ELISA did not guarantee positivity in the SPT. Human skin mast cells possess proteinase-activated receptor-2 (PAR-2) and can be activated by proteases, without the need for IgE action, as demonstrated (Siiskonen; Harvima, 2019). Additionally, mast cells can also be activated by purinergic signaling, independently or synergistically with allergens (Gao; Jacobson, 2017), which would explain sera from individuals negative in the IgE ELISA for Lm but positive in the SPT. Similarly, individuals negative in the SPT but positive in the ELISA can be explained by variations in the composition of allergenic extract components, which may vary according to the extraction method, time, and temperature, leading to decreased or complete loss of allergens, resulting in false interpretations in *in vivo* tests (Zimmer et al., 2022).

Additionally, we observed significant differences in the levels of specific IgE antibodies for Lm crude extract, unbound and bound fractions in the sera of the AT and NAT groups. This indicated that both extracts retained the crucial elements necessary for antibody recognition. Nevertheless, the patterns of the identified bands were different, indicating differences in the allergenic components. This aspect became even clearer when we demonstrated that participants in the NAT group were positive for unbound fraction or bound fraction, indicating that these extracts may contain a greater number of major allergens and increase the potential for antigenic recognition. In addition, recent studies have shown that numerous protein extracts contain non-allergenic proteins, termed 'ballast,' which have the ability to mask the major components contained in the crude extracts (Gogunskaya et al., 2022; Zimmer et al., 2022).

Our data showed that unbound and bound fractions could effectively inhibit the binding of sera from individuals in the AT group to Lm crude extract. Both unbound and bound fractions have the potential to be beneficial in AIT and in diagnosing those who are sensitized to Lm, possibly by maintaining the main allergens of Lm rather than Lm crude extract. It is better to use a genuine allergen that contains clinically relevant allergens and excludes irrelevant components for atopic patients, as immunotherapy can lead to new allergen sensitization. Additionally, the use of crude extracts in AIT is involved in the cause of side effects of treated patients. Therefore, it is more appropriate to use purified allergens, peptides, or fractions than crude allergenic extracts, especially in AIT (Prangtaworn et al., 2018; Eckl-Dorna et al., 2019; Zhernov et al., 2019; Correa et al., 2023). However, the complexity of natural allergenic components may not be accurately represented by purified allergens or allergenic peptides, which undergo allergenic modification after secretion and affect their allergenicity. Furthermore, these natural extracts contain minor allergens that are clinically important in

respiratory diseases (Aalberse, 2022), emphasizing the importance of the isolated fractions in this study.

Our results have shown that the fractions unbound to the ConA column, and the fractions bound to the ConA column are candidates for treatment by AIT and for diagnostics, such as SPT or detection of specific IgE in *in vitro* tests related to respiratory allergy caused by Lm pollen. Finally, future studies are needed to investigate the effects of unbound fraction and bound fraction compared to the crude extract of Lm in AIT, keeping in mind that these fractions can be prepared using a relatively simple procedure to isolate the major natural and genuine allergens from *L. multiflorum* pollen.

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CAPÍTULO III – Artigo a ser submetido (International Journal of Molecular Sciences)

Distinct IgG subclass profiles elicited by *Lolium multiflorum* glycoconjugates in atopic and non-atopic subjects

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Abstract: *Lolium multiflorum* (Lm), a European grass widely found in southern Brazil, is a significant agent causing allergic reactions in a large portion of the population. . This study assessed the responsiveness of IgG antibodies and their subclasses (IgG1, IgG2, IgG3, and IgG4) to the Lm crude extract (S1), as well as the unbound (S2) and bound (S3) fractions of the ConA column. 75 individuals were evaluated, 56 atopic (AT) and 19 non-atopic (NAT). It was shown that S3 (OD = 0.7497) and S1 (OD = 0.7774) presented higher levels of specific IgG to Lm when compared to S2 (OD = 0.6564), while the reactivity of IgG antibodies specific to Lm between AT and NAT was different only in S2 ($p = 0.0294$). It was observed that AT and NAT groups presented the same reactivity of high avidity IgG antibodies against Lm ($p = 0.26$). No differences were found in ELISA index (EI) between the subclasses when analyzing extract S1. Significant discrepancies were observed in the EI when comparing IgG1 with IgG3 ($p = 0.0298$), IgG1 with IgG4 ($p < 0.0049$), IgG2 with IgG3 ($p < 0.0001$), IgG3 with IgG4 ($p < 0.0001$) in S2. Regarding S3, there were differences in the EI between IgG1 and IgG4 ($p < 0.0001$), IgG2 and IgG3 ($p = 0.0119$), IgG2 and IgG4 ($p = 0.0074$), and IgG3 and IgG4 ($p < 0.0001$). For extract S1, a weak correlation was found between specific concentrations of IgG1 and IgG4 ($r = 0.4$; $p = 0.0006$). For S2, moderate correlations were found between IgG1 and IgG2 ($r = 0.68$; $p < 0.0001$), IgG1 and IgG4 ($r = 0.71$; $p < 0.0001$), and IgG2 and IgG4 ($r = 0.71$; $p < 0.0001$). Additionally, there were a weak correlation between IgG1 and IgG3 ($r = 0.32$; $p = 0.009$) and between IgG3 and IgG4 ($r = 0.27$; $p = 0.028$). For extract S3, moderate correlations were observed between IgG1 and IgG4 ($r = 0.63$; $p < 0.0001$). There were weak correlations between IgG1 and IgG2 ($r = 0.42$; $p = 0.0004$), between IgG1 and IgG3 ($r = 0.35$; $p = 0.0040$), and between IgG2 and IgG3 ($r = 0.30$; $p = 0.019$). These results show that both S2 and S3 are potential extracts for the diagnosis of IgG antibodies and their subclasses as well as for the treatment of allergic diseases caused by Lm pollen.

Keywords: Glycoconjugates, pollen, *Lolium multiflorum*, atopy, immunoglobulin G.

1. Introduction

Atopy is determined by the genetic predisposition of producing immunoglobulin (Ig) E against harmless substances known as allergens. (Verschoor; Von Gunten, 2019). House dust mites, dog and cat epithelium, grass pollen, and pollutants are among the allergens that can elicit or exacerbate the immune response, leading to the production of IgE in atopic individuals (Bousquet et al., 2008; Mahboubi Oskouei et al., 2017). The term "allergy" is commonly used to describe a spectrum of diseases, often but not always, mediated by IgE antibodies,

characterized by type I hypersensitivity reactions to allergens (Cookson, 1999; Arshad, 2006). Among these, rhinitis, and asthma stand as the most common prevalent allergic conditions globally, imposing significant public health and economic burdens due to their considerable morbidity and impact on quality of life. Rhinitis, marked by inflammation of the nasal mucosa, and asthma, characterized by airway inflammation and bronchial hyperresponsiveness, are particularly meaningful manifestations of allergic diseases (Sonia et al., 2018; Bousquet et al., 2020; Patel; Mims; Clinger, 2024).

Pollinosis is an allergic respiratory condition caused by the dispersion of pollen particles from grasses during specific seasons. Grass pollen allergens can trigger inflammatory allergic reactions when they surpass the epithelial barrier of the nose, resulting in symptoms (Mahboubi Oskouei et al., 2017; Basak; Bhattacharya, 2023). The most common symptoms are sneezing, rhinorrhea, itching, nasal obstruction and redness in the eyes in allergic patients (Small; Keith; Kim, 2018).

Lolium multiflorum (Lm) is a grass species in the Poaceae family that produces pollen and comes from to the southern part of Brazil. Pollen grains having proteins or glycoproteins that are accessible to the body can trigger an allergic response mediated by IgE antibodies. People who are sensitive to Lm pollen may experience symptoms during the spring months (in the Southern Hemisphere) when these plants are in bloom. Despite the prominence of this period, allergic patients often exhibited cross-reactivity to pollen from other grass species, such as *Phleum pratense*, posing challenges for both diagnosis and treatment (Sopelete et al., 2006; Bernardes et al., 2010b; Moreira et al., 2015).

Allergic reactions typically stem from an immune system imbalance triggering a type 2 (T2) immune response upon allergen exposure. Th2 cells release cytokines such as IL-4 and IL-13, which in turn stimulate B cells to produce allergen-specific IgE antibodies (Ernesto Akio et al., 2017; Gagnon et al., 2023; Nedeva et al., 2023; Zhou; Xu; Liu, 2023). Additionally, the production of allergen-specific IgG antibodies, particularly IgG1 subclass, also plays a role in allergic conditions. Among these, IgG1 is the most abundant subclass and may be involved in the positive regulation of the cellular binding complex of IgE and B cells. Its precise role in allergic diseases remains subject to debate. Recent studies have indicated that IgG1-producing B cells are critical for generating high-affinity pathogenic IgE (He et al., 2017). Conversely, it has also been shown that IgG1 can bind to allergens as well as to mast cell and basophil via FcγRIIb receptors, thereby inhibiting their degranulation during allergen immunotherapy (AIT), and subsequently alleviating allergic symptoms (Zinkhan et al., 2022). In addition, IgG4

plays a regulatory role by competing with IgE for the same binding site, similar to IgG1. This competition is an important marker for assessing the effectiveness of AIT by contributing to the development of tolerance and acting as a blocking element in allergen presentation (Lighaam; Rispens, 2016; Shamji et al., 2021b). Elevated levels of IgG4 and IgG1 are observed in allergic conditions, assuming that IgG subclasses can be valuable for diagnostic and AIT markers (Shamji et al., 2021b).

