UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

EPÍTOPOS MIMÉTICOS E AUTOANTÍGENOS APLICADOS AO IMUNODIAGNÓSTICO DA ARTRITE REUMATOIDE E ARTRITE IDIOPÁTICA JUVENIL OLIGOARTICULAR E POLIARTICULAR

Aluno: Galber Rodrigues Araujo

Orientador: Prof. Dr. Carlos Ueira Vieira

UBERLÂNDIA - MG
2015

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

EPÍTOPOS MIMÉTICOS E AUTOANTÍGENOS APLICADOS AO IMUNODIAGNÓSTICO DA ARTRITE REUMATOIDE E ARTRITE IDIOPÁTICA JUVENIL OLIGOARTICULAR E POLIARTICULAR

Aluno: Galber Rodrigues Araujo

Orientador: Prof. Dr. Carlos Ueira Vieira

Tese apresentada à Pós-Graduação em Genética e Bioquímica, Universidade Federal de Uberlândia, como parte dos requisitos para obtenção do título de Doutor em Genética.

UBERLÂNDIA - MG
2015

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

A663e
2015

Araujo, Galber Rodrigues,
Epítopos miméticos e autoantígenos aplicados ao Imunodiagnóstico da artrite reumatoide e artrite idiopática Juvenil oligoarticular e poliarticular / Galber Rodrigues Araujo. - 2015.
108 f. : il.

Orientador: Carlos Ueira Vieira.
Tese (doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica.
Inclui bibliografia.

1. Genética - Teses. 2. Artrite idiopática juvenil - Teses. 3. Phage Display - Teses. 4. Antígenos - Teses. I. Vieira, Carlos Ueira. II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 577.1

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

EPÍTOPOS MIMÉTICOS E AUTOANTÍGENOS APLICADOS AO IMUNODIAGNÓSTICO DA ARTRITE REUMATOIDE E ARTRITE IDIOPÁTICA JUVENIL OLIGOARTICULAR E POLIARTICULAR

ALUNO: Galber Rodrigues Araujo

COMISSÃO EXAMINADORA

Presidente: Dr. Carlos Ueira Vieira

Examinadores: Dr. Carlos Herique Martins da Silva

Dr. Luiz Ricardo Goulart

Dr. Marcelo De Franco

Dr. Marcelo Henrique Napimoga

Data da Defesa: 20/07/2015

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Dissertação/Tese foram contempladas

_____

Carlos Ueira Vieira

“A vitória está reservada para aqueles que estão dispostos a pagar o preço.”

Sun Tzu

Dedicatória

Dedico esta tese a todos os pacientes com artrite reumatoide e artrite idiopática juvenil que doaram um pouquinho de si para a realização deste trabalho.

Agradecimentos

A Deus por ter me acompanhado pelos caminhos que escolhi e por ter me permitido aprender, crescer e me fortalecer ao lado de pessoas incríveis que dividiram esta jornada comigo.

Ao meu orientador Prof. Dr. Carlos Ueira Vieira por ter acreditado no meu trabalho, por ter me dado a liberdade de ir atrás dos meu objetivos, assim como por tê-los apoiado. Muito obrigado por tudo que me proporcionou a aprender em todo esse tempo.

Ao Prof. Dr. Luiz Ricardo Goulart, muito obrigado por ter disponibilizado parte da estrutura necessária para a realização desse trabalho, por ter me ajudado em vários processos durante a etapa de escrita dos artigos, por ter me disponibilizado atenção sempre que precisei e por acreditar no meu potencial.

Aos professores Dr. João Gonçalves e Dr. João Eurico Fonseca por terem me recebido tão bem durante o meu período de doutorado sanduiche na Universidade de Lisboa, Portugal, e por terem contribuído muito para a finalização deste trabalho.

Aos meus pais José Marinho e Maria de Fátima, e a minha irmã Naiane por formarem o mais importante pilar que sustenta a minha estrutura. Obrigado por sempre me incluírem em suas orações, tenho recebido todas as bênçãos. Serei eternamente grato pela educação que recebi e pelos exemplos de honestidade, caráter e fé.

À Emilia, grande amiga, praticamente uma irmã, por ter estado ao meu lado sempre que precisei e por ter sido uma grande parceira em muitos experimentos, estudos e histórias. Muito obrigado por crescer junto comigo e por me dar a honra de ser seu amigo.

À Patrícia Tiemi, ser humano extraordinário, que me acalma com o simples fato de estar perto. Patchinha, muito obrigado pela paciência e por ter me inspirado a sempre querer saber mais sobre as coisas.

À Angela, mãe do príncipe Miguel, por fazer parte da minha vida, por me permitir fazer parte da sua, e por ter sempre a palavra certa na hora certa. Muito obrigado por tudo que me fez refletir após nossas sempre longas conversas. O simples fato de saber que você está feliz, me deixa muito feliz.

À Juliana e Thaise, por terem dado mais cor a muitos almoços regados a muitas conversas nas quais sempre me beneficiei. Muito obrigado pela amizade, professoras.

À Paula Santos, por toda a cumplicidade e alegria que sempre dividiu comigo.

A todos os amigos do Laboratório de Nanobiotecnologia incluindo: Aline, Bruna, Claudia, Jéssica, João Paulo, Karina, Lara, Larissa, Luciana, Mariana, Mayara (Chay), Patrícia Terra (companheira de viagem padrão ouro), Rafael, Victor e Yara, que, em algum momento, dividiram comigo momentos de angústia ou de alegria, e também muitas cumplicidades. Saibam que os admiro muito e que estarei na torcida para vê-los sempre nas minhas buscas ao PubMed.

Ao Cristian (Cris), por dividir a mesa de estudos comigo (inclusive neste momento), por me ensinar a ser mais cuidadoso, menos ansioso e por tornar meus dias em Lisboa mais vivos. Muito obrigado pelo companheirismo e por ter cuidado de mim sempre que precisei.

À professora Ana Maria Bonetti, dona de um coração enorme, pelo carinho que sempre teve comigo, pelo exemplo de dedicação ao trabalho e por toda experiência de vida e pesquisa que comigo compartilhou.

Aos amigos portugueses Adriano Gigante, Catarina Santos, Cátia Cantante, Luis Ferreira, Mariana Santa-Marta, Paula Matos, Pedro Perdigão, Raquel Antunes,

Sofia Fernandes, Sofia Pombo, Sofia Romano e Vasco Gonçalves, que conquistei durante meu período na Universidade de Lisboa. Muito obrigado por terem deixado o inverno mais suportável, o verão mais agradável e por terem me mostrado que compartilhamos muito mais do que apenas parte de nossa História.

Ao Dr. Carlos Henrique Martins da Silva e à Dra. Karla Pereira Fernandes do Hospital de Clínicas da Universidade Federal de Uberlândia, muito obrigado pela valiosa ajuda na obtenção das amostras biológicas de pacientes com artrite idiopática juvenil, que foram muito importantes para os trabalhos desenvolvidos pelo nosso grupo de pesquisa.

Aos membros da banca examinadora, criteriosamente escolhidos, por terem aceitado fazer parte da minha defesa e que, seguramente, irão acrescentar muitos dos seus conhecimentos para enriquecer a versão final deste trabalho.

À todos os membros da equipe do Biobanco do Instituto de Medicina Molecular do Centro Acadêmico de Medicina de Lisboa, muito obrigado por todo o tempo disponibilizado para separar amostras de soro e preencher planilhas com dados relevantes dos pacientes.

Aos técnicos do Biotério da Universidade Federal de Uberlândia pelo apoio na manutenção e manipulação dos camundongos utilizados nos experimentos *in vivo*.

À CAPES pelo apoio financeiro nos meus quatro anos de doutorado, inclusive no período no exterior com bolsa pelo programa Ciência sem Fronteiras.

À diretora Sandra Morelli, ao coordenador Carlos Ueira Vieira, à secretária Janaína, aos técnicos administrativos Madson e Ismair e aos Professores do Programa de Pós-Graduação em Genética e Bioquímica por trabalharem ardualmente em favor de um Programa cada vez melhor.

LISTA DE ABREVIATURAS E SÍMBOLOS

A	Alanina (aminoácido)
aa	Aminoácidos
Ac	Anticorpo
AI	Índice de Avidez
AIJ	Artrite Idiopática Juvenil
Anti-CAIII	Anticorpo Anti-Anidrase Carbônica III
Anti-CCP	Anticorpo Anti-Peptídeos Citrulinados Cíclicos
AR	Artrite Reumatoide
AUC	*Area Under the Curve*
BSA	Albumina do Soro Bovino
CA	Anidrase Carbônica
cDNA	Ácido Desoxirribonucléico complementar
CIA	Artrite induzida por colágeno
CII	Colágeno Tipo II
CRP	Proteína C-Reativa
D	Ácido aspártico (aminoácido)
DBA	*Dilute Brown non-Agout*
DNA	Ácido Desoxirribonucléico
E	Ácido glutâmico (aminoácido)
EDTA	Ácido etilenodiamino tetra-acético
ELISA	Ensaio Imunoenzimático
ESR	Velocidade de hemo-sedimentação
Fab	Fragmento ligante de antigeno
Fc	Região cristalizável
FR-	Fator Reumatoide negativo
FR+	Fator Reumatoide positive
G	Glicina (aminoácido)
G	Gramas
H	Histidina (aminoácido)
H	Hora

HLA	Antígeno Leucocitário Humano
I	Isoleucina (aminoácido)
Ig	Imunoglobulina
IgA	Imunogobulina A
IgD	Imunogobulina D
IgE	Imunogobulina E
IgG	Imunogobulina G
IgM	Imunogobulina M
IMM	Instituto de Medicina Molecular
K	Lisina (aminoácido)
L	Leucina (aminoácido)
LSE	Lúpus Sistêmico Eritematoso
M	Metionina (aminoácido)
M	Molar
mg	Miligrama
mL	Mililitro
mM	Milimolar
MS	Espectrometria de massas
N	Asparagina (aminoácido)
ng	Nanograma
NK	Células *Natural Killer*
NSAID	Anti-inflamatório não esteroidais
ºC	Graus Celsius
P	Prolina (aminoácido)
PD	*Phage Display*
PIII	Proteína III do capsídio de bacteriófagos filamentosos
PIX	Proteína IX do capsídio de bacteriófagos filamentosos
PMSF	Fluoreto de fenilmetilsulfonil
PVI	Proteína VI do capsídio de bacteriófagos filamentosos
PVII	Proteína VII do capsídio de bacteriófagos filamentosos
PVIII	Proteína III do capsídio de bacteriófagos filamentosos
Q	Glutamina (aminoácido)
R	Arginina (aminoácido)

RI	Índice de Reatividade
ROC	*Receiver Operating Characteristic*
RT	Temperatura ambiente
S	Serina (aminoácido)
T	Treonina (aminoácido)
UFU	Universidade Federal de Uberlândia
V	Valina (aminoácido)
VH	Domínio Variável de cadeia pesada
VL	Domínio Variável de cadeia leve
W	Triptofano (aminoácido)
Y	Tirosina (aminoácido)
µg	Micrograma
µL	Microlitro
%	Porcentagem

LISTA DE FIGURAS

Capítulo I - Fundamentação teórica

Capítulo II

Capítulo III

Capítulo IV

LISTA DE TABELAS

Capítulo I - Fundamentação teórica

Capítulo II

Capítulo III

Capítulo IV

SUMÁRIO

APRESENTAÇÃO

A artrite reumatoide (AR) é uma doença inflamatória crônica de causa ainda desconhecida, autoimune, que se caracteriza por inflamação em várias articulações e tecidos circundantes. A artrite idiopática juvenil (AIJ) refere-se a um grupo de doenças de causa ainda desconhecida, autoimunes, caracterizadas por artrite crônica que ocorrem em crianças até os 16 anos de idade. Compreende vários subtipos, sendo oligoarticular e poliarticular as formas mais comuns.

Apesar de alguns marcadores sorológicos e exames de imagem auxiliarem na detecção, o diagnóstico da AR e AIJ é basicamente clínico, se baseando no histórico do paciente e no exame físico feito pelo médico. Por se tratar de uma doença autoimune, pacientes com artrite apresentam anticorpos dirigidos contra componentes do próprio organismo, os autoanticorpos, que acabam servindo como biomarcadores da doença. Os dois autoanticorpos mais importantes utilizados para auxiliar no diagnóstico da AR e AIJ são os chamados fator reutamoide e anti-CCP (anticorpos contra proteína citrulinada).

Biomarcadores são definidos como parâmetros moleculares, anatômicos, fisiológicos, bioquímicos que podem ser utilizados para auxiliar no diagnóstico, acompanhar o progresso de doenças, ou prever e monitorar os efeitos do tratamento. Eles também podem ser associados com estágios específicos da doença. Biomarcadores podem ser selecionados de vários tipos de amostras biológicas e por várias técnicas diferentes. *Phage Display* (PD) é uma técnica rotineiramente utilizada para seleção de anticorpos e peptídeos com potencial para serem utilizados no diagnóstico de diversos tipos de doenças. Uma biblioteca de PD contém até 10^{10} diferentes clones de fagos, cada um exibindo em sua superfície, um tipo de peptídeo aleatório. No que se refere a doenças autoimunes, o grande número de peptídeos diferentes na composição da biblioteca faz do PD uma tecnologia poderosa na seleção de miméticos a autoantígenos.

Neste estudo, utilizamos a técnica de PD para selecionar peptídeos ligantes a imunoglobulinas G presentes no soro de pacientes com AR e AIJ e avaliar, através do imunoensaio ELISA, o potencial uso desses peptídeos como biomarcadores. No intuito de descrever novas ferramentas para auxiliar no diagnóstico da AIJ, a detecção de anticorpos direcionados contra o colágeno tipo II também foi analisada.

CAPÍTULO I
Fundamentação teórica

Resumo

Introdução: Artrite reumatoide (AR) e artrite idiopática juvenil (AIJ) são doenças reumáticas, de origem autoimune, sem causa conhecida, caracterizadas por inflamação que pode provocar danos articulares e, em alguns casos, danos em outras partes do corpos. Não há, até o momento, um teste único para o diagnóstico da AR e AIJ, que é baseado na história clínica do paciente, e em vários exames laboratoriais e de imagem. Neste contexto, a busca por novos biomarcadores com alta sensibilidade e especificidade para a AR e AIJ é de grande interesse.

Objetivos: O foco do presente estudo foi selecionar, caracterizar e validar alvos reconhecidos por anticorpos circulantes e que poderiam ser potencialmente descritos como autoantígenos da AR ou AIJ.

Metodologia: A tecnologia de *Phage Display* foi empregada para selecionar peptídeos ligantes a anticorpos circulantes de camundongos apresentando sinais de artrite após indução com colágeno tipo II (modelo CIA), e anticorpos circulantes de pacientes com AIJ. Análises *in silico*, espectrometria de massas e *Western blot* foram utilizadas para auxiliar na caracterização de peptídeos selecionados. Ensaios de ELISA, ELISA avidez e voltametria de pulso diferencial foram utilizados como plataforma para detecção de anticorpos direcionados contra os alvos propostos neste estudo. A acurácia no diagnóstico da AR e AIJ foi determinada pela curva ROC (*Receiver Operating Characteristics*).

Resultados: O peptídeo M12, selecionado contra IgGs circulantes de camundongos com sinais de artrite, foi capaz de discriminar pacientes com AR de pacientes com outras doenças autoimunes e indivíduos saudáveis ($p < 0.0001$) com alta acurácia, apresentando 91% de especificidade e 84.3% de sensibilidade. O peptídeo M12 foi identificado como mimético de uma região antigênica da proteína anidrase carbônica III, que já foi previamente identificada como um autoantígeno da AR. Os níveis de anticorpos contra o colágeno tipo II foram significativamente maiores em pacientes com AIJ quando comparados aos níveis obtidos por pacientes com espondilite anquilosante ($p = 0.006$) e indivíduos saudáveis ($p < 0.0001$). Além do mais, a detecção de anticorpos contra o colágeno tipo II foi mais frequente em pacientes com ≤ 6 meses de duração ($p =$

0.0007). Anticorpos apresentando alta avidez para o colágeno tipo II foram associados com a atividade da doença (p = 0.004). O peptídeo PRF+1, selecionado contra IgGs circulantes de pacientes com AIJ, foi capaz de discriminar pacientes com AIJ e AR de pacientes com outras doenças autoimunes e indivíduos saudáveis (p < 0.0001) com alta acurácia, apresentando 91% de especificidade e 61% de sensibilidade para AIJ, e 93% de especificidade e 94% de sensibilidade para AR. O biosensor eletroquímico desenvolvido para detecção de anticorpos contra o peptídeo PFR+1 provou ser uma forma rápida, barata e eficaz de discriminar amostras de soro de pacientes com JIA e RA de indivíduos saudáveis.

Conclusão: Em uma análise geral do conjunto de estudos aqui apresentados, é possível concluir que os antígenos utilizados para a detecção de anticorpos circulantes em pacientes com AIJ ou AR podem ser utilizados com alta acurácia para auxiliar no diagnóstico.

Palavras-chave: Artrite idiopática juvenil, artrite reumatoide, phage display, autoantígeno, sorodiagnóstico

Abstract

Introduction: Rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) are autoimmune rheumatic diseases of unknown cause characterized by inflammation, which can cause joint damage, and in some cases, damage to other parts of the body. To date, there is no single, definitive test for the diagnosis of RA and JIA, which is based on patient history, and various imaging and laboratory tests. In this context, the search for novel biomarkers with high sensitivity and specificity for RA and JIA is of great interest.

Objectives: The aim of this study was to select, characterize and validate targets recognized by circulating antibodies that could be potentially described as autoantigens of RA or JIA.

Methods: Phage Display technology was used to select peptides binding to circulating antibodies from mice presenting signs of arthritis after collagen type II induction (CIA model), and circulating antibodies from patients with JIA. *In silico* analysis, mass spectrometry and western blot were used to assist in the characterization of selected peptides. ELISA assays, ELISA avidity and differential pulse voltammetry was used as platform for detection of antibodies directed against the targets proposed in this study. Receiver Operating Characteristics (ROC) curve was determined for diagnostic accuracy for JIA and RA.