IgG2 and IgG3 likely exert an inhibitory function in allergies due to their high binding capacity to FcγRIIb receptors on mast cells and basophils, as opposed to IgG1 and IgG4. However, the precise role of IgG2 and IgG3 activation of these receptors in allergic rhinitis and asthma remains less well-described (Macglashan; Hamilton, 2016).

Allergenic extracts used in the diagnosis and treatment of allergic diseases are characterized by their complex and variable compositions. The presence of smaller allergens within these extracts can impact the results of allergic tests by potentially cross-reacting with specific antibodies. Recent studies have indicated that major allergens may be present in low concentrations within protein extracts (Yoshida et al., 2015; Twaroch et al., 2016; Valenta et al., 2018).

Our previous research has demonstrated the efficacy of ion exchange chromatography in isolating and concentrating a fraction derived from Lm crude allergenic extract, containing major allergens, including glycoproteins, with binding capacity to specific IgE and IgG4 serum antibodies in atopic patients (Correa et al., 2023). Furthermore, we have investigated the reactivity of fractions composed of glycoconjugates derived from house dust mite extracts using a ConA (*Canavalia ensiformis*) column, revealing specific antibody responses, including IgE, IgG1, and IgG4 (Almeida et al., 2006; Resende et al., 2019). However, to our knowledge, this method has not been previously conducted to assess antibody reactivity against Lm pollen extract. The objective of our study was to examine the reactivity of specific IgG class antibodies and their subclasses in the sera of individuals classified as AT and NAT groups to the crude extract of Lm pollen (S1) as well as its unbound (S2) and bound (S3) fractions obtained by using affinity chromatography on a ConA column.

2. Results

2.1. Grass pollen extract

Analysis of the crude extract of Lm (S1) revealed a concentration of 7,500 µg/mL by the bicinchoninic acid (BCA) method. Subsequently, S1 was used for affinity chromatography with Con A-Sepharose 4B matrix. The chromatogram showed two absorption peaks, with the first corresponding to fraction S2 and the second to fraction S3 (Figure 1). The eluate was collected and concentrated, and the protein concentration in the fraction S2 and S3 was determined. Determination of proteins by BCA resulted in a concentration of 6,200 µg/mL for S2 and 5,350 µg/mL for S3.

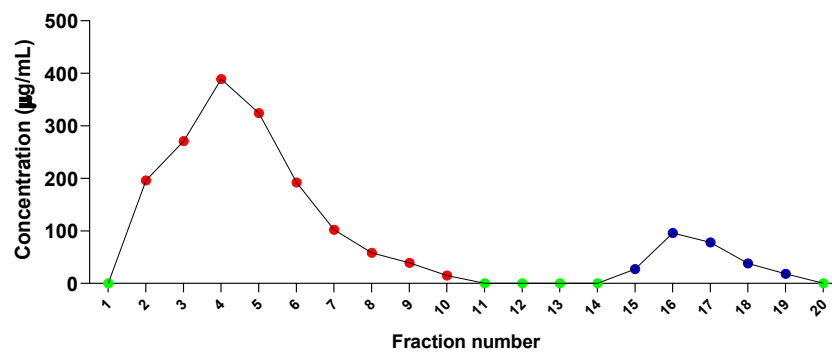


Figure 1. Fractionation profile of *Lolium multiflorum* extract on ConA column A. Green points indicate the blank, red points indicate the unbound fraction from the ConA column, while blue points indicate the bound fraction from the ConA column.

2.2. Study Participants

The study involved the participation of 75 participants, 56 AT, with SPT and/or EI positive for IgE antibodies against the Lm crude extract, and 19 NAT, negative for both. Among the study subjects, there was a prevalence of female in both AT (51.8%) and NAT (57.9%) groups, although there was no statistical difference among them (Table 1). Regarding the age, it was found that the average age of the AT was 32.9 years (18 to 60 years), while the average age of the NAT group was 29.5 years (18 to 58 years). The statistical analysis did not reveal any significant differences between the groups (Table 1). The median diameter of the wheal generated by the extract of *Lolium multiflorum* was 10 mm for the AT group and 0 mm for the NAT group ($p < 0.0001$), demonstrating a statistically significant difference between the groups. For SPT, in the AT group, the majority of subjects (78.6%) showed a positive reaction to the extract of Lm, while in the NAT group, no positivity was identified for this extract. In addition, the AT group (5.61) exhibited a larger median of the EI to specific IgE compared to the NAT group (0.75) (Table 1).

Table 1. Demographic and clinical characteristics of subjects categorized based on demographic and clinical characteristics of subjects categorized based on their history of pollinosis and SPT/EI results.

Characteristics	Atopic (AT) n=56	Non-atopic (NAT) n=19	p value
Sex			
Male	27 (48.2%)	8 (42.1%)	0.791
Female	29 (51.8%)	11 (57.9%)	
Mean	32.9 (18-60)	29.5 (18-58)	0.290
Lm wheal size (mm)			
Lm (median)	10.00	0	< 0.0001****
SPT			
Positive	44 (78.6%)	0 (0%)	< 0.0001****
Negative	12 (21.4%)	19 (100%)	
Lm-specific IgE			
EI (median)	5.61	0.75	< 0.0001****

L. multiflorum (Lm), Skin prick test (SPT), ELISA index (EI). * $p < 0.05$, **** $p < 0.0001$.

2.3. Analysis of *Lm* specific IgG

Upon determining the patients, we were interested in determining whether the anti-Lm IgG antibody concentrations against extracts S1, S2, and S3 differ among the AT and NAT groups. In order to accomplish this, we evaluated the IgG levels of all study participants in the three extracts. Our study revealed that the sera of the subjects had a higher mean reactivity towards S1 (OD = 0.7774) and S3 (OD = 0.7497) than to S2 (OD = 0.6564), but there was no significant difference between S1 and S3 (Figure 2A). Moreover, the anti-Lm IgG values were exclusively evaluated within the atopic and non-atopic groups. Thus, differences in the mean reactivity of S1 (OD = 0.7897) compared to S2 (OD = 0.6786) were observed in atopic individuals, but no significant difference was observed compared to S3 (OD = 0.7483) (Figure 2B). There was also a statistical difference in the reactivity of anti-Lm IgG to S1 (OD = 0.7384) and S3 (OD = 0.7540) compared to S2 (OD = 0.5860), but not between S1 and S3, when evaluating only non-atopic subjects (Figure 2C).

The reactivity of the IgG anti-Lm was compared between the AT and NAT groups for each of the three allergenic extracts. When tested against S2, the sera of the AT group presented a higher mean reactivity than the NAT group (Figure 2E), with a statistically significant difference (OD = 0.6824 vs. 0.5798; $p = 0.0294$), but not when tested against S1 (OD = 0.7918 vs. 0.7348; $p = 0.26$) (Figure 2D) and S3 (OD = 0.7534 vs. 0.7388; $p = 0.71$) (Figure 2F). Our findings revealed that S2 exhibited lower OD values for specific IgG than S1 and S3, even when the AT and NAT individuals were grouped (Figure 2A-C). This suggests reduced reactivity of S2 against these antibodies. Notably, when comparing AT and NAT individuals against the same extract, only S2 showed a significant difference between the groups, indicating its potential as a discriminatory fraction for IgG levels in allergic patients (Figure 2D-F).

An avidity ELISA test was performed to determine any differences in high avidity (IgG) specific antibodies to Lm between the AT and NAT groups. It is noteworthy that all participants tested had an avidity index (AI) above 50%. It has been shown no statistically significant difference ($p = 0.26$) between the AT (AI = 76.61) and the NAT group (AI = 69.14) against S1 (Figure 3).