Results: The M12 peptide selected against circulating IgGs from mice with signs of arthritis was able to discriminate RA patients from patients with other autoimmune diseases and healthy individuals ($p < 0.0001$) with high accuracy, presenting 91% specificity and 84.3% sensitivity. The M12 peptide was identified in an antigenic region of the protein carbonic anhydrase III, which has been previously identified as a RA autoantigen. The levels of antibodies to type II collagen were significantly higher in patients with JIA compared to levels obtained in patients with ankylosing spondylitis ($p = 0.006$) and healthy individuals ($p < 0.0001$). Moreover, the antibodies detection to type II collagen was more frequent in patients with ≤ 6 months duration ($p = 0.0007$). Antibodies displaying high avidity to collagen type II were associated with disease activity ($p = 0.004$). The PRF+1 peptide, selected from circulating IgGs from patients with JIA, was able to discriminate patients with JIA and RA from patients with other autoimmune

diseases and healthy individuals ($p < 0.0001$) with high accuracy, presenting 91% specificity and 61% sensitivity for JIA, and 93% specificity and 94% sensitivity for RA. The electrochemical biosensor designed to detect antibodies against the peptide PFR+1 proved to be a fast, cheap and effective way of discriminating serum samples from patients with RA or JIA from healthy individuals.

Conclusion: In a general analysis of the studies presented here, we conclude that the antigens used for the detection of circulating antibodies in patients with RA or JIA can be used with high accuracy to assist in diagnosis.

Key words: Juvenile idiopathic arthritis, rheumatoid arthritis, phage display, autoantigen, serodiagnosis

1. O sistema imunológico: uma breve revisão

O sistema imunológico é formado por uma rede de células, tecidos e órgãos que trabalham em conjunto para defender o organismo contra invasores, bem como manter a sua homeostase. Estes invasores são essencialmente microrganismos, pequenos organismos como bactérias, parasitas, e fungos que podem causar infecções. O corpo humano fornece um ambiente ideal para muitos desses microrganismos, e é trabalho do sistema imunológico mantê-los fora ou, destruí-los[1]. A era moderna da imunologia começou com a teoria da seleção clonal expressa de forma independente por David W. Talmage e Sir Frank Macfarlane Burnet[2; 3]. A teoria da seleção clonal postula que uma vez que um antígeno estranho entra no corpo, ele se liga a um único tipo de anticorpo selecionado a partir de um repertório ilimitado de anticorpos formados no início da vida do organismo. Isto explica a forma como o sistema imunitário é capaz de reconhecer e de responder a um número virtualmente inestimável de antígenos estranhos. A Figura 1 ilustra as diversas células que compõem o sistema imunológico.

O sistema imunológico tem sido conceitualmente dividido em dois sistemas: inato e adaptativo. O sistema imunológico inato representa uma resposta rápida a um número grande, mas limitado, de estímulos, e é a primeira linha de defesa contra patógenos, trabalhando para reconhecer componentes comuns para que mais respostas imunes possam ser sinalizadas na presença de agentes estranhos[4]. As principais células efetoras da imunidade inata são os macrófagos, neutrófilos, células dendríticas e células *Natural Killer (*NK). Fagocitose, liberação de mediadores inflamatórios, ativação de proteínas do sistema complemento, bem como síntese de proteínas de fase aguda, citocinas e quimiocinas são os principais mecanismos na imunidade inata[5]. O sistema imunológico adaptativo é um sistema antígeno-específico que gera memória imunológica a partir da ativação de células T e B, e possui como principal característica a produção de anticorpos específicos para responder a microrganismos invasores ou células infectadas[6]. Ou seja, em contraposição ao sistema inato, depende da ativação de células especializadas, os linfócitos, e das células por eles diferenciadas[4].

O sistema imune sadio deve manter o balanço entre a capacidade de responder a agentes infecciosos e de sustentar a autotolerância, não respondendo aos componentes próprios do organismo. A ausência de resposta adequada submete o indivíduo aos efeitos nocivos da invasão por patógenos, ao passo que o sistema respondendo de modo exacerbado pode gerar respostas inflamatórias prejudiciais[5].

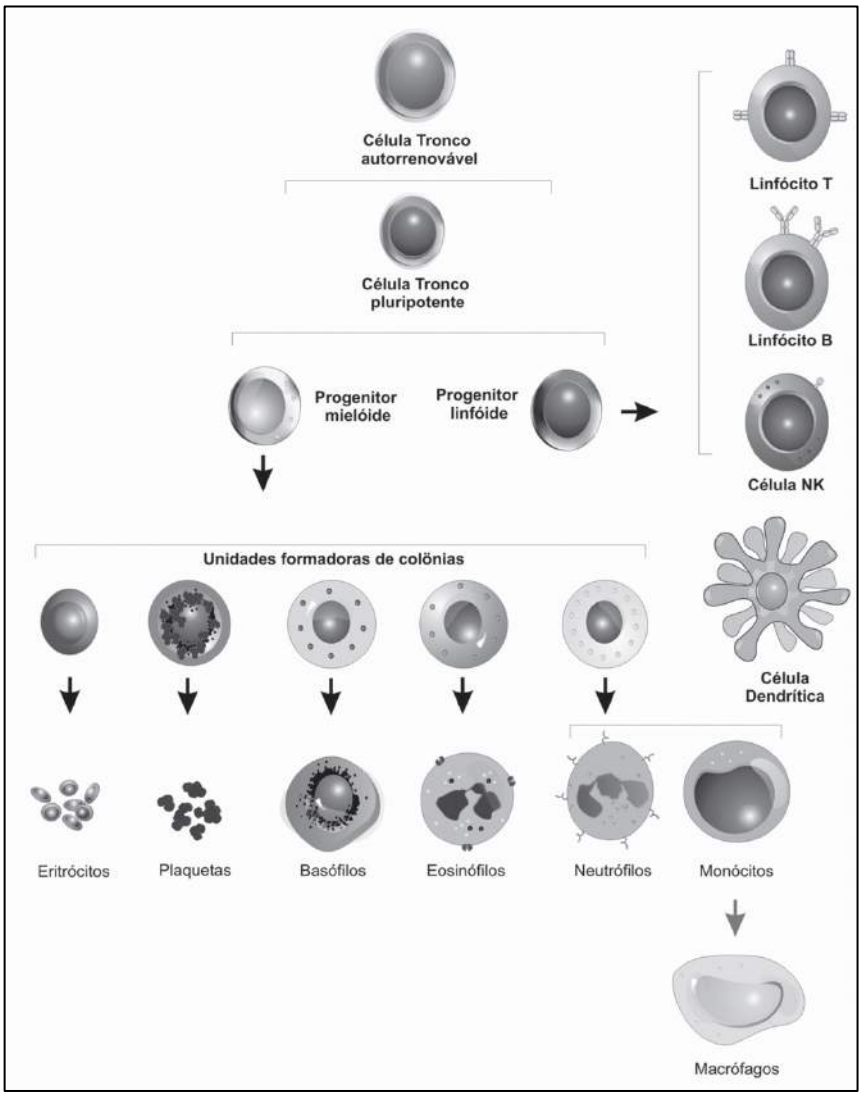


Figura 1. Painel ilustrando a origem das diversas linhagens de células que compõem o sistema imunológico. Fonte:[5]

1.1 Antígenos

Originalmente, o termo antígeno foi utilizado para designar qualquer molécula que fosse capaz de induzir a produção de anticorpos específicos pelas células B. Atualmente, no entanto, o termo é amplamente utilizado para indicar qualquer molécula que possa ser reconhecida pelos elementos do sistema imune adaptativo, ou seja, céulas T, células B ou ambas[7].

As respostas imunológicas podem também ser geradas contra substâncias menores, chamadas haptenos, se estes forem quimicamente acoplados a uma proteína transportadora maior, como por exemplo à albumina do soro bovino (BSA) ou outras matrizes sintéticas. Uma variedade de moléculas, tais como fármacos, açúcares simples, aminoácidos, peptídeos pequenos, fosfolipídios, ou triglicerídeos podem funcionar como haptenos. Assim, dado o tempo suficiente, qualquer substância estranha poderá ser identificada pelo sistema imunológico e ativar a produção de anticorpos específicos. No entanto, esta resposta imunológica específica é altamente variável e depende muito, em parte, do tamanho, estrutura e composição do antígeno. Os antígenos que induzem fortes respostas imunológicas são referidos como sendo fortemente imunogênicos[8].

A pequena região de um antígeno ao qual um anticorpo pode se ligar de forma específica é chamado de epítopo. Esta região compreende de 1 a 6 monossacarídeos ou 5 a 8 resíduos de aminoácidos na superfície do antígeno. Como moléculas antigênicas existem em espaço, o epítopo reconhecido por um anticorpo pode ser dependente da presença de uma conformação tridimensional específica (por exemplo, um sítio único formado pela interação de dois *loops* de proteínas nativas ou subunidades), ou o epítopo pode corresponder a uma região de sequência primária simples. Tais epítopos são descritos como conformacional e linear, respectivamente. A variedade de possíveis sítios de ligação é enorme, com cada local de potencial ligação de anticorpos apresentando suas próprias propriedades estruturais derivadas de ligações covalentes, ligações iônicas e interações hidrofílicas e hidrofóbicas[9].

Para ocorrer uma interação eficaz entre antígeno e anticorpo, o epítopo deve estar prontamente disponível para a ligação. Se a molécula alvo estiver desnaturada, como por exemplo por meio de fixação, redução, ou alterações de

pH ou durante a preparação para eletroforese em gel, o epítopo pode ser alterado e afetar sua capacidade de interagir com um anticorpo. Assim, o epítopo pode estar presente no antígeno em sua forma nativa, ou apenas em sua forma desnaturada. Por isso, uma anticorpo que funciona bem em análises de imunohistoquímica, pode não funcionar em análises de western blot por exemplo[8]. As características esperadas para um antígeno ser considerado um bom produto imunogênico, estão listadas na Tabela 1.

Tabela 1. Características esperadas para um antígeno ser considerado imunogênico.

Áreas de estabilidade estrutural e complexidade química dentro da molécula;
Extensões significativas sem apresentar muitos componentes repetidos;
Peso molecular mínimo entre 8.000 e 10.000 kDa (embora haptenos com pesos moleculares menores que 200 kDa possam ser utilizados na presença de uma proteína transportadora);
Habilidade para ser processado pelo sistema imunológico;
Regiões imunogênicas acessíveis ao mecanismo de formação de anticorpos;
Elementos estruturais que são suficientemente diferentes do hospedeiro;
Para peptídeos antigênicos, regiões contendo pelo menos 30% de aminoácidos imunogênicos (K, R, E, D, Q, N), hidrofilicidade significativa ou resíduos modificados.

Fonte:[9]

1.2 Anticorpos

Os anticorpos (Ac) ou imunoglobulinas (Ig) são moléculas produzidas por células denominadas linfócitos B e são capazes de localizar, reconhecer e ligar-se a antígenos específicos com a finalidade de inativar ou dar início à sua

eliminação. Possuem caráter glicoproteico e estão presentes no sangue circulante e na linfa.

Existem cinco classes de imunoglobulinas diferentes (IgA, IgD, IgE, IgG e IgM), sendo a IgG a mais abundante, chegando a 75% dos anticorpos totais do sangue, e mais utilizada para fins terapêuticos e biotecnológicos[7]. As imunoglobulinas possuem massa molecular de aproximadamente 150 kDa, compostos por dois tipos de cadeias polipeptídicas: pesada (H) e leve (L). Cada cadeia pesada é unida covalentemente a uma cadeia leve por uma ponte dissulfeto e, as duas cadeias pesadas, já ligadas às cadeias leves, são mantidas unidas covalentemente, também por pontes dissulfeto, formando o anticorpo[10]. Cada molécula de imunoglobulina tem duas regiões idênticas, chamadas de Fab, que são capazes de se ligar a antígenos, e uma região constante, chamada de Fc, que pode se ligar a receptores presentes na superfície de células, como macrófagos. De fato, é a estrutura da região Fc do anticorpo que determina a sua classe, qual células do sistema imunológico ele irá se ligar, e como ele irá agir[11] (Figura 2).

Anticorpos são comumente conjugados com enzimas ou fluoróforos em sua região Fc. A região Fc também ancora o anticorpo na placa para procedimentos de ELISA e é nessa região que se ligam anticorpos secundários em procedimentos de imunoprecipitação e imuno-histoquímica. Uma importante função biológica da região Fc é sua participação na degradação de antígenos estranhos. Quando já ligada ao seu antígeno específico, a região Fc ativa o sistema complemento e auxilia a fagocitose por se ligar aos macrófagos[9]. As imunoglobulinas podem ser fragmentadas por proteólise. Uma molécula de IgG quando submetida a clivagem por enzimas proteolíticas especificas é dividida em duas moléculas: uma delas comporta a região de ligação ao antígeno (Fab) e a outra a região constante (Fc). A fragmentação de IgG's às vezes é útil porque (1) fragmentos Fab não vão precipitar o antígeno em estudos *in vitro*; e (2) não será reconhecido pelas células do sistema imunológico em estudos *in vivo*, devido à falta da região Fc. Frequentemente, por causa do seu tamanho menor e por não demonstrar reações cruzadas (devido à perda da região Fc), os fragmentos Fab são radiomarcados para utilização em estudos funcionais. Curiosamente, os

fragmentos Fc são frequentemente utilizados como agentes de bloqueio em coloração histoquímica[9; 10].

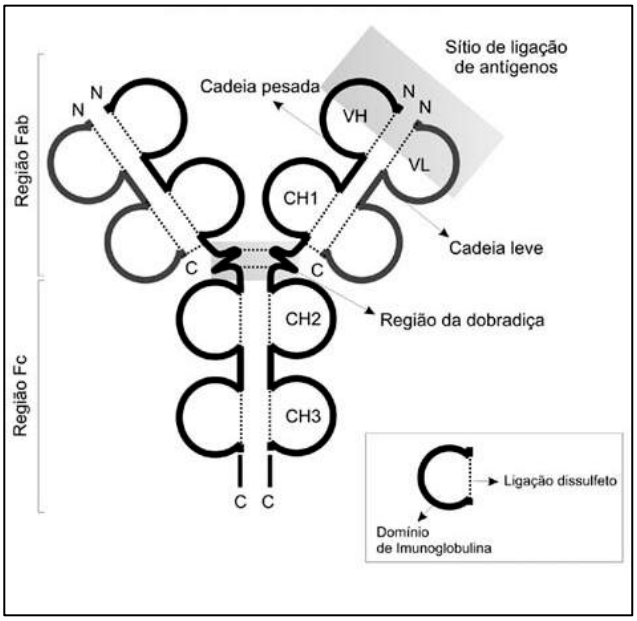


Figura 2. Esquema de uma molécula de imunoglobulina secretada. Fonte:[7]

A associação específica de antígenos e anticorpos é dependente de ligações de hidrogênio, interações hidrofóbicas, forças eletrostáticas e de van der Waals. Estes fatores são limitantes para algumas ligações fracas, de natureza não-covalente, mas algumas das associações entre antígenos e anticorpos podem ser bastante forte. Como os anticorpos, antígenos podem ser multivalentes, apresentando múltiplas cópias do mesmo epítopo, ou apresentando múltiplos epítopos que são reconhecidos por vários anticorpos. Interações envolvendo multivalência podem produzir complexos mais estabilizados, no entanto, multivalência também pode resultar em dificuldades de natureza estérica, reduzindo assim a possibilidade de ligação[9].

2. Doenças autoimunes

Doenças autoimunes são caracterizadas por condições crônicas iniciadas pela perda da tolerância imunológica a autoantígenos. Elas representam um grupo heterogêneo de distúrbios que afetam órgãos-alvo específicos ou múltiplos

sistemas[12]. A natureza crônica dessas doenças coloca o paciente sob constantes cuidados médicos, aumentando seus custos diretos e indiretos, e diminuindo a qualidade de vida. A incidência estimada para novos casos de doenças autoimunes é de aproximadamente 80 para cada 100.000 pessoas por ano e sua prevalência pode ir bem além de 3% da população mundial[13].

A maioria das doenças autoimunes afetam mulheres de meia-idade e estão entre as principais causas de morte nesse grupo de pacientes. Embora a incidência se difere entre países[14], vários estudos têm mostrado que, para alguns tipos de doenças autoimunes, associações com diversos fatores são encontradas entre as populações, como uma pré-disposição genética por exemplo. Doenças autoimunes podem ser hereditárias e são assim classificadas quando pessoas de um mesmo núcleo familiar desenvolvem a mesma doença, como é o caso do diabetes mellitus tipo I[15].

Inflamação e autoimunidade estão intimamente conectadas, seja no desenvolvimento ou na progressão das doenças autoimunes. A inflamação é mediada por citocinas pró-inflamatórias que são responsáveis pelo combate a um patógeno. Entretanto, em indivíduos que possuem uma desregulação do sistema imunológico, a resposta inflamatória severa pode contribuir para a patogenia das doenças autoimunes[16]. O aumento da prevalência de infecção no curso das doenças autoimunes é um aspecto importante entre a relação de infecção e autoimunidade, representando claramente que infecções são uma causa comum de morbidade e mortalidade em pacientes portadores de doenças autoimunes sistêmicas[17].

Doenças autoimunes não se iniciam no momento em que se tornam clinicamente aparentes, mas vários anos antes. Isto implica que existe uma possibilidade de prever a autoimunidade. Ao longo dos anos, vários fatores de risco têm sido associados com o início da doença. Entre eles os mais estudados são os fatores ligados ao sexo feminino[18], os alelos específicos do sistema HLA (antígeno leucocitátio humano) e não HLA[13; 19] e agentes ambientais[20; 21]. Além disso, a presença de autoanticorpos também pode prever manifestações clínicas específicas, gravidade e progressão da doença[22]. Muitos autoanticorpos têm capacidade preditiva e podem ser sorologicamente detectados muito antes do aparecimento dos sintomas clínicos da doença. Assim, a identificação desses

biomarcadores, associados com a história de autoimunidade familiar, e avaliação do seu valor preditivo pode ser muito útil para a medicina personalizada[23].