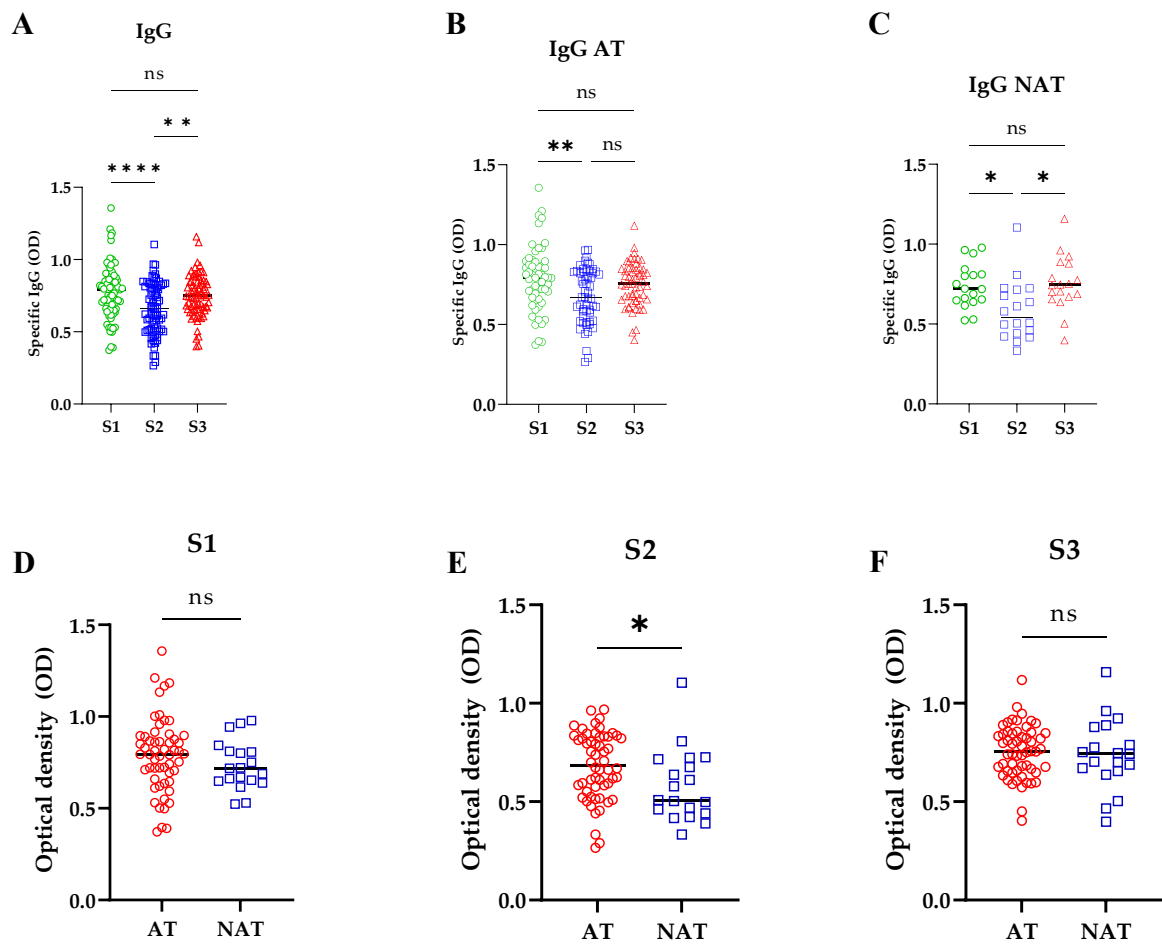


Figure 2. Levels of anti-Lm IgG antibodies against S1, S2, and S3 in AT and NAT individuals. (A). Anti-Lm IgG levels in sera of both atopic and non-atopic individuals compared to S1, S2, and S3. (B) Measurement of anti-Lm IgG serum levels in persons with atopic conditions targeting S1, S2, and S3. (C). Serum levels of anti-Lm IgG in non-atopic persons against S1, S2, and S3. (D). Serum levels of anti-Lm IgG in atopic and non-atopic persons against S1. (E). Serum levels of anti-Lm IgG in atopic and non-atopic persons against S2. (F). Serum levels of anti-Lm IgG in atopic and non-atopic persons compared to S3. Values are provided as optical density (OD). The black line indicates the median. Differences across groups were evaluated using the one-way ANOVA test with Tukey's multiple comparisons test and Mann-Whitney test. * = 0.05; ** = 0.01; **** = 0.0001.

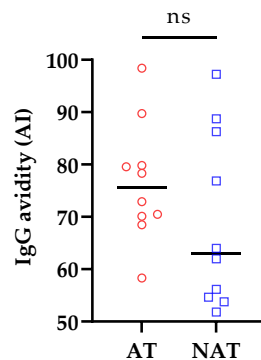


Figure 3. High avidity IgG levels for Lm in AT and NAT groups. The black line indicates the median. Differences between groups were assessed by Student's T test. Values are reported as avidity index (AI). ns = not significant.

2.4. Specific IgG for S1, S2 and S3

Differences in IgG reactivity was also investigated in relation to the antigen extracts S1, S2 and S3. For this purpose, we used the majority of the previously mentioned serum. First, the levels of IgG1, IgG2, IgG3 and IgG4 antibodies were compared for each of the three antigen extracts. As with S1, no differences in EI values between subclasses (Figure 4A). Even when the EI of the AT and NAT samples were examined independently, there was still no difference (Figure S1).

Surprising discrepancies in EI levels among different IgG subclasses were observed in the analysis of antigenic extracts S2 and S3. EI levels were significantly different when comparing IgG1 to IgG3 ($p = 0.0298$), IgG1 to IgG4 ($p = 0.0049$), IgG2 to IgG3 ($p < 0.0001$), IgG3 to IgG4 ($p < 0.0001$), but not between IgG2 and IgG4 ($p > 0.9999$) when discussing S2 (Figure 4B). Concerning S3 (Figure 4C), sera exhibited differences in EI levels when comparing IgG1 to IgG4 ($p < 0.0001$), IgG2 to IgG3 ($p = 0.0119$), IgG2 to IgG4 ($p = 0.0074$), and IgG3 to IgG4, but not between IgG1 and IgG2 ($p = 0.44$) and IgG1 and IgG3 ($p > 0.9999$).

Analyzing the sera AT and NAT separately with respect to S2 and S3 revealed significant variations. In the case of S2, there was a disparity in EI levels between IgG1 and IgG2 ($p = 0.0142$), IgG1 and IgG4 ($p = 0.0092$), IgG2 and IgG3 ($p < 0.0001$), and IgG3 and IgG4 ($p < 0.0001$) in the AT group. Additionally, with regard to S2, there was a difference between IgG3 and IgG4 ($p = 0.0037$) in the NAT group (Figure S1). EI levels for S3 revealed significant differences between IgG1 and IgG4 ($p < 0.0001$), IgG2 and IgG3 ($p < 0.0204$), IgG2 and IgG4 ($p = 0.0015$), and IgG3 and IgG4 ($p < 0.0001$) in the AT group. The NAT group

found discrepancies only when they compared the IgG1 and IgG2 subclasses ($p = 0.042$) (Figure S1).

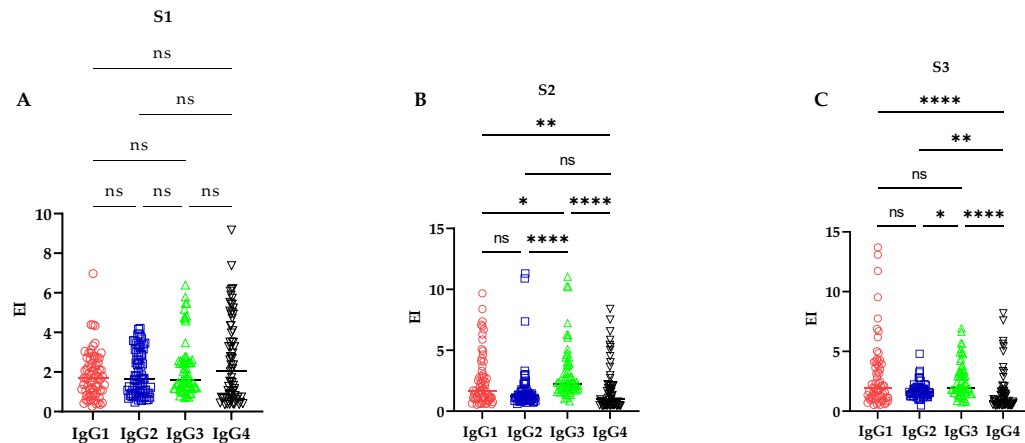


Figure 4. Levels of specific IgG subclasses for S1, S2, and S3 in AT and NAT groups. (A). Levels of specific IgG subclasses for S1. (B). Levels for specific IgG subclasses for S2. (C). Levels for specific IgG subclasses for S3. The black line indicates the median. Values are reported as Enzyme-Linked Immunosorbent Assay (ELISA) index (EI). Differences between groups were analyzed using one-way ANOVA with the Kruskal-Wallis test. ns = not significant, * = 0.05; ** = 0.01; *** = 0.001; **** = 0.0001.

Based on the differences in reactivity between the IgG subclasses for S2 and S3, we attempted to assess whether there was a difference between the three antigen extracts. Thus, a difference in EI values for specific IgG2 was found between S2 and S1 ($p = 0.0358$) and S3 and S2 ($p = 0.0068$) (Figure 5B). Differences were also observed between S2 and S1 ($p = 0.0032$) for specific IgG3 (Figure 5C) and between S1 and S2 ($p = 0.0302$), S1 and S3 ($p = 0.0469$) for specific IgG4 (Figure 5D), but no difference was found in EI values for specific IgG1 (Figure 5A).