2.1 Artrite idiopática juvenil

A Artrite Idiopática Juvenil (AIJ) é definida como uma doença de ocorrência antes dos 16 anos de idade, caracterizada primariamente pela presença de artrite persistente em uma ou mais articulações, por no mínimo 6 semanas, após exclusão de outras causas. É uma doença inflamatória crônica que, se não tratada precocemente, pode acarretar prejuízos permanentes[24].

De acordo com a "*International League of Associations for Rheumatology criteria*", a AIJ compreende sete subtipos, sendo oligoarticular e poliarticular os mais comuns[25]. Artrite que afeta até quatro articulações após os primeiros seis meses da doença é classificada como AIJ oligoarticular persistente. Embora por vezes considerada como uma condição benigna, o subtipo oligoarticular persistente pode ir da remissão completa após a interrupção do tratamento até o desenvolvimento de danos graves nas articulações afetadas. O subtipo AIJ oligoarticular estendida tem um pior prognóstico e inclui pacientes que após o diagnóstico, a artrite evolui para cinco ou mais articulações afetadas após os primeiros 6 meses da doença[26]. Artrite que afeta cinco ou mais articulações durante os primeiros 6 meses da doença é chamada de AIJ poliarticular, que é classificada como artrite poliarticular com fator reumatoide negativo (FR-) ou poliarticular com fator reumatoide positivo (FR+). Todos os subtipos de AIJ são de causas desconhecidas, e o diagnóstico baseia-se na avaliação conjunta do histórico médico, apresentação clínica e, alguns exames laboratoriais[27; 28].

Apesar de ser uma doença altamente heterogênica, é provável que haja alguma sobreposição genética, já que todos os subtipos de AIJ apresentam inflamação das articulações como a característica mais proeminente da doença[29].

A patogênese da AIJ envolve inflamação do revestimento sinovial, com o potencial de causar destruição da articulação, onde há infiltração da membrana sinovial por células inflamatórias causando a formação do *pannus*. O *pannus* é formado principalmente por células invasivas onde a membrana sinovial torna-se

espessa causando danos articulares e ósseos[24]. Acredita-se que os danos ósseos que podem ser observados em pacientes com AIJ são consequências da degradação da cartilagem causada por infiltrados de células, assim como anticorpos. A inflamação pode também causar maiores danos, tanto sistematicamente quanto localmente nas articulações afetadas[29].

Embora as manifestações clínicas na AIJ estejam relacionadas com o sistema imune adaptativo, trabalhos envolvendo autoantígenos que impulsionam a resposta humoral não têm recebido muita atenção. Eventos autoimunes, tais como células T auto-reativas e autoanticorpos já foram detectados na maioria dos subtipos de AIJ[30; 31]. Em contraste com a maioria das proteínas em circulação, os anticorpos são fáceis de serem mensurados, são menos sujeitos a variações no sangue, mais estáveis em amostras de soro, e podem ser facilmente detectados[32].

Estudos envolvendo autoanticorpos têm geralmente focado na artrite reumatoide (AR), e só depois então o desempenho desses autoanticorpos são investigados em pacientes com AIJ. Mesmo que evidências fornecidas por esta abordagem sejam limitadas, esses estudos têm fornecido um vislumbre dos mecanismos moleculares heterogêneos que contribuem para a patogênese dos vários subtipos de AIJ. A heterogeneidade da AIJ é exemplificada pelo fato de que os resultados obtidos em paciente com AR podem ser aplicados apenas a alguns subgrupos específicos de pacientes com AIJ. Por exemplo, o fator reumatoide, que tem uma sensibilidade de ~80% em paciente com AR, está presente somente em um pequeno subgrupo de pacientes com AIJ (subtipo poliarticular)[33]. A descoberta de autoanticorpos direcionados contra proteínas citrulinadas presentes na AR tem despertado enorme interesse por conta de seu desempenho em pacientes com AIJ[34]. No entanto, a descoberta de autoanticorpos que conferem maior especificidade e sensibilidade ao diagnóstico da AIJ é de grande interesse clínico.

2.2 Artrite Reumatoide

A artrite reumatóide (AR) é uma doença inflamatória sistêmica, autoimune, que afeta principalmente as articulações. Enquanto a taxa de prevalência exata de toda a população é desconhecida, os dados disponíveis sugerem que a AR afeta ~ 1% da população mundial, tornando-se uma das doenças reumáticas autoimunes mais comuns[35]. No Brasil, um estudo de 2004 sobre a prevalência de doenças reumáticas no país mostrou que a prevalência da AR foi de 0.46%[36]. A prevalência da AR parece ser menor nos países em desenvolvimento, o que pode estar relacionado ao número reduzido de estudos epidemiológicos realizados nestes países, ou mesmo à ausência de diagnóstico em pacientes de comunidades carentes pela dificuldade de acesso às unidades de saúde.

Embora tratamentos modernos para a AR possam levar muitos pacientes à remissão, o diagnóstico em estágios iniciais da doença é importante para prevenir danos irreversíveis ao revestimento sinovial e da cartilagem das articulações doentes, e também para prevenir a progressão da doença. Até o momento, não há ainda um bom indicador clínico disponível para o monitoramento do desenvolvimento a longo prazo da AR[37]. O Fator reumatoide é um autoanticorpo, mais comumente do tipo IgM, que se liga à região Fc de anticorpos IgG. A imunodetecção do fator reumatoide é o teste de sangue mais amplamente utilizado na classificação da artrite[38].

Nos atuais critérios de classificação[38], o diagnóstico da AR baseia-se na presença confirmada de sinovite em pelo menos uma articulação, ausência de um diagnóstico alternativo que explique melhor a ocorrência de sinovite, e realização de um escore total de ≥ 6 (de 10 possíveis) em um sistema de pontuação. A pontuação é derivada a partir de quatro critérios: o número e local das articulações afetadas (variando de 0 a 5), anormalidade sorológica (níveis elevados de fator reumatoide ou anticorpo anti-CCP; variando de 0 a 3), resposta de fase aguda elevada (variação de 0 a 1), e duração dos sintomas (< 6 vs ≥ 6 semanas; variando de 0 a 1). Afirma-se frequentemente que os níveis de fator reumatoide aumentam com a idade[39], mas não há muitos dados convincentes que comprovem esta afirmação. Cerca de 80% de todos os pacientes com AR acabará por ser soropositivo para fator reumatoide, enquanto apenas 40% são

positivos no início clínico da doença[40]. No entanto, não se sabe se os níveis elevados de fator reumatoide em indivíduos na população em geral sem artrite reumatoide está associado com o desenvolvimento posterior de AR[37].

Vários estudos demonstraram que existe uma fase "pré-clínica" da AR, durante o qual há a produção de autoanticorpos e marcadores inflamatórios anteriores ao início do aparecimento dos sinais e sintomas de doença articular que caracterizam clinicamente a doença[41; 42; 43] (Figura 3).

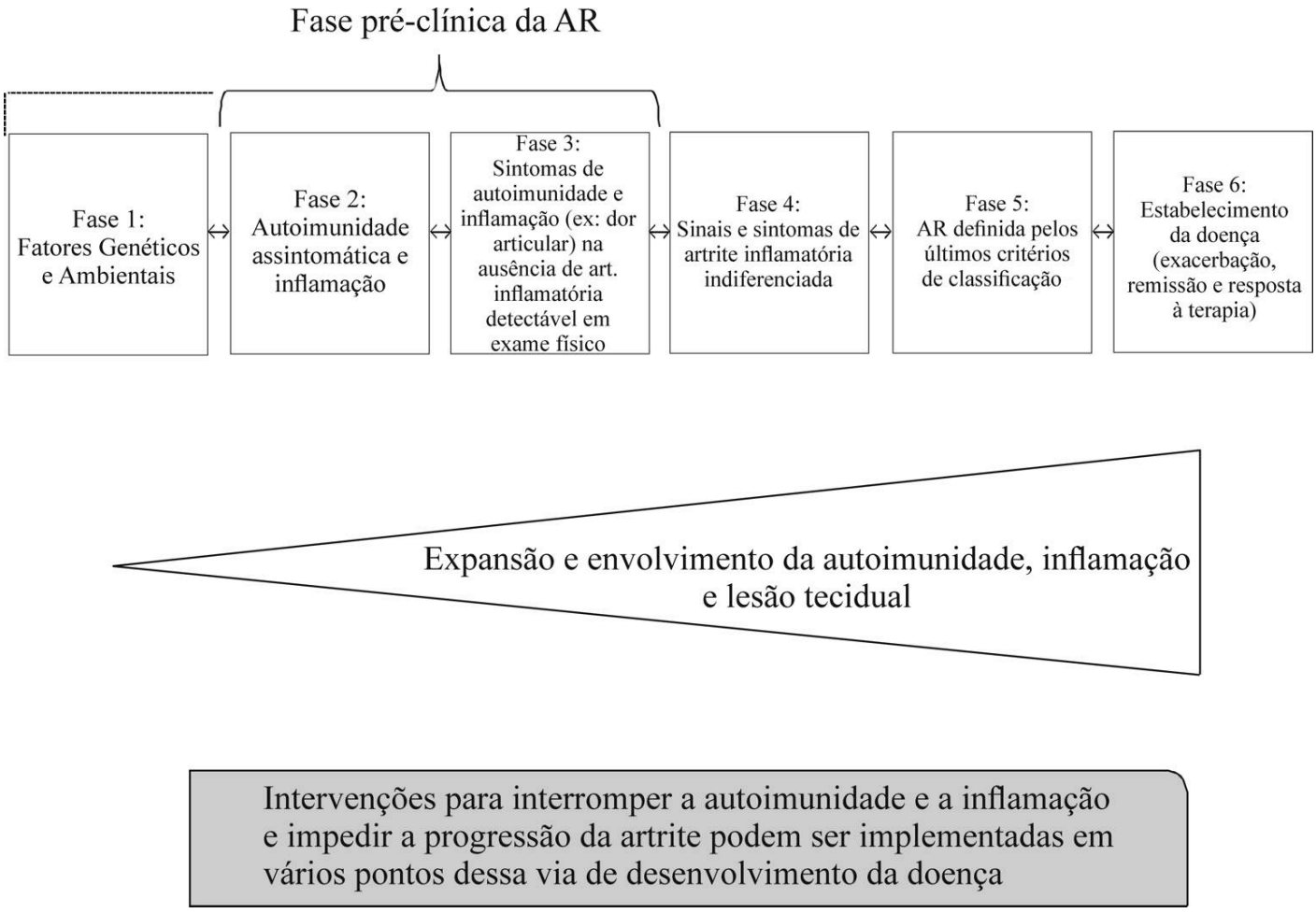


Figura 3. Fases do desenvolvimento da artrite reumatoide. Neste modelo de desenvolvimento da AR, a doença começa com riscos genéticos e ambientais (Fase 1), seguido por auto-imunidade e inflamação assintomática (por exemplo, produção de autoanticorpos, citocinas e quimiocinas; Fase 2), com progressão para sintomas que possam estar presentes na ausência de artrite inflamatória em exame físico (Fase 3), e eventual desenvolvimento de artrite inflamatória

indiferenciada (Fase 4) que pode progredir para a classificação da AR (Fase 5). A fase 6 é definida como a evolução da doença (por exemplo, as exacerbações, remissões, e resposta à terapia), após desenvolvimento de doença articular clinicamente aparente. Manifestações da doença na fase "pré-clínica" (fases 2 e 3) são identificáveis por meio de testes para detecção de biomarcadores e antes do desenvolvimento de artrite inflamatória (que é identificável através de um exame comum). Este processo de desenvolvimento da AR é caracterizado pela expansão da autoimunidade e inflamação, detectável por meio de avaliação de biomarcadores circulantes, embora que a Fase 1 também poderia ser incluído. As setas de cabeça dupla que indicam a progressão da doença podem ser interrompidas, ou invertidas, talvez por meio de intervenção farmacológica utilizando imunomoduladores, especialmente se iniciado antes do início do desenvolvimento de artrite inflamatória clinicamente aparente (Fase 4). Fonte:[35]

3 Autoantígenos

Um componente central no processo da autoimunidade são os autoantígenos, contra os quais o sistema imunológico produz autoanticorpos e células autorreativas. As regras que determinam o repertório de possíveis autoantígenos e que os diferenciam das moléculas não autoantigênicas são atualmente desconhecidas. Entre a grande variedade de moléculas humanas, apenas 1% foram observadas como prováveis autoantígenos[44]. Muitas doenças autoimunes compartilham os mesmos autoantígenos e os mesmos sintomas clínicos[45]. Notavelmente, os repertórios de autoanticorpos parecem semelhantes em humanos e modelos experimentais animais, como o camundongo[46]. Estes fatos e outras evidências sugerem que as propriedades autoantigênicas são governadas por regras ainda desconhecidas[47].

3.1 Colágeno tipo II

Colágenos são um grupo de macromoléculas estruturais que estão presentes no tecido conjuntivo incluindo osso, pele, cartilagem hialina, vasos

sanguíneos, membrana sinovial e fígado, entre outros. Quarenta e dois genes diferentes são responsáveis pela produção de, pelo menos, 28 tipos diferentes de colágeno, que já foram identificados em diferentes tecidos de vertebrados[48].

O colágeno tipo II (CII) é o tipo predominante na cartilagem articular. Mais de 50% de todas as proteínas da cartilagem articular e 90% da cartilagem hialina consistem do CII[49]. Também está presente no ouvido, laringe e traqueia, vítreo do olho, disco intervertebral e, durante o desenvolvimento, em muitos tecidos não condrogênicos[50]. Na AR, provavelmente na AIJ também (mecanismo ainda pouco explorado), o sistema imunológico reconhece o colágeno como uma substância estranha e produz anticorpos que o atacam e o destroem, o que resulta em uma inflamação crônica que pode gerar destruição articular, deformidade e perda de função[51]. Apesar de estar claro que, de alguma forma, autoanticorpos contra o colágeno tipo II participam na imunopatogênese da AR e AIJ, esses mecanismos ainda precisam ser esclarecidos.

Autoanticorpos contra o CII já foram detectados no soro de pacientes com diferentes subtipos de AIJ[52; 53; 54; 55], reforçando a hipótese de que a autoimunidade ao CII presente nas cartilagens desempenha um forte papel na patogênese da doença.

Artrite pode ser induzida por colágeno em algumas linhagens de roedores. A imunização com CII em camundongos dá origem a títulos significativos de anticorpos anti-CII. Três anticorpos monoclonais específicos contra CII (CII-C1, UL-1, e M2139) foram descritos e detalhadamente caracterizados pela interação molecular com a sua molécula alvo[56; 57; 58; 59], ligação *in vivo*[60], e sua patogenicidade e importância reguladora[61]. Camundongos da linhagem DBA / 1 desenvolvem poliartrite quando imunizados com CII de diferentes espécies. A artrite observada normalmente não é simétrica e qualquer combinação de patas / juntas pode ser afetada[62].

É de grande valor, portanto, desenvolver ensaios que não são dependentes somente de colágeno nativo extraído de tecidos, mas utilizar CII sintético ou recombinante de diferentes espécies que contenha diferentes epítopos que possam ser reconhecidos por anticorpos, visando aplicação no diagnóstico e monitoramento de doenças reumáticas[63]. Um artigo de revisão que complementa este tópico segue como anexo 1 desta tese.

3.2 Anidrase carbônica III

As anidrases carbônicas (CA) são enzimas que contém zinco, que desempenham um papel crítico na manutenção do pH intercelular/extracelular na maioria das células de mamíferos por catalisar a interconversão entre dióxido de carbono e bicarbonato. É um grupo de metaloenzimas e há pelo menos 15 isoformas diferentes presentes em células de mamíferos[64]. A anidrase carbônica III (CAIII) é expressa a um nível muito elevado no músculo esquelético, onde pode desempenhar um papel na eliminação de radicais de oxigênio e assim proteger as células do dano oxidativo[65].

Autoanticorpos contra a CAIII em pacientes com AR foram descritos pela primeira vez em 2007 por Robert-Pachot e colaboradores[66], onde a proteína recombinante foi utilizada como antígeno no ensaio de ELISA, com positividade em 16.7% nos pacientes analisados, enquanto que amostras do grupo controle não apresentaram anticorpos anti-CAIII. A sensibilidade desse novo autoantígeno para a AR, utilizando a técnica imunoblot 1D, foi de 17%. A especificidade foi elevada quando se comparada a outras doenças não autoimunes (100%), e baixa, quando comparada a algumas outras doenças autoimunes (67%).

Anticorpos anti-CAIII já foram detectados em 33.3% dos pacientes com lúpus sistêmico eritematoso (LSE), 13% com esclerose sistêmica, 20% com diabetes tipo I, 28% com doença de Addison e 5% com tireoidite autoimune. Portanto, apesar de serem detectados em maiores níveis em pacientes com AR, anticorpos contra a CAIII não estão restritos à AR. Em outro estudo, anticorpos anti-CAIII também foram detectados em pacientes com AR, LSE e diabetes mellitus tipo I[67]. Esses dados mostram que anticorpos anti-CAIII apresentaram baixa especificidade para a AR quando comparados com outras doenças autoimunes. Por ter essa característica promiscua, a descoberta de novos epítopos que conferem maior especificidade aos anticorpos anti-CAIII são de grande interesse para o diagnóstico da AR.

4 *Phage Display*

A tecnologia de *Phage Display* (PD) foi desenvolvida por G. Smith em 1985[68] como um método para apresentação de polipeptídios na superfície de bacteriófagos filamentosos. Desde então, este método tornou-se uma das formas mais eficazes para a produção de grandes quantidades de peptídeos, proteínas e anticorpos. Utilizando as técnicas de DNA recombinante, coleções de bibliotecas de peptídeos, variantes proteicos ou fragmentos gênicos (ou cDNA) podem ser apresentados na superfície de bacteriófagos e submetidos a inúmeras estratégias de seleção[69].

Fagos recombinantes expressando peptídeos randômicos em sua superfície podem ser selecionados por afinidade, onde os fagos selecionados podem ser amplificados em ciclos adicionais de crescimento em bactérias *E. coli* hospedeiras apropriadas[68].

A técnica de PD é baseada no uso de um bacteriófago filamentoso M13 capaz de infectar bactérias *E. coli* gram negativas. A partícula do fago é formada por uma fita simples de DNA circular envolta por uma capa proteica constituída por cinco proteínas (pIII, pVI, pVII, pVIII e pIX) (Figuras 4[A] e 4B). A maioria dos peptídeos e anticorpos são expressos nas proteínas pIII[70] e pVIII[71], presentes na superfície do fago M13, sendo a pIII a mais comum[72]. A pIII está relacionada com a infectividade do fago pela ligação ao *pilus* F da célula bacteriana. Ela apresenta três domínios (D1, D2 e D3) separados por resíduos de glicina. Estudos cristalográficos dos domínios D1 e D2 mostraram uma conformação semelhante à uma ferradura de cavalo[73] (Figura 4C). Devido a baixa quantidade de cópias da pIII em relação a pVIII as bibliotecas de peptídeos sintéticos fusionados na pIII são mais indicadas para descoberta de ligantes com alta afinidade, quando comparadas com as bibliotecas com peptídeos fusionados à pVIII[74].

O gene repórter *lacZ* presente no genoma do bacteriófago M13, facilita a distinção visual entre colônias bacterianas infectadas com fagos que carregam sequências exógenas (colônias azuis) e colônias não infectadas por partículas virais (colônias brancas)[75].

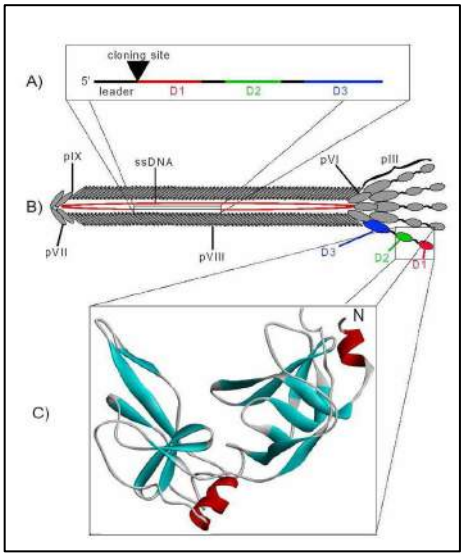


Figura 4. **Estrutura do fago filamentoso.** A) Composição do gene III, que codifica para a proteína de superfície pIII, mostrando o sítio de ligação de clonagem para introdução do gene adicional. B) Partícula viral com as proteínas pIII, pVI, pVII, pVIII e pXI. C) Cristalografia dos domínios D1 e D2 da proteína III, as alfa-hélices estão coloridas em vermelho e as fitas β em ciânico. Fonte[73]

A técnica de PD permite a criação de bibliotecas que contêm até 10^{10} variantes diferentes de moléculas expressas na superfície dos fagos e pode ser utilizada para seleção por afinidade de bibliotecas combinatórias de peptídeos para estudar interações proteína-ligante e caracterizar estes ligantes[76], interações receptor e sítio de ligação de anticorpos[77], definir epitopos para anticorpos monoclonais e selecionar alvos a partir de repertório de anticorpos[78]. Cada membro da biblioteca apresenta uma forma distinta, que determinará a capacidade de interação deste com uma molécula alvo. Quanto maior for o número de formas representadas na biblioteca, mais facilmente será encontrado um ligante afim[79].

O processo de seleção de ligantes a partir de uma biblioteca é chamado de *biopanning*. O processo de *biopanning* é baseado em repetidos ciclos de incubação, lavagem, amplificação e re-seleção dos fagos ligados ao alvo. Para o processo de seleção de ligantes, a molécula alvo pode ser imobilizada em diferentes plataformas como placas de microtitulação[80], membranas de PVDF[81],

imunotubos[82], beads magnéticos[83; 84] e até mesmo células inteiras[85]. Um ciclo completo de *biopanning* está representado na Figura 5.

O processo de eluição dos fagos ligados a alvos proteicos imobilizados compreende um ponto chave nos protocolos de seleção, algumas alterações realizadas nesta etapa durante os ciclos de seleção por afinidade, podem levar ao isolamento de clones com maior afinidade para a molécula alvo, como por exemplo, utilizar tampões de eluição com pH decrescente[86], utilizar solução com proteína alvo como tampão de eluição[87] e aumentar a estringência do tampão de lavagem em cada ciclo de seleção[88].

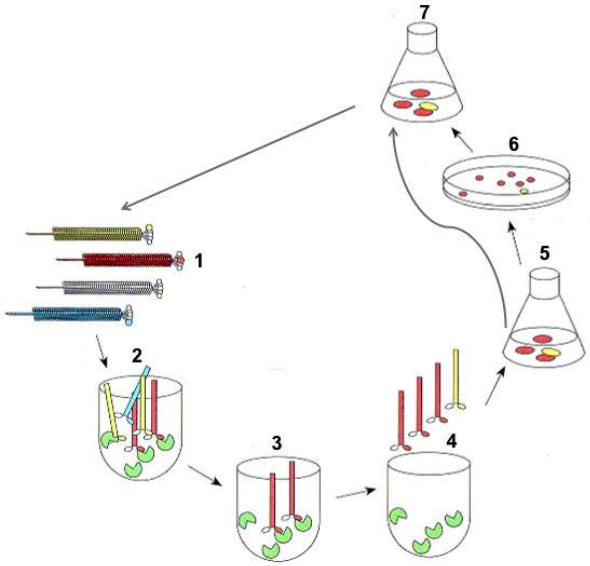


Figura 5. Esquema representativo do processo de *biopanning*. 1) Biblioteca de fagos. 2) Incubação da biblioteca de fagos com o alvo devidamente imobilizado. 3) Retirada dos fagos não ligantes por sucessivas lavagens. 4) Eluição dos fagos ligados ao alvo. 5) Infecção de *E. coli* com pili F com os fagos eluídos. 6) Titulação dos fagos eluídos. 7) Amplificação dos fagos eluídos contendo a população de fagos selecionados com maior afinidade pelo alvo.

A busca por novos componentes biotecnológicos que poderiam servir como medicamentos ou biomarcadores para o diagnóstico de várias doenças é um dos grandes desafios da ciência moderna. *Phage display* é considerada uma tecnologia poderosa para a obtenção de grandes quantidades de proteínas específicas, enzimas, anticorpos e peptídeos em um tempo relativamente curto[83], com impacto significativo em várias patologias.

5 OBJETIVOS

5.1 Objetivos Gerais

Investigar prováveis autoantígenos da artrite reumatoide e artrite idiopática juvenil e testar seu potencial uso como biomarcadores para serem utilizados como antígenos para detecção em imunodiagnóstico.

5.2 Objetivos específicos

Capítulo I: introdução à tese com detalhamento dos temas abordados nos artigos.

Capítulo II (Artigo I): utilizar o modelo murino de artrite DBA1/J associado à técnica de *Phage Display* para isolar e identificar biomarcadores que mimetizem autoantígenos da artrite reumatoide e testar seu potencial uso em imunodiagnóstico.

Capítulo III (Artigo II): investigar se anticorpos presentes no soro de crianças com artrite idiopática juvenil oligoarticular e poliarticular reconhecem o colágeno tipo II bovino e mensurar a avidez da interação antígeno-anticorpo.

Capítulo IV (Artigo III): selecionar, através da técnica de *Phage Display*, peptídeos miméticos à autoantígenos da artrite idiopática juvenil, testar sua eficiência como prováveis biomarcadores e propor um biosensor eletroquímico para detecção de anticopos.

6. Referências bibliográficas

[1] **Understanding the Immune System: How It Works**. Bethesda: NIAID Science Education, 2007.

[2] BURNET, F. M. The new approach to immunology. **N Engl J Med,** v. 264, p. 24-34, Jan 5 1961. ISSN 0028-4793 (Print) 0028-4793 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/13689160 >.

[3] TALMAGE, D. W. Immunological specificity, unique combinations of selected natural globulins provide an alternative to the classical concept. **Science,** v. 129, n. 3364, p. 1643-8, Jun 19 1959. ISSN 0036-8075 (Print) 0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/13668511 >.

[4] DELVES, P. J.; ROITT, I. M. The immune system. First of two parts. **N Engl J Med,** v. 343, n. 1, p. 37-49, Jul 6 2000. ISSN 0028-4793 (Print) 0028-4793 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10882768 >.

[5] CRUVINEL, W. M. et al. Sistema Imunitário – Parte I: Fundamentos da imunidade inata com ênfase nos mecanismos moleculares e celulares da resposta inflamatória. **Rev Bras Reumatol,** v. 50, n. 4, p. 434-461, 2010.

[6] PIETROPAOLO, M. et al. Primer: immunity and autoimmunity. **Diabetes,** v. 57, n. 11, p. 2872-82, Nov 2008. ISSN 1939-327X (Electronic) 0012-1797 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18971434 >.

[7] ABBAS, A. K.; LICHTMAN, A. H.; PILLAI, S. **Imunologia Celular e Molecular**. Sexta edição. São Paulo: Elsevier, 2008. 576 p.

[8] PILERI, S. et al. **Antibodies: A Laboratory Manual**. Segunda edição. New York: Cold Spring Harbor Laboratory, 1996.

[9] INC., C. I. **Introduction to antibodies**. Segunda edição. Billerica: Chemicon international, 2008.

[10] JANEWAY, C. A., JR. How the immune system works to protect the host from infection: a personal view. **Proc Natl Acad Sci U S A,** v. 98, n. 13, p. 7461-8, Jun 19 2001. ISSN 0027-8424 (Print) 0027-8424 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11390983 >.

[11] LAUREN, S. **How the immune system works**. Quarta edição. Hoboken: Library of Congress Cataloging-in-Publication Data, 2012.

[12] CARDENAS-ROLDAN, J.; ROJAS-VILLARRAGA, A.; ANAYA, J. M. How do autoimmune diseases cluster in families? A systematic review and meta-analysis. **BMC Med,** v. 11, p. 73, 2013. ISSN 1741-7015 (Electronic) 1741-7015 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23497011 >.

[13] COOPER, G. S.; STROEHLA, B. C. The epidemiology of autoimmune diseases. **Autoimmun Rev,** v. 2, n. 3, p. 119-25, May 2003. ISSN 1568-9972 (Print) 1568-9972 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12848952 >.

[14] YOUINOU, P. et al. Geo-epidemiology and autoimmunity. **J Autoimmun,** v. 34, n. 3, p. J163-7, May 2010. ISSN 1095-9157 (Electronic) 0896-8411 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20056534 >.

[15] CHO, J. H.; GREGERSEN, P. K. Genomics and the multifactorial nature of human autoimmune disease. **N Engl J Med,** v. 365, n. 17, p. 1612-23, Oct 27 2011. ISSN 1533-4406 (Electronic) 0028-4793 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22029983 >.

[16] DORIA, A.; SARZI-PUTTINI, P.; SHOENFELD, Y. Infections, rheumatism and autoimmunity: the conflicting relationship between humans and their environment. **Autoimmun Rev,** v. 8, n. 1, p. 1-4, Oct 2008. ISSN 1873-0183 (Electronic) 1568-9972 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18707029 >.

[17] CUTOLO, M. et al. Use of glucocorticoids and risk of infections. **Autoimmun Rev,** v. 8, n. 2, p. 153-5, Dec 2008. ISSN 1873-0183 (Electronic) 1568-9972 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18703175 >.

[18] QUINTERO, O. L. et al. Autoimmune disease and gender: plausible mechanisms for the female predominance of autoimmunity. **J Autoimmun,** v. 38, n. 2-3, p. J109-19, May 2012. ISSN 1095-9157 (Electronic) 0896-8411 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22079680 >.

[19] CRUZ-TAPIAS, P. et al. Shared HLA Class II in Six Autoimmune Diseases in Latin America: A Meta-Analysis. **Autoimmune Dis,** v. 2012, p. 569728, 2012. ISSN 2090-0430 (Electronic) 2090-0430 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22577522 >.

[20] BARRAGAN-MARTINEZ, C. et al. Organic solvents as risk factor for autoimmune diseases: a systematic review and meta-analysis. **PLoS One,** v. 7, n. 12, p. e51506, 2012. ISSN 1932-6203 (Electronic) 1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23284705 >.

[21] GEORGE, J.; LEVY, Y.; SHOENFELD, Y. Smoking and immunity: an additional player in the mosaic of autoimmunity. **Scand J Immunol,** v. 45, n. 1, p. 1-6, Jan 1997. ISSN 0300-9475 (Print) 0300-9475 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9010493 >.

[22] SHEPSHELOVICH, D.; SHOENFELD, Y. Prediction and prevention of autoimmune diseases: additional aspects of the mosaic of autoimmunity. **Lupus,** v. 15, n. 3, p. 183-90, 2006. ISSN 0961-2033 (Print) 0961-2033 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16634374 >.

[23] TOBON, G. J. et al. Are autoimmune diseases predictable? **Autoimmun Rev,** v. 11, n. 4, p. 259-66, Feb 2012. ISSN 1873-0183 (Electronic) 1568-9972 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22001417 >.

[24] RAVELLI, A.; MARTINI, A. Juvenile idiopathic arthritis. **Lancet,** v. 369, n. 9563, p. 767-78, Mar 3 2007. ISSN 1474-547X (Electronic) 0140-6736 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17336654 >.

[25] PETTY, R. E. et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. **J Rheumatol,** v. 31, n. 2, p. 390-2, Feb 2004. ISSN 0315-162X (Print) 0315-162X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14760812 >.

[26] HUEMER, C. et al. Patterns of joint involvement at onset differentiate oligoarticular juvenile psoriatic arthritis from pauciarticular juvenile rheumatoid arthritis. **J Rheumatol,** v. 29, n. 7, p. 1531-5, Jul 2002. ISSN 0315-162X (Print) 0315-162X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12136915 >.

[27] DANNECKER, G. E.; QUARTIER, P. Juvenile idiopathic arthritis: classification, clinical presentation and current treatments. **Horm Res,** v. 72 Suppl 1, p. 4-12, Nov 2009. ISSN 1423-0046 (Electronic) 0301-0163 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19940489 >.

[28] VAN ROSSUM, M. et al. Anti-cyclic citrullinated peptide (anti-CCP) antibodies in children with juvenile idiopathic arthritis. **J Rheumatol,** v. 30, n. 4, p. 825-8, Apr 2003. ISSN 0315-162X (Print) 0315-162X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12672206 >.

[29] DEANE, K. D. Can rheumatoid arthritis be prevented? **Best Pract Res Clin Rheumatol,** v. 27, n. 4, p. 467-85, Aug 2013. ISSN 1532-1770 (Electronic) 1521-6942 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24315049 >.

[30] SENNA, E. R. et al. Prevalence of rheumatic diseases in Brazil: a study using the COPCORD approach. **J Rheumatol,** v. 31, n. 3, p. 594-7, Mar 2004. ISSN 0315-162X (Print) 0315-162X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14994410 >.

[31] NIELSEN, S. F. et al. Elevated rheumatoid factor and long term risk of rheumatoid arthritis: a prospective cohort study. **BMJ,** v. 345, p. e5244, 2012. ISSN 1756-1833 (Electronic) 0959-535X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22956589 >.

[32] ALETAHA, D. et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. **Ann Rheum Dis,** v. 69, n. 9, p. 1580-8, Sep 2010. ISSN 1468-2060 (Electronic) 0003-4967 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20699241 >.

[33] GASTON, J. S. H. **Rheumatic diseases: immunological mechanisms and prospects for new therapies** University of Cambridge, 1999.

[34] KURIYA, B. et al. Validation of a prediction rule for development of rheumatoid arthritis in patients with early undifferentiated arthritis. **Ann Rheum Dis,** v. 68, n. 9, p. 1482-5, Sep 2009. ISSN 1468-2060 (Electronic) 0003-4967 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19015211 >.

[35] RANTAPAA-DAHLQVIST, S. et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. **Arthritis Rheum,** v. 48, n. 10, p. 2741-9, Oct 2003. ISSN 0004-3591 (Print) 0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14558078 >.

[36] NIELEN, M. M. et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. **Arthritis Rheum,** v. 50, n. 2, p. 380-6, Feb 2004. ISSN 0004-3591 (Print)

0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14872479 >.

[37] MAJKA, D. S. et al. Duration of preclinical rheumatoid arthritis-related autoantibody positivity increases in subjects with older age at time of disease diagnosis. **Ann Rheum Dis,** v. 67, n. 6, p. 801-7, Jun 2008. ISSN 1468-2060 (Electronic)
0003-4967 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17974596 >.

[38] PLOTZ, P. H. The autoantibody repertoire: searching for order. **Nat Rev Immunol,** v. 3, n. 1, p. 73-8, Jan 2003. ISSN 1474-1733 (Print)
1474-1733 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12511877 >.

[39] GILBERT, D. et al. Do naturally occurring autoantibodies participate in the constitution of the pathological B-cell repertoire in systemic lupus erythematosus? **J Autoimmun,** v. 9, n. 2, p. 247-57, Apr 1996. ISSN 0896-8411 (Print)
0896-8411 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8738970 >.

[40] COHEN, P. L.; MALDONADO, M. A. Animal models for SLE. **Curr Protoc Immunol,** v. Chapter 15, p. Unit 15 20, Feb 2003. ISSN 1934-368X (Electronic)
1934-3671 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18432900 >.

[41] RHO, J. H. et al. Human proteins with affinity for dermatan sulfate have the propensity to become autoantigens. **Am J Pathol,** v. 178, n. 5, p. 2177-90, May 2011. ISSN 1525-2191 (Electronic)
0002-9440 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21514432 >.

[42] KADLER, K. E. et al. Collagens at a glance. **J Cell Sci,** v. 120, n. Pt 12, p. 1955-8, Jun 15 2007. ISSN 0021-9533 (Print)
0021-9533 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17550969 >.

[43] EYRE, D. R. The collagens of articular cartilage. **Semin Arthritis Rheum,** v. 21, n. 3 Suppl 2, p. 2-11, Dec 1991. ISSN 0049-0172 (Print)
0049-0172 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1796302 >.

[44] BREWTON, R. G.; MAYNE, R. Mammalian vitreous humor contains networks of hyaluronan molecules: electron microscopic analysis using the hyaluronan-binding region (G1) of aggrecan and link protein. **Exp Cell Res,** v. 198, n. 2, p. 237-49, Feb 1992. ISSN 0014-4827 (Print)

0014-4827 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1729132 >.