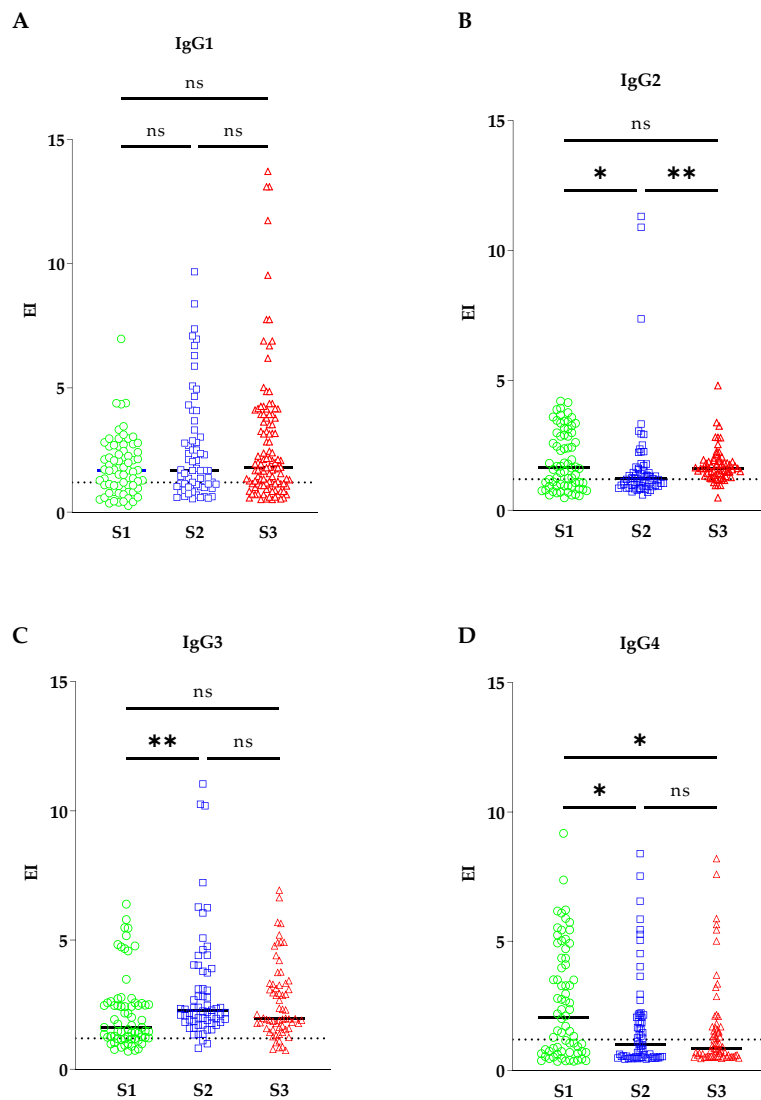


Figure 5. Levels of specific IgG subclasses for S1, S2, and S3. (A). Levels of specific IgG1 for S1, S2 and S3. (B). Levels of specific IgG2 for S1, S2 and S3. (C). Levels of specific IgG3 for S1, S2 and S3. (D) Levels of specific IgG4 for S1, S2 and S3. Values are reported as Enzyme-Linked Immunosorbent Assay (ELISA) index (EI). The black line indicates the median. The dashed line indicates the cutoff point. Differences between groups were analyzed using one-way ANOVA with the Kruskal-Wallis test. ns = not significant, * = 0.05; ** = 0.01.

We attempted to determine whether there were discrepancies between the subclasses of specific IgG in each extract of the AT and NAT groups. Consequently, we observed higher EI values for IgG1 (Figure 6A; $p = 0.0155$) and IgG4 (Figure 6D; $p = 0.0120$) against S1 in AT subjects compared to NAT, but not for IgG2 (Figure 6B) and IgG3 (Figure 6C). No differences were observed between the AT and NAT groups when comparing EI levels with S2 (Figure 7A-7D). In addition, it was also possible to observe a difference in EI for specific IgG4 against S3, with higher levels of this antibody in the NAT group compared to AT (Figure 8D; $p = 0.0348$); however, no differences were observed between the other subclasses (Figure 8A-8C).

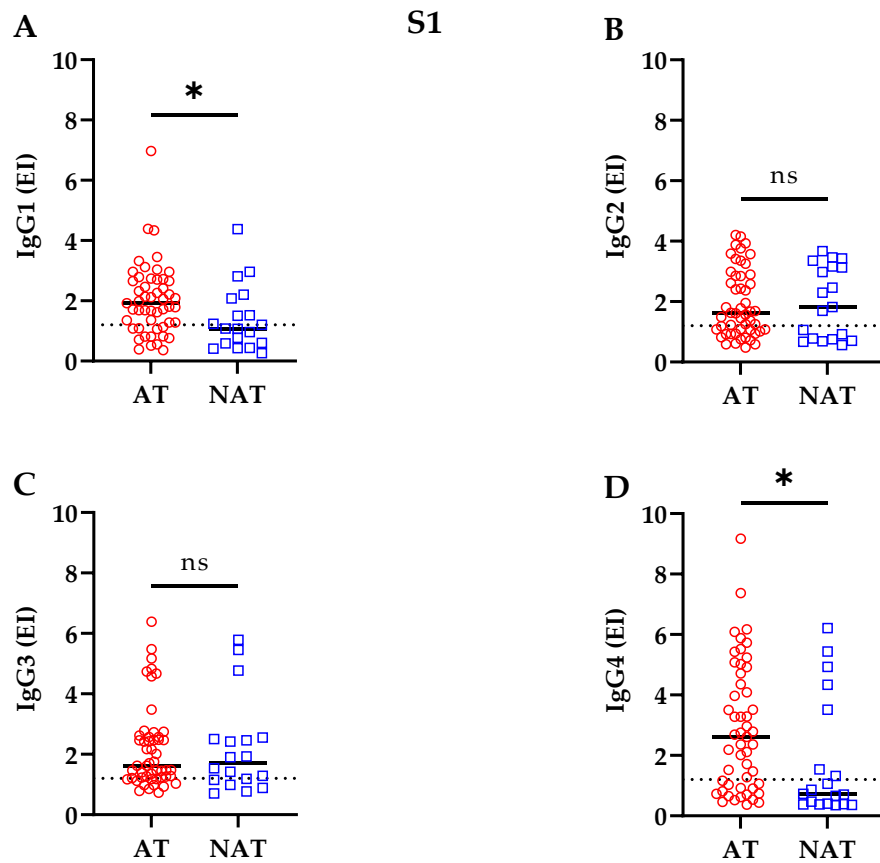


Figure 6. Levels of specific IgG1, IgG2, IgG3 and IgG4 subclasses for S1 in AT and NAT. (A). Levels of specific IgG1 for S1 in AT and NAT. (B). Levels of specific IgG2 for S1 in AT and NAT. (C). Levels of specific IgG3 for S1 in AT and NAT. (D) Levels of specific IgG4 for S1 AT and NAT. Values are reported as Enzyme-Linked Immunosorbent Assay (ELISA) index (EI). The black line indicates the median. The dashed line indicates the cutoff point. ns = not significant, * $p < 0.05$.

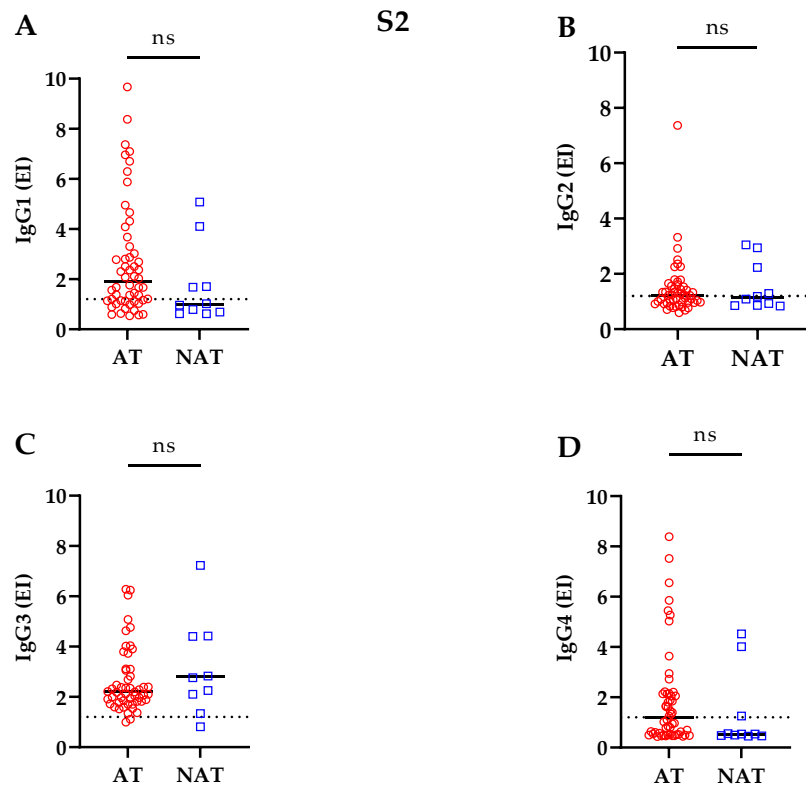


Figure 7. Levels of specific IgG1, IgG2, IgG3 and IgG4 subclasses for S2 in AT and NAT. (A). Levels of specific IgG1 for S2 in AT and NAT. (B). Levels of specific IgG2 for S2 in AT and NAT. (C). Levels of specific IgG3 for S2 in AT and NAT. (D) Levels of specific IgG4 for S2 in AT and NAT. The dashed line indicates the cutoff point. ns = not significant.