[45] VAN ROON, J. A.; LAFEBER, F. P.; BIJLSMA, J. W. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. **Arthritis Rheum,** v. 44, n. 1, p. 3-12, Jan 2001. ISSN 0004-3591 (Print)
0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11212172 >.

[46] LINDSLEY, C. B. et al. IgG anticollagen antibodies in sera of patients with childhood rheumatic diseases [abstract]. **Arthritis & Rheumatism,** v. 23, p. S56, 1980.

[47] GILLIAM, B. E.; CHAUHAN, A. K.; MOORE, T. L. Evaluation of anti-citrullinated type II collagen and anti-citrullinated vimentin antibodies in patients with juvenile idiopathic arthritis. **Pediatr Rheumatol Online J,** v. 11, n. 1, p. 31, 2013. ISSN 1546-0096 (Electronic)
1546-0096 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23987731 >.

[48] BERNTSON, L. et al. Anti-type II collagen antibodies, anti-CCP, IgA RF and IgM RF are associated with joint damage, assessed eight years after onset of juvenile idiopathic arthritis (JIA). **Pediatr Rheumatol Online J,** v. 12, p. 22, 2014. ISSN 1546-0096 (Electronic)
1546-0096 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24944545 >.

[49] ROSENBERG, A. M.; HUNT, D. W.; PETTY, R. E. Antibodies to native and denatured type II collagen in children with rheumatic diseases. **J Rheumatol,** v. 11, n. 4, p. 425-31, Aug 1984. ISSN 0315-162X (Print)
0315-162X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/6332907 >.

[50] HOLMDAHL, R. et al. Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. **Arthritis Rheum,** v. 29, n. 3, p. 400-10, Mar 1986. ISSN 0004-3591 (Print)
0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2421741 >.

[51] DOBRITZSCH, D. et al. Crystal structure of an arthritogenic anticollagen immune complex. **Arthritis Rheum,** v. 63, n. 12, p. 3740-8, Dec 2011. ISSN 1529-0131 (Electronic)
0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22127694 >.

[52] RAPOSO, B. et al. Epitope-specific antibody response is controlled by immunoglobulin V(H) polymorphisms. **J Exp Med,** v. 211, n. 3, p. 405-11, Mar 10 2014. ISSN 1540-9538 (Electronic)
0022-1007 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24534192 >.

[53] NANDAKUMAR, K. S. et al. Arthritogenic antibodies specific for a major type II collagen triple-helical epitope bind and destabilize cartilage independent of inflammation. **Arthritis Rheum,** v. 58, n. 1, p. 184-96, Jan 2008. ISSN 0004-3591 (Print)
0004-3591 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18163493 >.

[54] HOLMDAHL, R. et al. Multiple epitopes on cartilage type II collagen are accessible for antibody binding in vivo. **Autoimmunity,** v. 10, n. 1, p. 27-34, 1991. ISSN 0891-6934 (Print)
0891-6934 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1720677 >.

[55] ROWLEY, M. J.; NANDAKUMAR, K. S.; HOLMDAHL, R. The role of collagen antibodies in mediating arthritis. **Mod Rheumatol,** v. 18, n. 5, p. 429-41, 2008. ISSN 1439-7595 (Print)
1439-7595 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18521704 >.

[56] BENDELE, A. Animal models of rheumatoid arthritis. **J Musculoskelet Neuronal Interact,** v. 1, n. 4, p. 377-85, Jun 2001. ISSN 1108-7161 (Print)
1108-7161 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15758488 >.

[57] LINDH, I. et al. Type II collagen antibody response is enriched in the synovial fluid of rheumatoid joints and directed to the same major epitopes as in collagen induced arthritis in primates and mice. **Arthritis Res Ther,** v. 16, n. 4, p. R143, 2014. ISSN 1478-6362 (Electronic)
1478-6354 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25005029 >.

[58] SUPURAN, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. **Nat Rev Drug Discov,** v. 7, n. 2, p. 168-81, Feb 2008. ISSN 1474-1784 (Electronic)
1474-1776 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18167490 >.

[59] ZIMMERMAN, U. J. et al. Anti-oxidative response of carbonic anhydrase III in skeletal muscle. **IUBMB Life,** v. 56, n. 6, p. 343-7, Jun 2004. ISSN 1521-6543 (Print)
1521-6543 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15370882 >.

[60] ROBERT-PACHOT, M. et al. Carbonic anhydrase III: a new target for autoantibodies in autoimmune diseases. **Autoimmunity,** v. 40, n. 5, p. 380-9, Jul 2007. ISSN 0891-6934 (Print)
0891-6934 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17612900 >.

[61] LIU, C. et al. Carbonic anhydrases III and IV autoantibodies in rheumatoid arthritis, systemic lupus erythematosus, diabetes, hypertensive renal disease, and heart failure. **Clin Dev Immunol,** v. 2012, p. 354594, 2012. ISSN 1740-2530 (Electronic)
1740-2522 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23049597 >.

[62] SMITH, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. **Science,** v. 228, n. 4705, p. 1315-7, Jun 14 1985. ISSN 0036-8075 (Print)
0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/4001944 >.

[63] BRATKOVIC, T. Progress in phage display: evolution of the technique and its application. **Cell Mol Life Sci,** v. 67, n. 5, p. 749-67, Mar 2010. ISSN 1420-9071 (Electronic)
1420-682X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20196239 >.

[64] CHO, W.; FOWLER, J. D.; FURST, E. M. Targeted binding of the M13 bacteriophage to thiamethoxam organic crystals. **Langmuir,** v. 28, n. 14, p. 6013-20, Apr 10 2012. ISSN 1520-5827 (Electronic)
0743-7463 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22404231 >.

[65] HESS, G. T. et al. M13 bacteriophage display framework that allows sortase-mediated modification of surface-accessible phage proteins. **Bioconjug Chem,** v. 23, n. 7, p. 1478-87, Jul 18 2012. ISSN 1520-4812 (Electronic)
1043-1802 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22759232 >.

[66] BARBAS, C. F. I. et al. **Phage Display: A Laboratory Manual.** New York: Cold Spring Harbor Laboratory 2001.

[67] HOLLIGER, P.; RIECHMANN, L.; WILLIAMS, R. L. Crystal structure of the two N-terminal domains of g3p from filamentous phage fd at 1.9 A: evidence for conformational lability. **J Mol Biol,** v. 288, n. 4, p. 649-57, May 14 1999. ISSN 0022-2836 (Print)
0022-2836 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10329170 >.

[68] RODI, D. J.; MAKOWSKI, L. Phage-display technology--finding a needle in a vast molecular haystack. **Curr Opin Biotechnol,** v. 10, n. 1, p. 87-93, Feb 1999. ISSN 0958-1669 (Print)
0958-1669 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10047512 >.

[69] MESSING, J. New M13 vectors for cloning. **Methods Enzymol,** v. 101, p. 20-78, 1983. ISSN 0076-6879 (Print)
0076-6879 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/6310323 >.

[70] HOFFMANN, S. et al. Competitively selected protein ligands pay their increase in specificity by a decrease in affinity. **Mol Biosyst,** v. 6, n. 1, p. 126-33, Jan 2010. ISSN 1742-2051 (Electronic)
1742-2051 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20024074 >.

[71] ZHANG, J. et al. Phage-derived fully human antibody scFv fragment directed against human vascular endothelial growth factor receptor 2 blocked its interaction with VEGF. **Biotechnol Prog,** v. 28, n. 4, p. 981-9, Jul 2012. ISSN 1520-6033 (Electronic)
1520-6033 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22581629 >.

[72] ARAUJO, T. G. et al. A novel highly reactive Fab antibody for breast cancer tissue diagnostics and staging also discriminates a subset of good prognostic triple-negative breast cancers. **Cancer Lett,** v. 343, n. 2, p. 275-85, Feb 28 2014. ISSN 1872-7980 (Electronic)
0304-3835 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24099914 >.

[73] BRÍGIDO, M. M.; MARANHÃO, A. Q. Bibliotecas apresentadas em fagos. **Biotecnologia Ciência & Desenvolvimento**, n. 26, p. 44-51, 2002.

[74] FELICIANO, N. D. et al. Bacteriophage-fused peptides for serodiagnosis of human strongyloidiasis. **PLoS Negl Trop Dis,** v. 8, n. 5, p. e2792, 2014. ISSN 1935-2735 (Electronic)
1935-2727 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24874206 >.

[75] LIU, B. et al. Towards proteome-wide production of monoclonal antibody by phage display. **J Mol Biol,** v. 315, n. 5, p. 1063-73, Feb 1 2002. ISSN 0022-2836 (Print)
0022-2836 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11827476 >.

[76] GARET, E. et al. Rapid isolation of single-chain antibodies by phage display technology directed against one of the most potent marine toxins:

Palytoxin. **Toxicon,** v. 55, n. 8, p. 1519-26, Jul 2010. ISSN 1879-3150 (Electronic)
0041-0101 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20223256 >.

[77] ALVES, P. T. et al. Revisiting the CD14: Epitope mapping by Phage Display. **Immunobiology,** v. 219, n. 11, p. 822-9, Nov 2014. ISSN 1878-3279 (Electronic)
0171-2985 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25154906 >.

[78] SANTOS, P. S. et al. Functional epitope core motif of the Anaplasma marginale major surface protein 1a and its incorporation onto bioelectrodes for antibody detection. **PLoS One,** v. 7, n. 3, p. e33045, 2012. ISSN 1932-6203 (Electronic)
1932-6203 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22427942 >.

[79] NORONHA, E. J.; WANG, X.; FERRONE, S. Isolation of human tumor-associated cell surface antigen-binding scFvs. **Methods Mol Biol,** v. 178, p. 227-33, 2002. ISSN 1064-3745 (Print)
1064-3745 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11968492 >.

[80] D'MELLO, F.; HOWARD, C. R. An improved selection procedure for the screening of phage display peptide libraries. **J Immunol Methods,** v. 247, n. 1-2, p. 191-203, Jan 1 2001. ISSN 0022-1759 (Print)
0022-1759 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11150550 >.

[81] GASKIN, D. J. et al. Phage display combinatorial libraries of short peptides: ligand selection for protein purification. **Enzyme Microb Technol,** v. 28, n. 9-10, p. 766-772, Jun 7 2001. ISSN 1879-0909 (Electronic)
0141-0229 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11397457 >.

[82] RHYNER, C. et al. Phage display of human antibodies from a patient suffering from coeliac disease and selection of isotype-specific scFv against gliadin. **Immunology,** v. 110, n. 2, p. 269-74, Oct 2003. ISSN 0019-2805 (Print)
0019-2805 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14511241 >.

[83] BAZAN, J.; CALKOSINSKI, I.; GAMIAN, A. Phage display--a powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. **Hum Vaccin Immunother,** v. 8, n. 12, p. 1817-28, Dec 1 2012. ISSN 2164-554X (Electronic)
2164-5515 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22906939 >.

CAPÍTULO II

Improved serological detection of rheumatoid arthritis: a highly antigenic mimotope of Carbonic Anhydrase III selected in a murine model by phage display*

*Este capítulo está formatado como Artigo Original de acordo com as normas do periódico *Arthritis Research & Therapy* (com algumas alterações estruturais para melhor se adequar ao formato da Tese)

Improved serological detection of rheumatoid arthritis: a highly antigenic mimotope of Carbonic Anhydrase III selected in a murine model by phage display

Galber Rodrigues Araujo[1,2*], Emília Rezende Vaz[1], Patricia Tieme Fujimura[1], João Eurico Fonseca[3,4], Lucélia Maria de Lima[1], Helena Canhão[3,4], Gabriela Venturini[5], Karina Helena Morais Cardozo[6], Valdemir Melechco Carvalho[6], Marcelo Henrique Napimoga[7], Luiz Ricardo Goulart[1,8], João Gonçalves[2,9], Carlos Ueira-Vieira[1]

[1]Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

[2]iMed.UL – Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

[3]Rheumatology Research Unit, Institute of Molecular Medicine, Lisbon, Portugal

[4]Rheumatology Department, Santa Maria Hospital, Lisbon Academic Medical Center, Lisbon, Portugal

[5]Laboratory of Genetics and Molecular Cardiology, Heart Institute, University of São Paulo Medical School, São Paulo, SP, Brazil

[6]Fleury group, São Paulo, SP, Brazil

[7]Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas, SP, Brazil

[8]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA

[9]IMM – Institute of Molecular Medicine, Lisbon, Portugal

*Corresponding author:

E-mail: galber.araujo@gmail.com

Abstract

Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects around 1% of the human population worldwide. RA diagnosis can be difficult by the fact that there is no definitive test for its detection. Therefore, the aim of this study was to identify biomarkers that could be used for RA diagnosis.

Methods: Sera from collagen-induced arthritis (CIA) mouse model were used to select potential biomarkers for RA diagnosis by Phage Display technology. *In silico* and *in vitro* analyses were performed to characterize and validate the selected peptides. Samples were classified into three groups: RA, two other immune-mediated rheumatic diseases (systemic lupus erythematosus – SLE and ankylosing spondylitis – AS) and healthy controls (HC). ELISA assay was carried out to determine antibody levels, and diagnostic parameters were determined by constructing Receiver Operating Characteristic (ROC) curves. Mass spectrometry and western blot were performed to identify the putative autoantigen that was mimicked by a highly reactive mimotope.

Results: After three rounds of selection, fourteen clones were obtained and tested for immunoreactivity analysis against sera from RA and HC groups. The phage-fused peptide with the highest immunoreactivity (M12) was synthesized, and was able of efficiently discriminate RA patients from SLE, AS and HCs ($p < 0.0001$) by ELISA. The specificity and sensitivity of anti-M12 antibodies for RA diagnosis were 91% and 84.3%, respectively. The M12 peptide was identified as a peptide that mimics a predicted antigenic site of the carbonic anhydrase III (CAIII) protein, a ubiquitous biomarker that has been identified in patients with other diseases.

Conclusion: M12 is the first peptide associated to CAIII protein that may be used as an antigen for antibodies detection to aid in RA diagnosis with high sensitivity and specificity.

Keywords: Rheumatoid Arthritis, CIA mice model, Carbonic Anhydrase III, Autoantibodies

Introduction

Rheumatoid arthritis (RA), the most common inflammatory autoimmune disease, affects 0.8% of the adult population worldwide [1]. RA diagnosis is largely a clinical one, relying, particularly in the early stages, on the history and examination of the patient, with tests (blood or imaging) sometimes helping to confirm the diagnosis [2]. Serological support to diagnosis has, up to now, been restricted to the determination of rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPAs), where assays using cyclic citrullinated peptides (CCP) as antigen for ACPAs detection have gained wide acceptance [3]. RF presents higher sensitivity as compared to anti-CCP antibodies for the established disease, with a relatively low specificity. In fact, the RF antibody is not specific for RA due to cross-reactivity with many other inflammatory diseases as well as in elderly healthy individuals [4]. ACPAs are considered a valuable serological biomarker for RA [5] and the diagnostic performance of different generations of CCPs (CCP1; CCP2 and CCP3) have been evaluated in many different studies [6-8]. Differences in cut-off values, specificities and sensitivities exist between the three different generations and also between different assays used to antibodies detection. However, anti-CCP2 showed better performance characteristics with values of sensitivity ranging from 41% to 92.2% and specificity ranging from 65% to 100% [9]. At present, the detection of antibodies against CCP2 by ELISA is the most widely used assay in studies involving ACPAs worldwide. The combination of RF and anti-CCP2 assays demonstrate a positive predictive value close to 100%, which is much higher than the value of either of the tests alone [10]. The presence of RF and anti-CCP has been associated with progressive and destructive disease [11, 12]. Seronegativity in both early and established RA remains a major limitation of these two biomarkers, highlighting the need for new complementary markers that could improve diagnostic sensitivity [13]. Because of the low sensitivity or specificity of the current serological tests, the quest for new efficient auxiliary biomarkers in RA is of clinical relevance.

Animal models of arthritis have contributed to the overall knowledge on RA physiopathology and to the identification of important mediators of inflammation. The Collagen-induced arthritis (CIA) mouse model has proven to be a valuable experimental model for inflammatory RA studies [14-17]. After immunization with

type II collagen (CII), DBA/1J mice develop a severe polyarthritis mediated by an autoimmune response that shares many features with human RA [18].

With the goal of identifying new clinically useful biomarkers for RA, we have explored the CIA mouse model and Phage Display (PD) technology to isolate peptides that can mimic RA autoantigens. PD technology has been widely used by our group and others to screen targeting peptides in drug discovery and biomarkers selection, and has been highly effective in discovering peptides with affinities to virtually any target [19-24]. Short peptide sequences selected by PD libraries with high affinity to antibodies, receptors or proteins may present potential applications in diagnostics or therapeutics kits and vaccines [25, 26]. Using the cDNA phage display library for autoantigen selection, were recently identified novel autoantibodies in early and seronegative RA patients with sensitivities ranging from 2% to 29%, and specificities ranging from 95% to 100%. These autoantibodies can be found in 44% to 67% ACPA negative RA patients [27].

This investigation describes the identification of a short peptide selected by PD technology against sera from CIA mice. This short peptide was characterized by *in silico* and *in vitro* strategies, and further tested as a potential biomarker for RA diagnosis in comparison to other rheumatic diseases and healthy controls. Its predicted antigenic epitope target was deduced by mass spectrometry and western blot analyses.

Material and Methods

Study subjects

For this study, we used serum samples from patients who fulfilled the RA diagnostic criteria according to the 2010 classification [28]. The demographic and laboratory characteristics of all studied subjects are presented in Table 1. Well-characterized serum samples used in this study were requested from Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. Samples from healthy subjects who did not have any arthritis symptoms were used as the healthy control (HC) group. To provide data on assay specificity, samples from patients with other rheumatic diseases (non-RA) were also analyzed. The subjects enrolled in this study were classified into three groups: RA: 172 patients (151 females and 21

males, mean age of 53.7 ± 11.2); HC: 113 subjects (79 females and 34 males, mean age of 58.8 ± 8.7); and Non-RA: 32 patients with other immune-mediated rheumatic diseases that consisted of 19 systemic lupus erythematosus (SLE) and 13 ankylosing spondylitis (AS) patients. For RA patients, data recorded were: age at disease diagnosis, disease duration, medication status, presence of RF and anti-CCP, erythrocyte sedimentation rate (ESR), levels of C-reactive protein (CRP), tender and swollen joint counts were obtained at the time of blood sample collection. Blood samples were allowed to clot and then centrifuged at 4,000 rpm for 10 min. All sera samples were filtrated with microcell filter (0.22 μm) to eliminate red blood cell fragments and bacteria, and then frozen at -80°C immediately until analysis. The parts of the study which involved human subjects was approved by the ethics committees of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria and the Hospital Garcia de Orta, Lisbon, Portugal, and conducted in accordance with the regulations governing clinical trials such as the Declaration of Helsinki (2008). Informed consent form was signed by all subjects enrolled in this study before any protocol procedure was carried out.