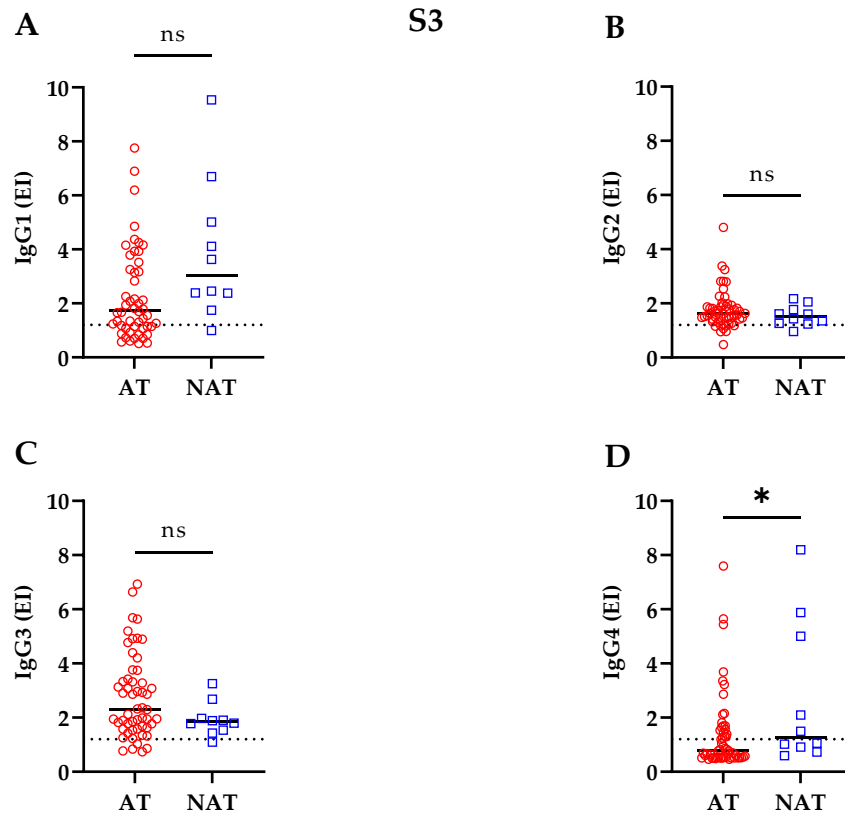


Figure 8. Levels of specific IgG1, IgG2, IgG3 and IgG4 subclasses for S1, S2, and S3 in AT and NAT. (A). Levels of specific IgG1 for S3 in AT and NAT. (B). Levels of specific IgG2 for S3 in AT and NAT. (C). Levels of specific IgG3 for S3 in AT and NAT. (D) Levels of specific IgG4 for S3 in AT and NAT. Values are reported as Enzyme-Linked Immunosorbent Assay (ELISA) index (EI). The black line indicates the median. The dashed line indicates the cutoff point. ns = not significant. * $p < 0.05$.

Based on these results, it was necessary to determine whether there was a correlation between the IgG subclasses. The analysis revealed a weak correlation between the specific concentrations of IgG1 and IgG4 for S1 ($r = 0.4$, $p = 0.0006$), but no other correlation was found (Figure 9A). In S2, IgG1 and IgG2 ($r = 0.68$; $p < 0.0001$), IgG1 and IgG4 ($r = 0.71$; $p < 0.0001$) and IgG2 and IgG4 ($r = 0.71$; $p < 0.0001$) were found to be moderately correlated with each other, but there was also a weak correlation between IgG1 and IgG3 ($r = 0.32$; $p = 0.009$) and IgG3 and IgG4 ($r = 0.27$; $p = 0.028$) (Figure 9B). Moderate correlations for S3 between IgG1 and IgG4 ($r = 0.63$; $p < 0.0001$) and weak correlations between IgG1 and IgG2 ($r = 0.42$; $p = 0.0004$), between IgG1 and IgG3 ($r = 0.35$; $p = 0.0040$), and between IgG2 and IgG3 ($r = 0.30$; $p = 0.019$) (Figure 9C).

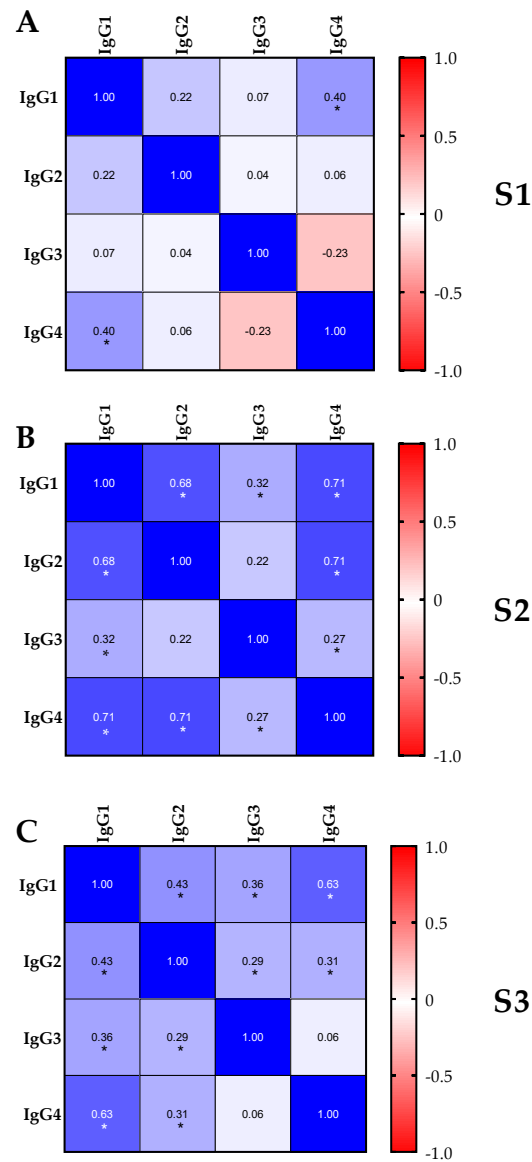


Figure 9. Correlation matrix between the levels of specific IgG subclasses against S1, S2 and S3. (A) Correlation matrix between the levels of the specific IgG subclasses against S1. (B) Correlation matrix between the levels of the specific IgG subclasses against S2. (C) Correlation matrix between the levels of specific IgG subclasses and S3. The correlation between the groups was calculated using the Spearman correlation. * = $p < 0.05$.

3. Discussion

Understanding the pollen antigens, particularly those derived from *Lolium multiflorum* (Lm), is crucial given its significance as a major allergen source in southern Brazil. The ability of this plant to elicit a type 2 (T2) immune responses emphasizes the importance of further elucidating its antigens and the mechanisms by which they trigger allergic reactions (Moreira et al., 2015; Correa et al., 2023). In this study, our objective was to develop an effective method for separating and concentrating pollen antigens, particularly glycoconjugates, to better understand their role in respiratory allergic reactions. Building upon previous research demonstrating the importance of pollen antigens in allergic responses, our approach aimed to provide novel insights into the immunological mechanisms underlying allergic reactions induced by Lm pollen.

The pivotal role of glycoconjugates, including glycoproteins, in allergy sensitization, diagnosis, and treatment, emphasizes their importance in this field (Visez et al., 2021; Xu et al., 2023). The identification and characterization of these compounds offer valuable insights into the mechanisms underlying pollen allergy, particularly in the case of *Lolium multiflorum*, because their components are poorly understood (Correa et al., 2023). It is noteworthy that some glycoproteins exhibit cross-reactivity with cross-carbohydrate determinants (CCDs) present in a variety of natural allergens, complicating diagnosis, and treatment strategies (Homann; Schramm; Jappe, 2017; Lu et al., 2022). Our study aimed to elucidate the specific interactions between glycoconjugates and the immune system in pollen allergy, contributing to improved diagnostic and therapeutic approaches for allergic diseases.

We used affinity chromatography with a Con A, a lectin derived from *Canavalia ensiformis*, which selectively binds carbohydrates such as mannose and glucose (Resende et al., 2019), to isolate S1 extract, of which fractions S2 and S3 was derived. These extracts and fractions were used to investigate the potential differences in IgG antibody binding between atopic (AT) and non-atopic (NAT) individuals. We observed that the specific IgG reactivity in serum was lower against S2 than against the other extracts, indicating that non-binding column components have a lower capacity to induce such antibodies. Furthermore, our study observed detectable levels of anti-Lm IgG in all subjects. IgG plays a fundamental role in immune tolerance by activating induced regulatory cells (iTreg) and facilitating the migration of specialized dendritic cells to lymph nodes, inducing Treg naive in iTreg cells (Gocki; Bartuzi, 2016). Additionally, IgG provides protection role to offspring through transmission via plasma, breast milk, and umbilical cord blood, thus, offspring of mothers with high levels of allergen-

specific IgG are not sensitized until the age of 5 years (Shamji et al., 2021b). Here, we found similar detectable levels of high-avidity IgG in both the AT and NAT groups, indicating that antigen exposure can elicit a strong IgG response even in non-atopic individuals, which is consistent with previous observations (Hochwallner et al., 2011).

Because of the different roles of the IgG subclasses in allergies (Macglashan Jr; Alvarez-Arango; Tversky, 2021), we tested their levels against the three different extracts. IgG1 is of particular interest because of its dual function in allergic reactions. While some authors found no difference in IgG1 levels between allergic and non-allergic individuals, others have suggested a potential role for IgG1 in effectively inhibit basophil activation (Lewandowicz-Uszyńska; Pasternak; Pentoś, 2021; Zinkhan et al., 2022; Nelson; Wu, 2023). The results of our study showed variations in the levels of IgG1 in comparison to other types of IgGs when tested against S2 and S3, but not S1. This suggests that there may be variances in the reactivity of IgG1 to various pollen extracts. Nevertheless, there were no discernible disparities in the levels of IgG1 when analyzed individually between S1, S2, and S3 within the identical group. Interestingly, we observed differences in IgG1 levels between the AT and NAT groups for S1, but not for S2 and S3, suggesting a possible diagnostic tool for distinguishing between allergic and non-allergic individuals. In addition, we observed correlations between IgG1 and IgG4 for S1, between IgG1, IgG2, IgG3 and IgG4 for S2 and S3, demonstrating the potential of these fractions for the detection IgG subclasses in serum and their relevance as allergy marker. Therefore, although blocking activity tests using functional assays such as IgE-Fab or basophil activation are necessary, it has been shown that there is an increase of up to 100 times in IgG antibody levels after AIT (Tan; Layhadi; Shamji, 2022), the use of the extracts found here, with good efficacy in detecting IgGs, may favor their use in detecting markers of efficacy of pollen extract therapy.

Limited knowledge is available on the immune mechanisms of IgG2 and IgG3 in allergic diseases. Although previous studies have been shown their inhibitory effects on histamine and other mediators in basophils and mast cells by activating FcγRIIb (CD32) and inhibiting FcεRI, as well as their ability to bind to allergens and prevent the binding of IgE. Notably, IgG3 has demonstrated more effective activation of CD32 than IgG2 and IgG1 in reactions with antigens and antibodies in human basophils. In addition, IgG2 has been shown to increase following sublingual immunotherapy (SLIT) against grass pollen (Macglashan; Hamilton, 2016; Burton et al., 2018b; Burton et al., 2018a; Heeringa et al., 2020). In our study, we observed consistent reactivity of IgG2 and IgG3 against S1, but without statistical

differences. Against S2, IgG2 had lower reactivity than IgG3 but not compared to IgG1 and IgG4, while IgG3 had higher reactivity than IgG1 and IgG4. The reactivity of IgG2 for S3 was lower than that of IgG3, but it was higher than that of IgG4, and it did not differ significantly from IgG1. IgG2 showed lower reactivity against S2 compared to other extracts, but no difference between S1 and S3. In contrast, IgG3, in comparison to S1, had a weaker reactivity against S2, and there was no distinction in immunoglobulin reactivity between S1 and S3, or between S2 and S3. Additionally, no difference in IgG2 and IgG3 reactivity levels between the AT and NAT groups was found. IgG2 and IgG3 did not correlate with any antibody in S1. IgG2 correlated positively with all IgG subclasses in S3 and with IgG1 and IgG4 in S2, while IgG3 correlated with IgG1 and IgG4 in S2 and with IgG1 and IgG2 in S3. According to this evidence, S2 and S3 exhibit diverse specificities for IgG subclasses and a strong binding with these immunoglobulins, which could prove helpful in clinical diagnosis and immunotherapy (Heeringa et al., 2020; Durham; Shamji, 2023). Moreover, it is noteworthy that both the AT and NAT groups produced similar levels of Lm-specific IgG2 and IgG3, which makes this the first study to test the detection of these antibodies against Lm. Thus, additional studies are needed to examine the impact of these immunoglobulins on patients undergoing AIT.

As previously mentioned, IgG4 antibodies are closely associated with allergic diseases and AIT. They have the specificity to bind to FcεRI and thus compete with the binding of IgE to the allergen and prevent the release of preformed mediators by basophils and mast cells. In addition, IgG4 does not activate complement and binds weakly to Fcγ receptors, giving this immunoglobulin a blocking effect (Platts-Mills et al., 2021; Rispens; Huijbers, 2023). Here, it was found that the concentration of IgG4 was lower than that of IgG1 and IgG3, but not that of IgG2 against S2. Moreover, IgG4 was found to have a significantly lower antibody level against S3, but there was no discernible difference from S1 with respect to the subclasses of IgG. It is noteworthy that S1 had higher IgG4 reactivity than any other extract, but there was no distinction between S2 and S3. According to the results, the reactivity of specific IgG4 was different between AT and NAT in S1 and S3, but not in S2, suggesting the potential use of both S1 and S3 in Lm allergy diagnosis and treatment. A positive correlation was also observed between the reactivities of IgG4 and IgG1 specific for S1, between IgG4 and all subclasses of IgGs specific for S2 and between IgG4 and IgG1 and IgG2 specific for S3. It is important to mention that no subjects with AIT were used in this study, which could increase the level of IgG4 antibodies specific to S1, as well as in S2 and S3, as already observed in the literature (Heeringa et al., 2020; Figo et al., 2023).

In summary, our study demonstrates the ability of fractions S2 and S3 to bind specific antibodies, highlighting their potential for diagnosing and treating Lm-sensitized patients. These findings suggest that fractions S2 and S3 could serve as viable alternatives to crude extract (S1) in clinical practice, offering potential benefits in terms of improved diagnostic and treatment. Moreover, our results emphasize the importance of considering the prolonged use of extracts without clinical relevance, which may inadvertently promote new sensitization to allergens. Moving forward, future analyses should focus on verifying IgE reactivity using immunoenzymatic techniques and conducting structural elucidation of the proteins in fractions S2 and S3. These efforts will enhance our understanding of their functions and pave the way for developing more effective diagnostic and therapeutic strategies for pollen allergy.

4. Materials and Methods

4.1 Lolium multiflorum protein extraction

L. multiflorum was identified and registered by the Herbarium of the University of Caxias do Sul under the model HUUCS 56105, available at "<https://specieslink.net/search>". Grass pollen was collected in Caxias do Sul, Rio Grande do Sul, Brazil during the flowering time of the plant from September to November. The pollen was sieved (pore 0.50 mm/Tyler 32, Granutest-Telastem Sieves, Brazil) to remove possible contaminants. After drying, the pollen was placed on white paper to enhance its purity. The collected material was then sent to the Allergy and Clinical Immunology Laboratory of the Federal College of Uberlandia (UFU).

Protein extraction from Lm pollen was performed according to the protocol established by our laboratory, with modifications (Sopelete et al., 2006). In brief, approximately 1 g of Lm pollen was homogenized in 9 mL of 0.01 M phosphate-buffered saline (PBS) with a pH of 7.2, supplemented with the protease inhibitor PMSF (2 mmol). The resulting mixture was macerated under ice conditions and then shaken overnight at 4°C. The mixture was then centrifuged at 15,000 x g for 30 minutes at 4°C. Next, the collected supernatant was dialyzed overnight with PBS. After dialysis, the extract was centrifuged again and the resulting supernatant was collected, aliquoted and stored at -70°C for later use in assays. The total protein concentration of the Lm extract was determined using the bicinchoninic acid (BCA) protein quantification method as described by Smith et al. (Smith et al., 1985). After storage, the extract was labelled crude extract.

4.2. *Concanavalin A Affinity chromatography*

Affinity chromatography was carried out in accordance with the manufacturer's instructions, as previously mentioned (Resende et al., 2019). In brief, a Con A-Sepharose 4B matrix (5 ml, GE Healthcare, Uppsala, Sweden) was packed, washed (1 mmol MgCl₂ and 1 mmol CaCl₂ in PBS) and equilibrated (20 mmol Tris, Ph 7.4 containing 0.5M NaCl). 2 mg/mL of S1 were placed in the column for 2h to allow binding of the binding portion. Subsequently, washing with equilibration buffer was carried out until the complete elution of the S2, measured by ultraviolet absorbance (UV) at 280 nm. After that, an elution buffer (with 0.5 M glucose) was applied onto the column. Eluate collection was continued until the complete volume of the S3 was completely applied, measured by UV absorbance at 280 nm.