Table 1 Demographic and laboratory characteristics of the studied population

	RA	HC	SLE	AS
Mean age ± SD	53.7 ± 11.2	58.8 ± 8.7	55.7 ± 5.7	49.5 ± 7.5
Gender (F/M)	151/21	79/34	16/3	4/9
Caucasian, *n* (%)	146 (85 %)	104 (92 %)	18 (95 %)	11 (85 %)
Mean disease duration (years) ± SD	8.1 ± 8.4		9.2 ± 6.5	12 ± 9.1
Rheumatoid factor positives (*n*)	102 (59.3 %)		†	†
CRP ± SD (mg/dL)	2.4 ± 1.1		3.1 ± 0.9	2.7 ± 0.8
ESR ± SD (mm/hr)	32 ± 26		33.4 ± 19.5	†
Anti-CCP positives (n)	75 (43.6 %)		†	†
Biologic therapy (n of patients in treatment)	62 (36 %)		4 (21 %)	3 (23 %)
NSAIDs (*n* of patients in treatment)	127 (74 %)		9 (47 %)	5 (38 %)

Abbreviations: *RA* rheumatoid arthritis, *HC* healthy control, *SLE* systemic lupus erythematosus, *AS* ankylosing spondylitis, *SD* standard deviation, *F* female, *M* male, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *anti-CCP* Anti-cyclic citrullinated peptide antibodies, *NSAIDs* nonsteroidal anti-inflammatory drugs. CRP value of ≥ 0.8 mg/dL is considered elevated. ESR value of ≥ 15 mm/hr is considered elevated. † Values not available

CIA Mice

A total of twelve male DBA/1J mice (8–10 weeks) were housed at the animal facility of the Federal University of Uberlândia (Uberlândia-MG, Brazil) where they fed *ad libitum*. This study was carried out in strict accordance with the recommendations contained in terms for the use of animals in research and teaching of the Federal University of Uberlândia (Uberlândia, MG, Brazil), in compliance with the National Guidelines, as set forth by the Institutional Animal Care (Law number 11.794, 2008). The study protocol was approved by the Animal Ethics Committee from the Federal University of Uberlândia (UFU) under the number CEUA/UFU 059/10.

Arthritis induction and assessment of CIA mice

Arthritis was induced in the isogenic murine model DBA/1J (males; 8-10 weeks old) as described previously with some modifications [18]. Briefly, a commercial bovine CII (Becton Sigma-Aldrich, St. Louis, MO) emulsified in complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) was used for mice immunization. DBA/1J mice (n = 9) were immunized by intradermal injections at the base of the tail with 100 µg of bovine CII. For the control group, mice (n = 3) were immunized with PBS in CFA. Mice were boosted at day 30 with 50 µg bovine CII in CFA. After immunization, mice were examined twice a week when paw edema was measured using manual calipers and arthritis signs were visually scored by evaluating joint inflammation, using an established scoring system from 0 to 4 [29], where: 0 = no evidence of erythema and swelling, 1 = erythema and mild swelling confined to the tarsals or ankle joints, 2 = erythema and mild swelling extending from the ankle to the tarsals, 3 = erythema and moderate swelling extending from the ankle to metatarsal joints, 4 = erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb. Around sixty days after immunization, mice presenting acute arthritis (score 4) were sacrificed under anesthesia. Blood (for sera extraction) and inflamed joints were then extracted for peptides selection through PD technology.

Total protein extraction from inflamed joints of CIA mice

Inflamed joints were removed from CIA mice, macerated with liquid nitrogen and suspended in extraction buffer (20mM Tris-HCl pH7.2, 10mM EDTA, 2mM EGTA, 250mM sucrose, 1mM DTT, 1mM Benzamidine, 1mM PMSF). The resulting material was transferred to a microtube, and centrifuged at 20,000 x *g* for 30 minutes at 4°C. The supernatant was collected and concentration of the extracted proteins was determined by the Bradford method [30]. As many inflammatory cytokines indicative of RA are expressed in the cartilage, ligament, pannus, articular capsule etc., of CIA mice [31], we used these proteins to dissociate the selected phage clones from target antibodies by competitive elution.

Purification and isolation of Immunoglobulin G from sera

Immunoglobulin G (IgG) purification and isolation from sera of mice with acute arthritis and naïve were performed with Dynabeads® Protein G, purchased from Invitrogen (Carlsbad, CA), following the manufacturer's instructions. Briefly, a total of 50 µl of magnetic beads were washed twice with TBS-T 0.1% (Tris-Buffered Saline, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 plus 0.1% Tween 20) and then incubated with 100 µl of pooled serum for 1 h at room temperature (RT). After binding, the beads-IgG complex was blocked with TBS plus 5% BSA at 37ºC for 1 h, washed three times with TBS-T 0.1% and then resuspended in 200 µl of TBS.

Mimotopes selection through Phage Display

For mimotopes (phages expressing peptides on its surface) selection a PhD-12mer phage display peptide library kit (New England Biolabs, Beverly, MA, USA) was screened against IgG purified from CIA mice. This is a combinatorial library of random dodecapeptides fused to the N-terminus of the minor coat protein (pIII) of M13 phages. The library consists of 2.7×10^9 diverse sequences that were amplified once to yield about 50 copies of each peptide sequence.

Based on scientific evidences showing that CIA mice and RA patients share several pathological features; circulating autoantibodies to common targets [29], and also because arthritis can be much easier monitored in experimental animal models than in human due the complexity of the symptoms, we decided to perform the mimotopes selection against IgG purified from CIA mice.

The strategy adopted for the mimotopes selection consisted in a subtractive step to remove nonspecific phages by pre-incubating the phage peptide library with IgG purified from naïve mice. In order to remove nonspecific phages, a volume of 7×10^8 beads/ml coupled with IgG purified from naïve mice serum was incubated with 1×10^{11} phage particles from the PhD-12 library in 200 µl of TBS-T 0,1% solution for 30 min at RT. After paramagnetic beads precipitation by using the Magnetic Particle Concentrator (Dynal MPC™ - Invitrogen), unbound phages were collected and incubated with beads coupled with IgG purified from CIA mice presenting signs of acute arthritis, following incubation for 30 min at RT. The unbound phages were discarded this time by washing ten times with TBS-T 0.1%. For competitive elution, the bound phages were incubated for 30 min with 10 µg of the total protein extracted from inflamed joints of CIA mice. The eluted phages were amplified in *E. coli* ER2738 strain (New England Biolabs, Beverly, MA, USA) and purified by PEG-NaCl precipitation. After each of the three rounds of selection, individual bacterial colonies containing amplified phage clones were grown in a microtiter plate and titrated as described elsewhere [32].

DNA extraction and sequencing

Phages DNA were isolated from 1-ml overnight cultures by precipitation with 1/6 vol PEG/NaI (20% w/w, polyethylene glycol 8000) and iodide buffer [10 mm Tris-HCl (pH 8.0), 1 mm EDTA, and 4 m NaI]. Phage DNA was precipitated with absolute ethanol, followed by wash with 70% ethanol, and resuspended in 20 µl of Milli-Q water. Electrophoresis was performed on 0.8% agarose gel stained with ethidium bromide solution in order to verify DNA quality. Sequencing reactions were carried out by using the DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare), with the primer -96 M13 (5'-OH CCC TCA TAG TTA GCG TAA CG-3') following the manufacturer's instructions and detection was performed in a MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer.

Bioinformatic analysis

A tool that can be found on the Sequence Manipulation Suite collection of JavaScript programs [33] was utilized to obtain the reverse complementary

sequences of the DNA extracted from phages. Amino acid sequences were deducted by ExPASy Proteomics and Sequence Analysis tool (http://web.expasy.org/translate/) [34]. For sequence similarity analysis, multiple sequence alignment was performed by ClustalW2 online server (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Three-dimensional structure prediction was performed using The Pepitope Server (http://pepitope.tau.ac.il/) [35], and Immune Epitope Database and Analysis Resource (http://tools.iedb.org/mainp/). PyMOL (available at http://www.pymol.org) plugin was used for showing the peptide surface. Antigenicity prediction was carried out using Kolaskar and Tongaonkar antigenicity scale (http://tools.immuneepitope.org/bcell/) [36].

Immunoreactivity of the selected mimotopes by Phage-ELISA

For immunoreactivity measurements of selected mimotopes against sera from RA and HC groups, a phage-ELISA assay was performed. A ninety-six-well Maxisorp™ microtiter plate (NUNC, NY, USA) was coated in triplicates with anti-M13 monoclonal antibody (Amersham Biosciences, Little Chalfont, UK) diluted (1:100) in carbonate buffer (0.1 M $NaHCO_3$, pH 8.6) overnight at 4ºC. The plate was washed once with TBS-T 0.5% and blocked for 1h at 37ºC with 5% BSA diluted in TBS. Additionally, the plate was washed twice and incubated with culture supernatant containing amplified phage particles (~ 10^{11} pfu/µL) for 1h at 37ºC. The plate was washed three times followed by incubation with serum pools of RA and HC groups diluted (1:100) in TBS-T 0.5% plus 5% BSA for 1h at 37ºC. The plate was washed three times more with TBS-T 0.5% followed by incubation with HRP-conjugated rabbit anti-human IgG (Roche Applied Science) diluted (1:5000) in TBS-T 0.5% plus 5% BSA for 1h at 37ºC. The ELISA plate was washed three times, revealed with OPD SigmaFast™ (Sigma-Aldrich) and read at 492 nm. The reactivity obtained by the wild-type M13 phage without displaying any peptide (performed for each sample tested) was used for data adjustment, where the final optical density (OD) values obtained for each mimotope was adjusted by subtracting the corresponding OD values obtained by the wild-type M13 phage.

Purification of human anti-M12 antibody and M12 mimotope by immunoprecipitation

For purification of IgG antibodies that bind to the M12 mimotope, 1×10^{11} of M12 phage particles were covalently bound to Dynabeads® (Invitrogen). Thereafter, a volume of 50 µL of the solution containing the beads-M12 complex was separately incubated with 100 µL of pooled sera from RA patients and HCs for 1h at RT under shaking for IgG:M12 binding. After incubation, the complex was washed ten times with TBS-T 0.1% and the unbound non-specific IgG present in the supernatant were discarded. The IgG that bound with high affinity to the M12 phage clone were eluted with 100 µL of elution buffer (0.2 M Glycine-HCl, pH 2.2 and BSA 1 mg/mL) after incubation for 10 min at RT, followed by neutralization with 15 µL of 1 M Tris-HCl (pH 9.1). The eluted IgG were incubated with 50 µL of magnetic beads, as previous described. Subsequently, the solution containing the bead-IgG complex was incubated with 1 µg of total protein extracted from inflamed joints of CIA mice for 1h at RT, under shaking condition for protein binding. The unbound proteins were discarded by washing ten times with TBS-T 0.1%, followed by elution of the bound proteins with 100 µl of 0.8 M acetic acid (pH 2.0). Proteins eluted were dried and submitted to mass spectrometry analysis.

Western blot analysis

Total proteins extracted from inflamed joints of CIA mice (1 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 1 h with 3% BSA in PBS, and then rinsed three times in washing buffer containing PBS-T 0.1%. Thereafter, the membrane was incubated overnight at 4°C with IgG purified from sera of RA patients and HCs diluted at 100 µg/ml in PBS plus 3% BSA, washed three times and incubated for 1 h with rabbit anti-human IgG conjugated with peroxidase (Roche Applied Science) diluted at 1:5000 in PBS plus 3% BSA. The subsequent washing steps and detection procedures were performed according to the ECL Plus manual (GE Healthcare).

Mass Spectrometry

Protein digestion: dried proteins were suspended in 30 µL of 0.2% RapiGest and vortexed for 5 min. Protein digestion was carried out as described elsewhere with few modifications [37]. Briefly, 10 µL of 50 mM Ammonium Bicarbonate was added to the protein suspension to a final volume of 40 µL. Protein samples were denatured with 25 µL of 0.2% (w:v) RapiGest SF for 15 min at 80°C, reduced with 2.5 µL of 100 mM dithiothreitol at 60°C for 30 min, alkylated with 2.5µL of 300 mM iodoacetamide at RT, and enzymatically digested at 37°C overnight with trypsin at a 1:100 (w/w) enzyme:protein ratio. Then, 10 µL of 5% trifluoroacetic acid was added to the digestion mixture to hydrolyze the RapiGest, and samples were incubated at 37°C for 90 min. The tryptic peptide solution was then centrifuged at 14,000 rpm for 30 min at 6°C, and the pH of the supernatant was adjusted to 2.6 by the addition of 10 µL 3% Acetonitrile 0,1% Formic Acid.

Liquid chromatography/mass spectrometry analysis: separation of peptides was performed using nano liquid chromatography employing reversed-phase. Peptides were injected into nanoLC through a nanoACQUITY system (Waters). Samples were first trapped in a Symmetry C18 5 µm, 180mm x 20mm column (Waters) with 0.1% TFA in 3% acetonitrile, then peptides were eluted from the trap column to a HSS T3 1.8 µm, 75 µm × 15 cm analytical column (Waters) using as mobile phase A: water with 0.1 % formic acid and B: 0.1% formic acid in acetonitrile. Mass spectrometric acquisition was achieved in a Synapt MS Q-TOF mass spectrometer equipped with a NanoLockSpray source in the positive ion mode (Waters, Manchester, UK). For all measurements, the mass spectrometer was operated in the ‘V’ mode with a typical resolving power of at least 12,500. Data-independent scanning (MSE) experiments were performed by switching between low (3 eV) and elevated collision energies (15–50 eV) applied to the trap ‘T-wave’ cell filled with argon. Scan times of 0.8 s were used for low- and high-energy scans from m/z 50 to 2000.

Protein identification and database analysis: protein identification was performed in Global Server v.2.5 (PLGS) with mouse UniProtKB Complete Proteome database. Maximum missed cleavages by trypsin allowed were up to one, fixed modification by carbamidomethylation (cysteine) and variable modifications by acetyl N-terminal and oxidation (methionine) were considered.

The precursor and fragment ion mass error tolerances were adjusted to 10 and 20 ppm, respectively (default values). The criteria used for a positive protein match were at least three fragment ions per peptide, seven fragment ions per protein, and at least one peptide per protein hit. A false-positive discovery rate was allowed up to 4%.

Peptide design and synthesis

After bioinformatics analysis of the selected clones, the M12 peptide sequence was designed and chemically synthesized by GenScript USA Inc (Piscataway, NJ, USA). The peptide was constructed with 17 residues (CNVNSKSPVERITGGGS), with amidation of the C-terminal and conjugation of the Bovine Serum Albumin to cysteine at the N-terminus, a design strategy to increase sensitivity and decrease cross-reactivity in ELISA assay [38].

Anti-M12 antibodies detection by ELISA

Specific ELISA test was carried out to determine the M12 synthetic peptide reactivity to sera from patients with RA, SLE, AS and HCs. Ninety-six-well Maxisorp^{TM} microtiter plates (NUNC, NY, USA) were coated with synthetic M12 (1.5 µg/ml) in carbonate buffer (0.1 M $NaHCO_3$, pH 8.6), and incubated overnight at 4°C. After blocking with 3% BSA in PBS at 37°C for 1h, 100 µL/well of sera from the different groups were diluted in blocking buffer (1:100) and incubated for 1h at 37°C under gentle agitation. After four washes with PBS-T 0.05%, HRP-conjugated rabbit anti-human IgG (Roche Applied Science) diluted (1:5000) in the blocking buffer was incubated for 1h at 37°C under gentle agitation. ELISA plate was washed three times with PBS-T 0.05%, revealed with OPD SigmaFast^{TM} (Sigma-Aldrich) and read at 492 nm. All samples were tested in triplicates. The optimum point of reaction for anti-M12 antibodies detection was determined using the receiver operating characteristic (ROC) curve, where a cut-off point was determined as the value of the parameter corresponding to the highest possible sensitivity without losing specificity. To calculate the ROC curve, sensitivity and specificity we consider the control groups as a single group. Each serum sample was tested without M12 as negative control. The final OD values obtained for each RA, HC, SLE or AS samples were adjusted by subtracting the corresponding OD

value obtained by the negative control. After data adjustment, OD values obtained for each sample from all groups were divided by the cut-off value for data normalization. The values obtained are expressed as reactivity index (RI), where: samples presenting RI ≥ 1 were considered positives.

Statistical analysis

Unpaired *t* test with Welch's correction was used to evaluate the differences of the sera reactivity in ELISA assays among groups for phage clones and the synthetic peptide. Sensitivity and specificity parameters were calculated based on the receiver operating characteristic (ROC) curve analysis, and Fisher's exact test was used for categorical data. To estimate the positive predictive accuracy, the area under the curve (AUC) was also determined. Pearson's correlation was used for analysis among variables. All statistical analyses were performed using GraphPad Prism 5.0 software. *P* values less than 0.05 were considered statistically significant.

Results

Arthritis induction

Arthritis induction was efficient in CIA mice. A total of 80% of male DBA/1J mice developed acute arthritis around 60 days after CII immunization. The severity and incidence of arthritis were assessed as described in materials and methods. No manifestation of arthritis was observed in mice of the control group treated with PBS in CFA. A schematic workflow illustrating the steps employed in this study is shown in Figure 1.

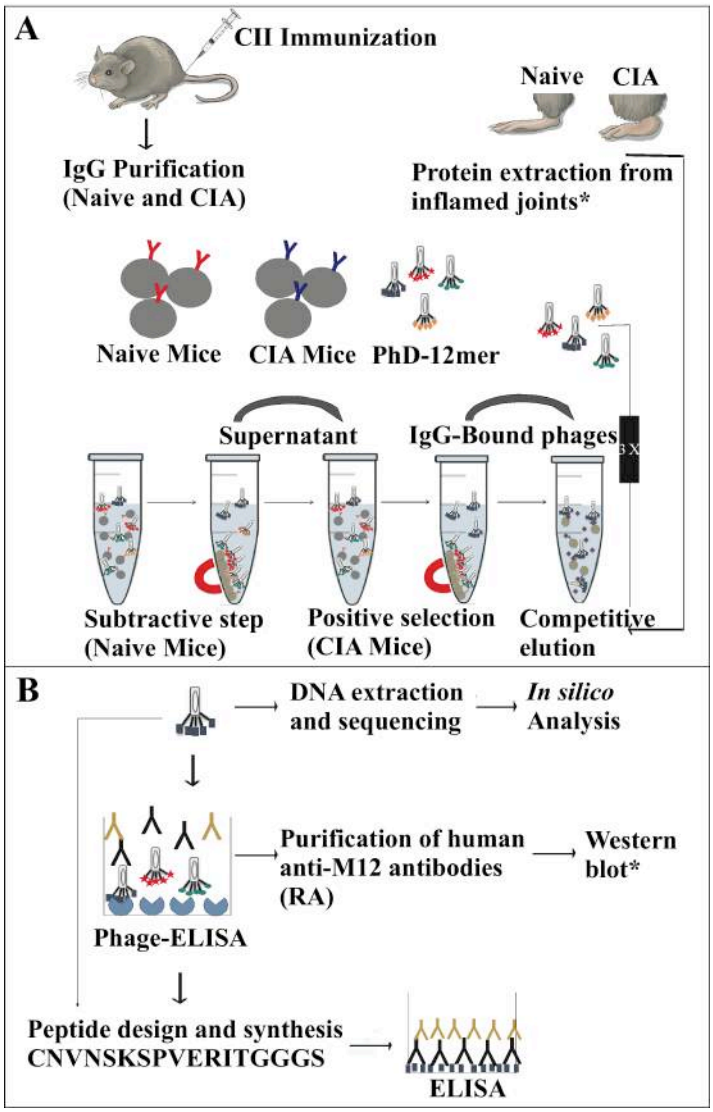


Figure 1 Schematic workflow illustrating the steps involved in this study. **(**A**)** Selection step, and **(**B**)** validation step. Arthritis was induced in CIA mice by immunization with CII. IgG were purified by protein G beads from serum of mice

presenting acute arthritis and naïve. A Phage Display library was used to select mimotopes against purified IgG from CIA mice (3 cycles of biopanning), with a subtractive step against IgG from naïve mice. IgG-bound phages from CIA mice were competitively eluted by incubation with total proteins extracted from inflamed joints. Total proteins extracted from inflamed joints were also used for anti-M12 antibodies recognition in Western blot. Immunoreactivity of each selected mimotopes was tested by phage-ELISA. After DNA extraction, *in silico* and *in vitro* analysis, the most reactive mimotope (M12) was identified as a peptide that mimics a predicted antigenic site of the human CAIII protein. Validation of the M12 synthetic peptide as a possible RA autoantigen was carried out by ELISA assay.

Selection of mimotopes by Phage Display

The enrichment of phages recovered after each round of biopanning was determined by output/input ratio. The increase from 8×10^4 in the first round to 4×10^5 after three rounds of affinity selection, showed a clear enrichment of phage particles (Table 2). These data indicate a successful affinity selection of phages that specifically recognized IgG present in sera of DBA1/J mice with acute arthritis. A total of thirty-seven randomly selected mimotopes were obtained after three rounds of biopanning using a phage displayed 12-mer random peptide library. From the thirty-seven mimotopes selected, fourteen presented different sequences. Alignment analysis revealed some consensus sequence between the mimotopes selected (Figure 2A), indicating that these motifs were positively selected during the biopanning. The selected mimotopes showed different reactivities when tested against human sera by phage-ELISA, and all of them were able to discriminate RA patients from HCs. The reactivity values were very similar for all tested mimotopes, except for M12, which showed the highest values of absorbance (Figure 2B) and the highest difference compared to the other clones ($p < 0.05$). Due to the higher reactivity compared to the other mimotopes; the common motif shared with other five clones, and the highest ratio RA:HC, we focused on M12 peptide for further investigation. However, the other mimotopes selected will be explored in future studies.

Table 2 Enrichment of phage for each round of selection from phage display peptide library.

Round	Number of phage particles		
	Input (cpu[a])	Output (cpu)	Ratio (Output/Input)
1st	1×10^{11}	8×10^{4}	8×10^{-7}
2nd	1×10^{11}	9×10^{3}	9×10^{-8}
3rd	1×10^{11}	4×10^{5}	4×10^{-6}

[a]cpu = phage units

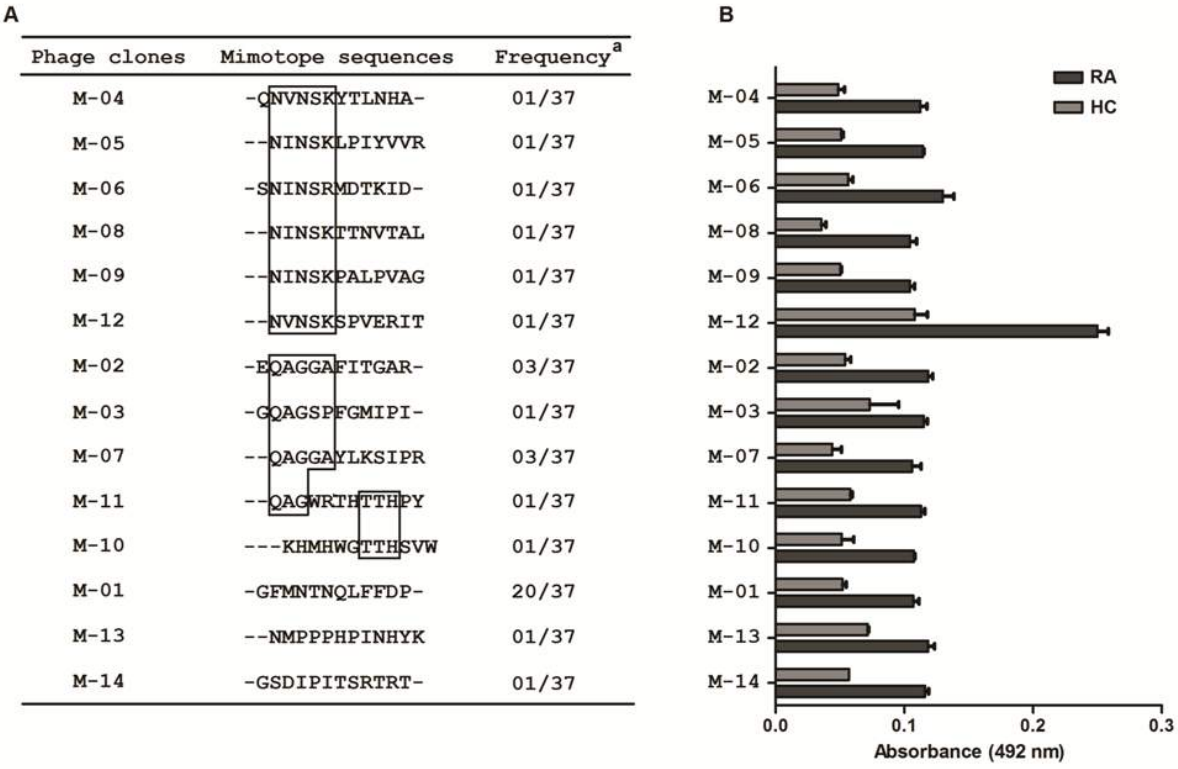


Figure 2 Perfomance of the mimotopes selected by Phage Display. (**A**) Multiple sequence alignment with all selected mimotopes showing their consensus sequences and frequency, and (**B**) reactivity obtained though the interaction of the mimotopes selected and pooled sera from RA patients and HCs. [(a)] Frequency is defined as the ratio of the number of phage clones expressing a common peptide sequence to that of the total phage clones obtained in the biopanning.

Western blot analysis

SDS-PAGE fractionated proteins from inflamed joints of CIA mice were transferred to a nitrocellulose membrane. Western blot revealed that purified IgG from RA patients against the M12 mimotope recognized a protein with a molecular weight of approximately 29 kDa (Figure 3).

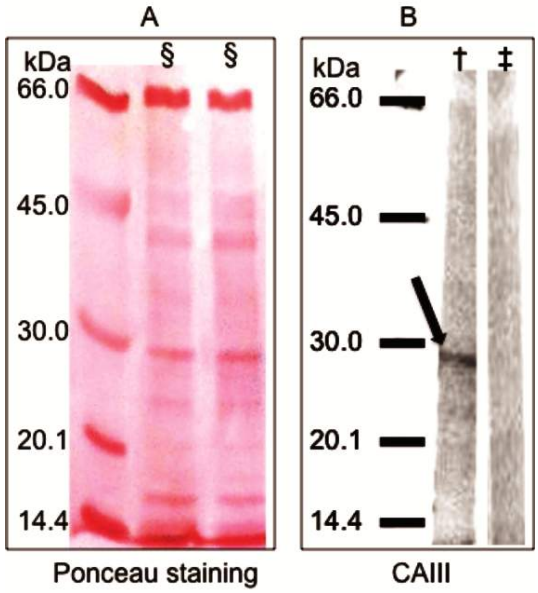


Figure 3 Western blot analyses. (**A**) The Ponceau staining shows 1 µg of total proteins extracted from inflamed joints of CIA mice (§) separated by 10% SDS-PAGE. (**B**) The same membrane stained with Ponceau was probed with anti-M12 antibodies purified from pooled sera of RA patients (†) and HCs (‡). Anti-M12 antibodies displayed strong reactivity with a protein extracted from inflamed joints of CIA mice presenting mass of approximately 29 kDa (arrow). The Carbonic anhydrase III (CAIII) was identified as the protein target that is mimicked by the M12 mimotope. Membrane was cropped in order to allow differential incubation with anti-M12 antibodies purified from RA patients and HCs.

Antigen target and epitope prediction

Mass spectrometry (MS) analysis identified 15 putative proteins associated with anti-M12 IgG, where 14 proteins presented few peptide matches, with very low scores and sequence coverage and none of them presented a molecular mass close to 29 kDa. Therefore, based on the molecular weight of the protein target by anti-M12 antibodies observed in the Western blot results (29 kDa), the alignment of putative proteins with the M12 peptide sequence, and the sequencing coverage

by MS, we have identified the Carbonic anhydrase III (CAIII) [Uniprot/Swiss-Prot:P16015] as the protein target that the M12 peptide mimics. The CAIII protein showed 49.62% of sequencing coverage, including the peptide sequence, which was aligned by conserved and semi-conserved amino acids (aa) residues between 24-35 positions (KGDNQSPIELHT).

The multiple sequence alignment revealed several homologous sequences between M12 peptide and the CAIII protein sequences from mouse and human. Nine (75%) conserved or semi-conserved aa residues of the M12 peptide sequence matched a domain of the CAIII protein (Figure 4A). A 3D structural alignment was performed to predict the putative epitope site of the M12 peptide in the CAIII protein structure [PDB:3UYN], which confirmed its surface exposure and corroborate to the possible antibody-binding region in the external sequences of the predicted protein (Figure 4B). Five (41.6%) aa residues (SPVET) of the M12 peptide was matched to an antigenic region of the human CAIII protein (Figure 5).

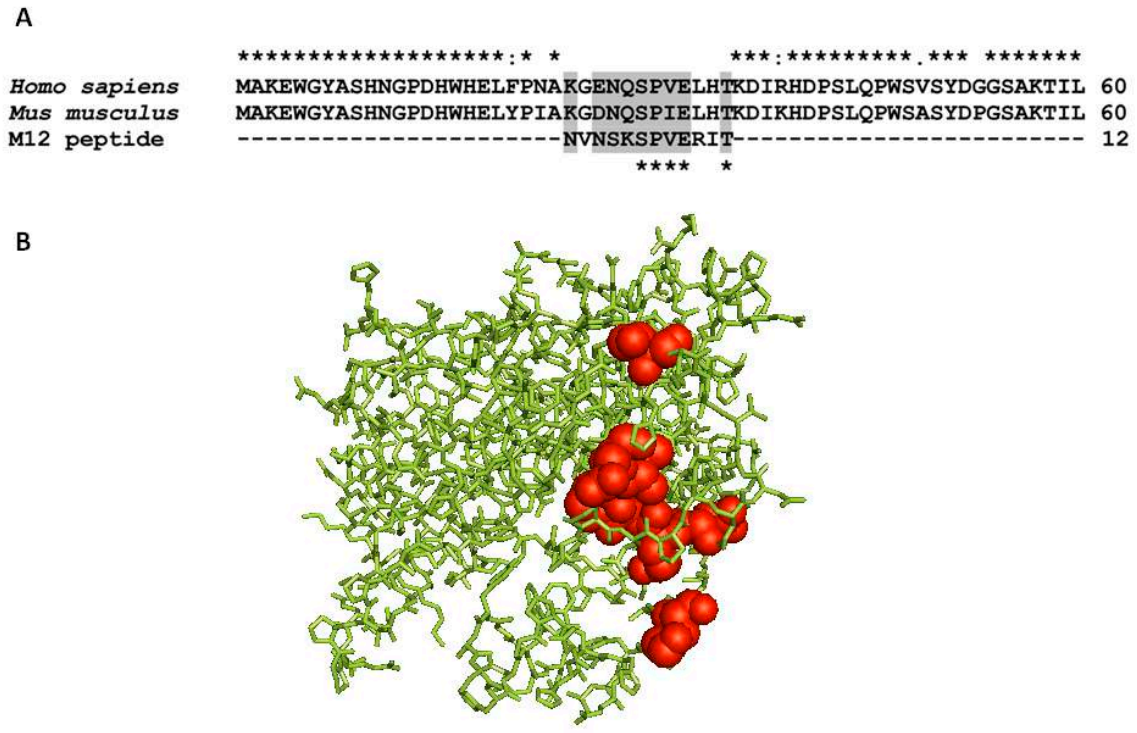


Figure 4 Peptide localization. (**A**) Multiple alignments of M12 peptide and deduced amino acid sequences of carbonic anhydrase III from *Homo sapiens* and *Mus musculus*. Conserved (star marked) and semi-conserved residues are gray highlighted. Valine (V) amino acid is star marked because it matches a position

between M12 peptide and human CAIII protein. (**B**) Model of 3D structure predicted in the PyMol server software for the human carbonic anhydrase III with M12 peptide localization.

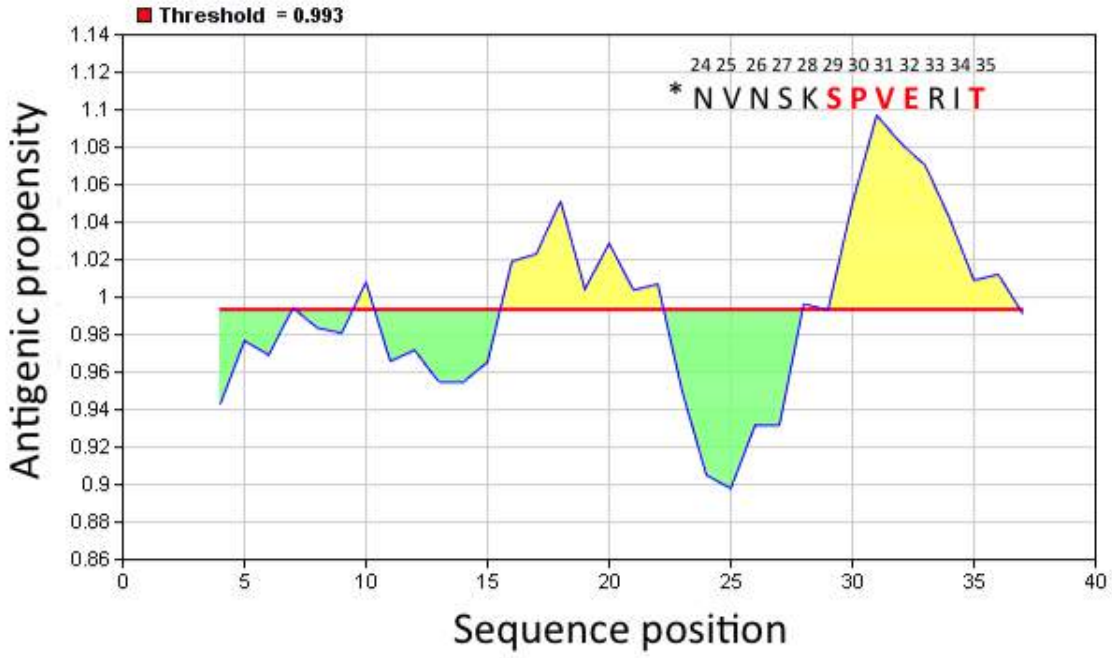


Figure 5 Antigenicity predictions by using Kolaskar-Tongaonkar Algorithm. Threshold (0.993); average (0.993); minimum antigenicity (0.898); maximum antigenicity (1.097). Regions with antigenic propensity scale upper 0.993 are antigenic. Window size and center position were 7 and 4, respectively. *****Localization of M12 peptide in the CAIII protein (aa 24-35). Matched aa residues are presented in red.

Evaluation of the M12 peptide as a possible biomarker for RA diagnosis

The M12 synthetic peptide was tested by ELISA with individual sera obtained from 172 RA patients, 113 HCs, 19 SLE and 13 AS in order to evaluate its potential diagnostic. The cut-off point was determined as the value of the parameter corresponding to the highest sensitivity without lowering the specificity. One hundred forty-five out of the 172 (84.3%) RA patients were positive to anti-M12 antibodies. The synthetic molecule was able to efficiently discriminate sera from RA patients and HCs ($p < 0.0001$), SLE ($p < 0.0001$) and AS ($p < 0.0001$) (Figure 6A). The ROC curve analysis constructed based on the control groups was significant ($p < 0.0001$; AUC = 0.946). Based on the determined cut-off point, M12

synthetic peptide presented specificity of 91% and sensitivity of 84.3% (Figure 6B). For humoral immune response evaluation, 55 (32%) serum samples from RA patients were simultaneously positive for RF, anti-CCP and anti-M12 antibodies; while 45 (26.2%) samples were positive only for anti-M12 and 12 (7%) were negative for the three antibodies (Table 3).