4.3. *Subjects*

The study involved the recruitment of individuals, male and female, aged between 18 and 60 years with or without typical symptoms for allergic rhinitis and asthma in the cities of Caxias do Sul, RS, and Uberlândia, MG. Subjects gave their informed consent and the Ethics Committee in Human Research of the Federal University of Uberlândia CAAE: 09978918.6.0000.5152 (protocol number 3.342.127) approved this study. No patients were receiving treatment for AIT.

4.4. *Blood samples*

Approximately 10 mL of blood was collected from each participant. The serum samples were obtained through centrifugation. The resulting sera were divided into aliquots and stored at -20°C until the serologic tests were performed.

4.5. *Skin Prick Test (SPT) and specific-IgE*

The SPT for the assessment of immediate hypersensitivity was determined as described elsewhere (Sopelete et al., 2006). The Lm pollen extract was used for the test, with a negative control (saline solution) and a positive control (histamine hydrochloride at 10 mg/mL). Individuals having a positive SPT result more than 3 mm or EI higher than 1.2 for Lm extract. The EI value was calculated by dividing the optical density (OD) of the sample by the cutoff.

The cutoff value was established by calculating the mean of two negative controls and then adding three standard deviations. Individuals from Caxias do Sul, RS and Uberlândia, MG, who showed negative results in both SPT and EI for Lm extract were selected in the NAT group.

4.6. *Specific-IgG*

IgG specific antibodies to Lm was conducted based on the literature (Pajuaba; Silva; Mineo, 2010), with adaptations. High-affinity microplates were coated with S1, S2, or S3 at a concentration of 5 µg/mL overnight. Subsequently, the plates were washed with 0.05% PBS-T. The washing procedure followed all other steps of the assay. Sera from AT and NAT patients were diluted in 1% PBS-T-BSA at a ratio of 1:64 and incubated for 1 hour at 37°C in a humid chamber. They were then incubated with the anti-IgG-peroxidase complex (1:10,000) for 1 hour at 37°C in a humid chamber. After the final wash step, the TMB chromogenic solution (3,3', 5,5; -tetramethylbenzidine) (Thermo Fisher Scientific Inc., MA, USA) was added for 10 minutes, followed by the addition of H₂SO₄ (2N) solution to halt the reaction. Optical density (OD) was determined at 405 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA). The results were expressed in terms of optical density (OD).

4.7. *Avidity ELISA*

IgG antibodies' avidity to Lm was performed, with minor modifications (Pajuaba; Silva; Mineo, 2010). High-affinity microplates (Corning) were coated, blocked, and washed following the IgG ELISA protocol. Sera from AT and NAT patients were diluted in 1% PBS-T-BSA at a ratio of 1:256 and incubated in quadruplicate for 1 hour at 37 °C in a humid chamber. Subsequently, one duplicate was incubated for 10 minutes with 6M urea at room temperature, while the other duplicate was incubated only with PBS-T-BSA 1%. The plate was then washed four times with PBS-T and incubated with the anti-IgG-peroxidase complex (1:10,000) for 1 hour at 37°C in a humid chamber. After the final wash step, the TMB (3,3', 5,5; - tetramethylbenzidine) chromogen solution (Thermo Fisher Scientific Inc., MA, USA) was added for 10 minutes, followed by the addition of H₂SO₄ (2N) solution. Optical density (OD) was determined at 450 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA). Results were expressed in terms of OD. The results were further expressed as

an avidity index (AI) and calculated for each serum sample dilution according to the following formula: $AI = (OD \text{ of the sample with urea} / OD \text{ without urea}) * 100$.

4.8. *Specific-IgG1 and IgG4*

IgG1 and IgG4 subclasses specific antibodies to Lm were measured using the ELISA, as previously described (Moreira et al., 2015). High affinity plates (Corning 96-well EIA/RIA) were coated with S1, S2 or S3 at a concentration of 20 µg/ml overnight at 4 °C. The plates were then rinsed with phosphate buffer containing 0.05% Tween (PBS-T) and blocked with non-fat milk bovine (M) in 1% PBS-T for 1 hour at room temperature. Subsequently, 1% PBS-T-M was used as a diluent, washing with PBS-T after each stage of the process. The serum samples were diluted 1:5 and incubated for two hours at 37°C in a humid environment. Subsequently, the biotinylated human anti-IgG1 or anti-IgG4 secondary antibody (1:1,000; Sigma-Aldrich) was added for 1 hour at 37°C. In the final stage, the streptavidin-peroxidase conjugate was added at a dilution of 1:1,000 for 30 minutes at room temperature. The reaction was disclosed by the addition of 2,2'-diazine of ethylbenzothiazoline sulfonic acid (ABTS; Sigma-Aldrich) to 0.01M in 0.07M citrate-phosphate buffer, pH 4.2, containing 0.03% hydrogen peroxide. Optical density (OD) was evaluated at 405 nm using an Epoch Microplate Spectrophotometer (Biotek Instruments, Inc., VT, USA). The results were expressed in EI.

4.9. *Specific-IgG2 and IgG3*

IgG2 and IgG3 subclasses specific antibodies to Lm and fractions were measured using ELISA, as previously described (Moreira et al., 2015). Plates were coated with S1, S2 or S3 at a concentration of 20 µg/ml overnight at 4 °C. The plates were then rinsed with phosphate salt buffer containing 0.05% Tween (PBS-T) and blocked with bovine soroalbumin (BSA) at 1% PBS-T for 1 hour at room temperature. Following this, 1% PBS-T-BSA was utilized as a diluent, and PBS-T was utilized for washing after each stage of the process. The serum samples were diluted 1:5 and kept incubating for two hours at 37 °C with humidity. Subsequently, the biotinylated human anti-IgG2 or anti-IgG3 secondary antibody (1:1,000; Sigma-Aldrich) was added for 1 hour at 37 °C. In the final stage, the streptavidin-peroxide conjugate was added at a dilution of 1:1,000 for 30 minutes at room temperature. The reaction was disclosed and analyzed, as previously.

4.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.4 software (GraphPad Software, Inc. CA, USA). The differences between the gender and wheal size were analyzed by Fisher's exact test. Student's t-test, Mann-Whitney test, ANOVA or Kruskal-Wallis's test were used for age, mean wheal size, avidity index (AI), optical density (OD) and ELISA index (EI). All results were considered statistically significant when $p < 0.05$.

Supplementary Materials:

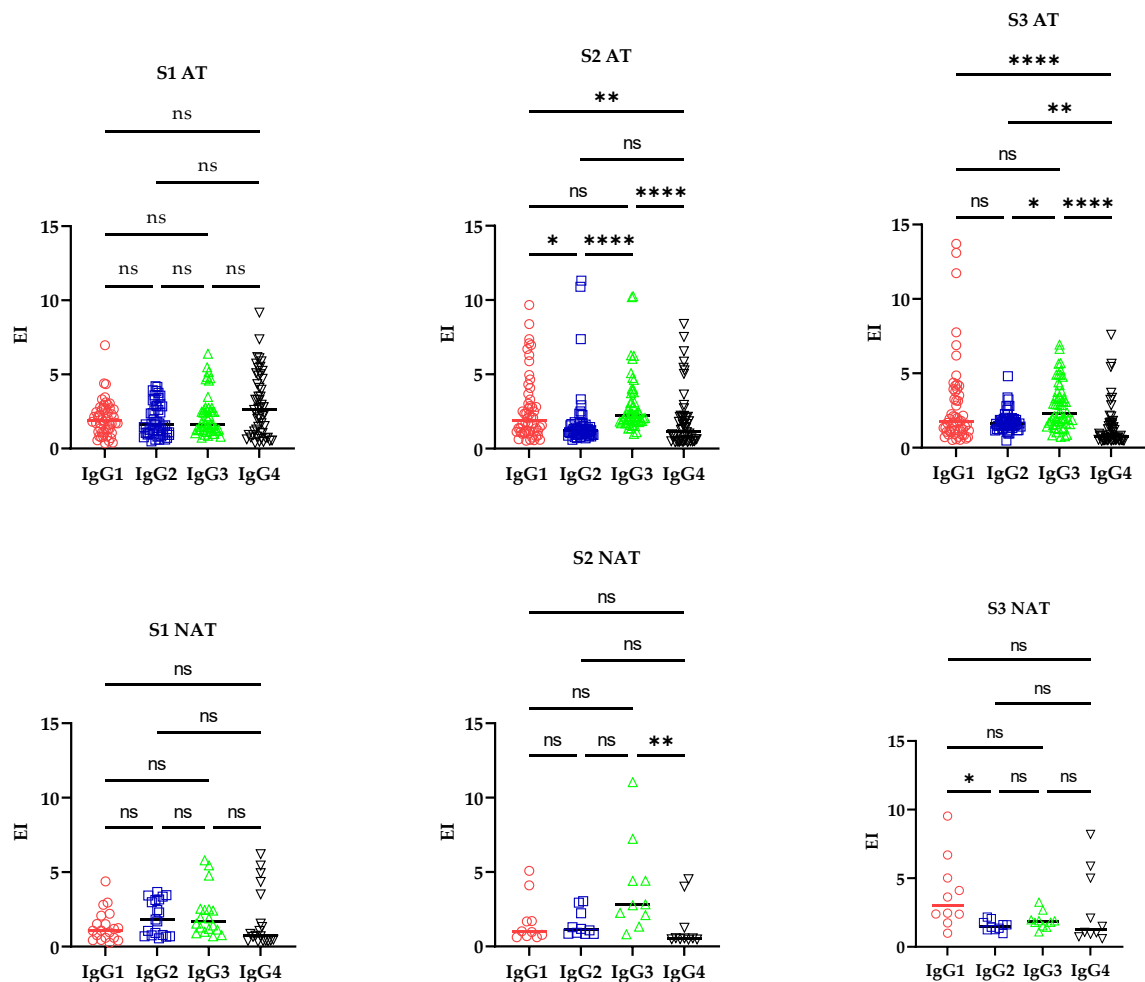


Figure S1. Levels of specific IgG subclasses for S1, S2, and S3 in AT and NAT groups. (A). The black line indicates the median. Values are reported as ELISA index (EI). Differences between groups were analyzed using Kruskal-Wallis test. ns = not significant, * = 0.05; ** = 0.01; **** = 0.0001.

Author Contributions: “Conceptualization, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; methodology, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; validation, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; formal analysis, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; investigation, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; resources, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; data curation, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; writing—original draft preparation, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; writing—review and editing, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; visualization, A.S.C., L.A.R.O., T.A.J., B.P.T., M.M.B., F.A.M., R.O.R., J.S.M., and E.A.T; supervision, J.S.M., R.O.R. and E.A.T; project administration, J.S.M., R.O.R. and E.A.T; funding acquisition, J.S.M., R.O.R. and E.A.T. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: The research project was approved by the Ethics Committee for Research of the Federal College of Uberlândia under protocol number 3.342.127. Participants were required to sign the informed consent form (ICF) to participate in the study.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Considerações Finais

A presente tese investigou profundamente os alérgenos do pólen da gramínea *Lolium multiflorum* (Lm), com foco na caracterização de seus componentes e nas aplicações clínicas para diagnóstico e tratamento de doenças alérgicas respiratórias. A partir dos três manuscritos que compõem este trabalho, podemos destacar alguns pontos essenciais e conclusivos:

1) No primeiro manuscrito, demonstrou-se que a cromatografia de troca iônica, especificamente a fração S2 isolada, foi eficaz na identificação de alérgenos clinicamente relevantes em pacientes sensibilizados. Tanto o extrato S1 quanto o S2 apresentaram reatividade semelhante de IgE e IgG4, sugerindo que ambos são adequados para distinguir indivíduos atópicos e não atópicos. A análise por espectrometria de massas revelou a presença de alérgenos do grupo 1 e 5, reforçando a importância do S2 como candidato promissor para imunoterapia específica.

2) No segundo manuscrito, a cromatografia de afinidade com Concanavalina A permitiu o isolamento das frações não ligada e ligada, que mostraram perfis eletroforéticos distintos e reatividade significativa de IgE em pacientes atópicos. Essas frações demonstraram efeitos inibitórios no extrato bruto de Lm, indicando potencial tanto para diagnóstico quanto para tratamento de alergias respiratórias induzidas por Lm.

3) No terceiro manuscrito, investigou-se a resposta de anticorpos IgG e suas subclasses (IgG1, IgG2, IgG3 e IgG4) ao extrato bruto de Lm, bem como às frações S2 e S3. Observou-se que as frações S2 e S3 apresentaram diferenças na reatividade de IgG entre pacientes atópicos e não atópicos, destacando-se especialmente as variações significativas nos índices ELISA entre as subclasses de IgG em ambas as frações. Esses resultados indicam que S2 e S3 são promissores para o diagnóstico e tratamento de doenças alérgicas mediadas por IgG relacionadas ao pólen Lm.

Em conjunto, os estudos realizados nesta tese fornecem *insights* valiosos para a compreensão dos mecanismos imunológicos envolvidos nas alergias ao pólen de Lm, além de estabelecerem fundamentos sólidos para o desenvolvimento de abordagens terapêuticas mais direcionadas e eficazes. O avanço no conhecimento sobre os alérgenos específicos e suas interações com o sistema imunológico é crucial para melhorar o manejo clínico e a qualidade de vida dos pacientes afetados por essas condições alérgicas complexas.

Conclusões

CAPÍTULO I

- A cromatografia de troca iônica foi eficaz no isolamento da fração S2 contendo os componentes principais com relevância clínica em pacientes sensibilizados a alérgenos de polens da gramínea *L. multiflorum*.
- O extrato S1 e a fração S2 foram igualmente eficazes na separação de indivíduos atópicos e não atópicos do pólen de *L. multiflorum* utilizando as técnicas de ELISA e Immunoblotting;
- A sensibilidade e a especificidade dos testes ELISA para identificar anticorpos IgE específicos frente a S1 e S2 foram similares;
- O componente imunodominante mais importante foi a banda de 32 kDa presente em ambos os extratos S1 e S2, que demonstrava reatividade aos anticorpos IgE e IgG4 específicos para os polens de *L. multiflorum*.
- A análise de espectrometria de massas revelou que a banda de 32 kDa contém alérgenos do grupo 1 e 5, enquanto a banda de 58 kDa contém uma proteína hipotética de *Oryza sativa*.
- A fração S2, que contém os alergênicos principais clinicamente relevantes, pode ser uma alternativa ao extrato total (S1) para diagnóstico in vivo (TCP) ou in vitro (testes sorológicos) e para imunoterapia específica com alérgenos.

CAPÍTULO II

- A cromatografia de afinidade foi eficaz no isolamento de duas frações, uma não ligada e outra ligada a coluna de ConA, contendo os componentes principais com relevância clínica em pacientes sensibilizados a alérgenos de polens da gramínea *L. multiflorum*.
- Os perfis eletroforéticos do extrato bruto de Lm, da fração não ligada e da fração ligada diferiram nos padrões de bandas, indicando componentes proteicos distintos dentro de cada fração.
- O extrato bruto de polens de Lm, a fração não ligada e a fração ligada foram eficazes na separação de indivíduos atópicos e não atópicos ao pólen de *L. multiflorum* utilizando as técnicas de ELISA e Immunoblotting;
- A reatividade da IgE foi avaliada usando técnicas de ELISA e Immunoblotting, revelando bandas específicas no soro de indivíduos com AT contra extrato bruto de Lm,

fração não ligada e fração ligada, enquanto nenhuma reatividade foi observada no soro de NAT.

- As bandas de 50, 33 e 26 kDa foram reativas no Immunoblotting contra o extrato bruto de polens de Lm, a banda de 33 kDa foi reativa contra a fração não ligada a coluna de ConA, enquanto as bandas de 58, 33 e 31 kDa foram reativas contra a fração ligada a coluna de ConA.
- As frações não ligadas e ligadas obtidas por cromatografia de afinidade com Concanavalina A mostraram efeitos inibitórios no extrato bruto de Lm, sugerindo que essas frações poderiam ser candidatas potenciais para o tratamento e diagnóstico de alergias respiratórias causadas por Lm.

CAPÍTULO III

- A cromatografia de afinidade foi eficaz no isolamento de duas frações, S2 e S3, contendo os componentes com grande relevância para pacientes sensibilizados a alérgenos de polens da gramínea *L. multiflorum*.
- Foram evidenciadas diferenças na reatividade de anticorpos IgG específicos entre os dois grupos, particularmente nas frações S2 e S3.
- Foi demonstrado que em todos os pacientes testados, o nível de IgG de alta avidéz superou os 50%.
- Houve diferenças significativas no índice ELISA (EI) ao comparar diferentes subclasses de IgG nas frações S2 e S3. Em S2, variações foram observadas entre IgG1 com IgG3, IgG1 com IgG4, IgG2 com IgG3 e IgG3 com IgG4. Da mesma forma, em S3, diferenças foram encontradas entre IgG1 e IgG4, IgG2 e IgG3, IgG2 e IgG4 e IgG3 e IgG4.
- As correlações entre as subclasses de IgG variaram entre as diferentes frações. Para S1, uma correlação fraca foi observada entre IgG1 e IgG4, enquanto correlações moderadas foram observadas para S2 entre IgG1 e IgG2, IgG1 e IgG4 e IgG2 e IgG4. Além disso, houve correlações fracas entre IgG1 e IgG3 e entre IgG3 e IgG4. Para o extrato S3, correlações moderadas foram observadas entre IgG1 e IgG4.
- No geral, os resultados sugerem que tanto S2 quanto S3 são extratos promissores para o diagnóstico de anticorpos IgG e suas subclasses, bem como para o tratamento de condições alérgicas induzidas pela exposição ao pólen Lm.

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