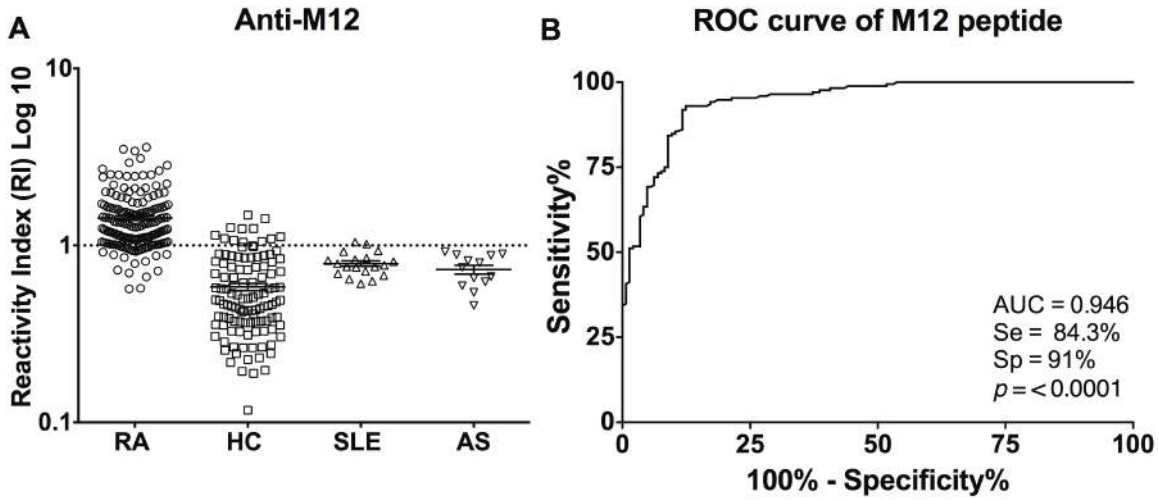


Figure 6 Anti-M12 antibodies detection by ELISA. Detection of anti-M12 antibodies in sera from RA patients (n = 172), healthy controls (n = 113), SLE (n = 19) and AS (n = 13) patients and its respectively ROC curve. (**A**) Sera from the four groups individually tested for their ability to bind to synthetic M12 peptide. Horizontal line = cut-off value; error bars show mean and standard deviation. (**B**) ROC curve constructed based on the control groups. The area under the curve (AUC), sensitivity (Se), specificity (Sp) and corresponding *P*-value are indicated inside the graph.

Table 3 Antibodies positivity in the RA population.

Antibodies detection			Positivity	
Rheumatoid factor	Anti-CCP	Anti-M12	No.	%
+	-	-	8	4.6
-	+	-	0	0
-	-	+	45	26.2
+	+	-	7	4
+	-	+	32	18.6
-	+	+	13	7.6
+	+	+	55	32
-	-	-	12	7
Total			172	100

+ = positive; − = negative

Correlation of anti-M12 humoral response with clinical variables

The weakly associated variables with anti-M12 levels from Gaussian populations (Pearson) analysis were disease duration (r = 0.1753) and the use of biological therapy (r = -0.2934) (Table 4).

Table 4 Clinical variables associated with detection of anti-M12 antibodies in patients with RA.

Variables	Anti-M12		
	Pearson *r*	*P*	*R* squared
Ethnicity	†	†	†
Gender	†	†	†
Age	†	†	†
Disease duration	0.1753	<0.05	0.03072
Rheumatoid factor	†	†	†
ESR	†	†	†
CRP	†	†	†
Anti-CCP	†	†	†
Tender joints	†	†	†
Swollen joints	†	†	†
Biologic therapy	−0.2934	<0.005	0.08616
NSAIDs	†	†	†

Abbreviations: *F/M* Female/Male, *P/N* Positive/Negative, *NSAIDs* nonsteroidal anti-inflammatory drugs. Correlations were performed from Gaussian populations (Pearson) with a confidence interval of 95 %. † Values not statistically significant.

Discussion

In order to identify new biomarkers for the improvement of RA diagnosis, we have used the CIA mice model and PD technologies to select peptides that could mimic RA autoantigens. This approach allowed us to discover a new peptide with 12 amino acids (M12) that encompasses an antigenic domain of the Carbonic anhydrase III protein. The use of the synthetic M12 peptide as an autoantigen in ELISA assay confirmed the presence of specific anti-M12 antibodies in sera of patients with RA with high specificity and sensitivity.

We have used the DBA1/J mice as a model for inflammatory RA, where arthritis can be induced in susceptible mouse strains by immunization with CII [39]. DBA/1J mice are CIA models of particular interest, since this strain is not known to have any immunologic aberrations or other pathologic defects [40]. CIA mice are widely used as an animal model for RA studies [14-17, 41-43]. We have chosen this experimental model due to the fact that genomic similarities between mice and humans are quite high, reaching 98% [44]; it is much easier to monitor arthritis in mice than in human, and also because it is a unique experimental opportunity to increase our understanding of human arthritis. We confirmed the efficiency of the arthritis induction in our CIA mice strain, when a total of 80% of DBA/1J developed signs of arthritis around sixty days after CII immunization. We have used a subtractive phage display selection against IgG present in sera of CIA mice aiming to select new RA biomarkers. We have chosen this technology due to its successful applications in biomarkers discovery, especially when short random peptides are selected against specific target molecules, such as those selected against IgG present in sera for antigen discovery [45, 46].

The highly reactive peptide M12 was able to efficiently discriminate RA patients from healthy controls as well as from patients affected by SLE and AS, two other immune-mediated rheumatic diseases. In fact, M12 peptide was not the most enriched mimotope after rounds of phages amplification. On the other hand, the most frequent selected mimotope will not necessarily be the most reactive. Selection is independent, what means that increasing one does not eliminate the effect of the other [47]. Many events during viral infection may contribute to increasing the amplification of specific phages. The biological reasons for abundances and growth advantage of some mimotopes have been discussed in

many reports and include events like the phages ability of binding to bacterial pili; and interference with packing or infection [48-50]. The M12 peptide sequence was further characterized by reverse binding using its specific IgG antibodies to capture and identify its antigen target by mass spectrometry, which led us to the CAIII protein. The CAIII protein is a member of a multigene family that encodes carbonic anhydrase isozymes. It is an abundant muscle protein and plays an important role in facilitated CO_2 diffusion and diverse processes involving H+ and -HCO_3 transport [51]. The CAIII gene encodes a protein of 260 aa and a molecular weight of approximately 29 kDa, which is well conserved throughout human and mice. This protein has already been identified as an autoantigen expressed in the synovial membrane of RA patients [52] and its circulating autoantibodies have been found in many diseases [53]. Kolaskar and Tongaonkar antigenicity scale is a semi-empirical method that makes use of physicochemical properties of aa residues and their frequencies of occurrence in experimentally known segmental epitopes and is used to predict antigenic determinants on proteins. Using this parameter, this method that can predict antigenic determinants with 75% accuracy [36] has been used in many studies [54-56]. Amino acid residues of the M12 peptide matched conservative and semi-conservative residues in a predicted antigenic and exposed site of a putative epitope of the human CAIII protein, which probably gave this peptide the ability to be recognized by specific IgG during the mimotopes selection. This hypothesis is supported by the fact that semi-conserved aa residues present similar physicochemical properties with the original residue allowing antibodies binding [57]. Of interest, a synthetic peptide between 24-54 aa residures of the human CAIII, that encompasses the M12 epitope region, has been commercially used to generate anti-CAIII antibodies in rabbits (Product ID: ABIN391954). This fact probably reinforces the importance of CAIII protein in RA and the potential use of this peptide as an antigen for antibodies detection to aid in diagnosis.

A wide range of serum biomarkers has been assessed to improve diagnosis and prognosis of RA. However, only the RF and anti-CCP antibodies have gained wide acceptance [10]. The use of recombinant CAIII protein as an antigen has confirmed the presence of specific anti-CAIII antibodies in RA sera [52, 53]; however, its sensitivity considering the entire CAIII protein was only 17%. In fact,

when compared with healthy controls, the specificity of circulating autoantibodies was high (100%), but reached only medium to low specificity when compared with other autoimmune diseases, such as SLE (67%) [52]. The cross-reactivity and lack of sensitivity of the whole CAIII protein suggests the presence of multiple epitopes sharing common regions with other proteins, as well as the presence of immunodominant epitopes that surpass the response to the M12 critical epitope, which may have generated either false positive results or an insufficient reactivity. On the other hand, the use of the M12 peptide conferred more sensitivity and specificity in the detection of RA, since it was able to discriminate RA patients from HCs, patients with AS and SLE, with high accuracy.

Several studies have measured the three different generations of anti-CCP antibodies in RA patients in comparison with control groups. These antibodies present specificities in the range of 65% to 100% (mean of 93.4%) and sensitivities in the range of 42% to 92.2% (mean of 64.3%) [9, 58-65]. RF testing presents specificities in the range of 85% to 90.29% and sensitivities in the range of 46.26 to 90% [66-68]. The diagnostic parameters for the M12 peptide for RA diagnosis presented specificity of 91% and sensitivity of 84.3%, which is very close to the mean of values reported for anti-CCP and RF antibodies, the two most currently used serum biomarkers for RA. On the other hand, anti-M12 antibodies were detected in 26.2% of samples that are negative for anti-CCP and RF. We speculate that the high percentage of positive samples for anti-M12 antibodies might be due the different tests used to cut-off calculation, or even the patients disease activity at the time of samples collection or individual response to therapy. In this context, high levels of anti-CCP and RF antibodies in RA patients have already been associated with an insufficient response to therapy [69, 70], while anti-M12 antibodies were weakly associated with therapeutic response in our RA cohort, what may help to explain the percentage of positive samples only for M12. It is widely accepted that the identification of RA at early stages and consequently the implementation of effective treatment strategies can significantly improve patients prognosis [71]. Anti-M12 antibodies showed a weak association with disease duration, suggesting its potential use as a biomarker in different stages during the process of RA development.

Conclusions

We have selected and identified a peptide that is able of detecting specific circulating IgG in the serum of RA patients with high specificity and sensibility. Although antibodies against synthetic M12 peptide were detected in patients with early and established RA, its potential use in the diagnosis at different stages of the disease remains to be studied during follow-up in a well-caracterized cohort of patients with early RA, and also, its specificity as a serum biomarker should be further studied in a much larger cohort of patients with other inflammatory diseases. This peptide mimics a predicted antigenic region of the human Carbonic anhydrase III by linear sequence analysis and could be used as antigen for detection of specific RA autoantibodies.

List of abbreviations

RA: Rheumatoid Arthritis; AS: Ankylosing Spondylitis; SLE: Systemic Lupus Erythematosus; RF: Rheumatoid Factor; IgG: Immunoglobulin G; ACPA: Anti-Citrullinated Peptide Antibodies; CCP: Cyclic Citrullinated Peptides; CII: Type II Collagen; CAIII: Carbonic anhydrase III; CIA: Collagen-Induced Arthritis; PD: Phage Display; CRP: C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; AUC: Area Under the Curve; ROC: Receiver Operating Characteristic (curve).

Competing interest

GRA; PTF; LRG and CUV are co-inventors of a patent protecting the use of the 14 peptides selected in this study and its sequence for diagnostic use. The remaining authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: GRA; JEF; ERV; LRG; JG and CUV. Performed peptides selection: GRA; PTF and LML. Performed immunoassays: GRA; ERV and PTF. Performed *in silico* analysis: GRA and PTF. Performed and analyzed mass spectrometry: GV; KHMC and VMC. Contributed to statistical analyses: GRA; PTF and HC. Contributed with reagents, materials, serum samples, and analysis tools: JEF; HC; MHN; LRG; JG and CUV. Contributed to

write the paper: GRA; ERV; PTF; JEF; GV; LRG; MHN and CUV. All authors read, improved and approved the final manuscript.

Funding

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Foundation, Ministry of Education of Brazil (CSF SDW-2027/13-5) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Acknowledgements

The authors thank all members of the Biobanco-IMM from the Lisbon Academic Medical Center, for the work spent to separate blood samples and fill multiple worksheets containing relevant patient data and Renato Mendonça Borges for the assistance in designing the workflow figure.

References

1. Rindfleisch JA, Muller D: **Diagnosis and management of rheumatoid arthritis**. *American family physician* 2005, **72**(6):1037-1047.
2. National Collaborating Centre for Chronic Conditions (UK). **Rheumatoid Arthritis: National Clinical Guideline for Management and Treatment in Adults**. London: Royal College of Physicians; 2009.
3. van Venrooij WJ, van Beers JJ, Pruijn GJ: **Anti-CCP antibodies: the past, the present and the future**. *Nature reviews Rheumatology* 2011, **7**(7):391-398.
4. van Venrooij WJ, Hazes JM, Visser H: **Anticitrullinated protein/peptide antibody and its role in the diagnosis and prognosis of early rheumatoid arthritis**. *The Netherlands journal of medicine* 2002, **60**(10):383-388.
5. Rantapaa-Dahlqvist S: **Diagnostic and prognostic significance of autoantibodies in early rheumatoid arthritis**. *Scandinavian journal of rheumatology* 2005, **34**(2):83-96.
6. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ: **The diagnostic properties of**

rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis and rheumatism* 2000, **43**(1):155-163.

7. Coenen D, Verschueren P, Westhovens R, Bossuyt X: **Technical and diagnostic performance of 6 assays for the measurement of citrullinated protein/peptide antibodies in the diagnosis of rheumatoid arthritis**. *Clinical chemistry* 2007, **53**(3):498-504.
8. Vander Cruyssen B, Cantaert T, Nogueira L, Clavel C, De Rycke L, Dendoven A, Sebag M, Deforce D, Vincent C, Elewaut D *et al*: **Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays**. *Arthritis research & therapy* 2006, **8**(4):R122.
9. Goeb V, Jouen F, Gilbert D, Le Loet X, Tron F, Vittecoq O: **Diagnostic and prognostic usefulness of antibodies to citrullinated peptides**. *Joint, bone, spine : revue du rhumatisme* 2009, **76**(4):343-349.
10. Taylor P, Gartemann J, Hsieh J, Creeden J: **A systematic review of serum biomarkers anti-cyclic citrullinated Peptide and rheumatoid factor as tests for rheumatoid arthritis**. *Autoimmune diseases* 2011, **2011**:815038.
11. van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Toes RE, Huizinga TW: **Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis**. *Arthritis research & therapy* 2005, **7**(5):R949-958.
12. Syversen SW, Gaarder PI, Goll GL, Odegard S, Haavardsholm EA, Mowinckel P, van der Heijde D, Landewe R, Kvien TK: **High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study**. *Annals of the rheumatic diseases* 2008, **67**(2):212-217.
13. Mjaavatten MD, van der Heijde DM, Uhlig T, Haugen AJ, Nygaard H, Bjorneboe O, Kvien TK: **Should anti-citrullinated protein antibody and rheumatoid factor status be reassessed during the first year of followup in recent-onset arthritis? A longitudinal study**. *The Journal of rheumatology* 2011, **38**(11):2336-2341.

14. Park KH, Mun CH, Kang MI, Lee SW, Lee SK, Park YB: **Treatment of collagen-induced arthritis using immune modulatory properties of human mesenchymal stem cells**. *Cell transplantation* 2015.
15. Chow LN, Choi KY, Piyadasa H, Bossert M, Uzonna J, Klonisch T, Mookherjee N: **Human cathelicidin LL-37-derived peptide IG-19 confers protection in a murine model of collagen-induced arthritis**. *Molecular immunology* 2014, **57**(2):86-92.
16. Eneljung T, Tengvall S, Jirholt P, Henningsson L, Holmdahl R, Gustafsson K, Gjertsson I: **Antigen-specific gene therapy after immunisation reduces the severity of collagen-induced arthritis**. *Clinical & developmental immunology* 2013, **2013**:345092.
17. Ding X, Hu J, Li J, Zhang Y, Shui B, Ding Z, Yao L, Fan Y: **Metabolomics analysis of collagen-induced arthritis in rats and interventional effects of oral tolerance**. *Analytical biochemistry* 2014, **458**:49-57.
18. Rosloniec EF, Cremer M, Kang AH, Myers LK, Brand DD. **Collagen-induced arthritis**. *Curr Protoc Immunol.* 2010, **89**:15.5:15.5.1–15.5.25.
19. Wang M, Li X, Chen J, Zhou Y, Cao H, Wu X, Jiang H: **Screening and evaluating the mimic peptides as a useful serum biomarker of ankylosing spondylitis using a phage display technique**. *Rheumatology international* 2011, **31**(8):1009-1016.
20. Sergeeva A, Kolonin MG, Molldrem JJ, Pasqualini R, Arap W: **Display technologies: application for the discovery of drug and gene delivery agents**. *Advanced drug delivery reviews* 2006, **58**(15):1622-1654.
21. Samoylova TI, Morrison NE, Globa LP, Cox NR: **Peptide phage display: opportunities for development of personalized anti-cancer strategies**. *Anti-cancer agents in medicinal chemistry* 2006, **6**(1):9-17.
22. Alves PT, Fujimura PT, Morais LD, Goulart LR: **Revisiting the CD14: epitope mapping by Phage Display**. *Immunobiology* 2014, **219**(11):822-829.
23. Reis CF, Carneiro AP, Vieira CU, Fujimura PT, Morari EC, Silva SJ, Goulart LR, Ward LS: **An antibody-like peptide that recognizes malignancy among thyroid nodules**. *Cancer letters* 2013, **335**(2):306-313.

24. Santos PS, Sena AA, Nascimento R, Araujo TG, Mendes MM, Martins JR, Mineo TW, Mineo JR, Goulart LR: **Epitope-based vaccines with the Anaplasma marginale MSP1a functional motif induce a balanced humoral and cellular immune response in mice**. *PloS one* 2013, **8**(4):e60311.
25. Scott JK, Smith GP: **Searching for peptide ligands with an epitope library**. *Science* 1990, **249**(4967):386-390.
26. Huang J, Ru B, Dai P: **Bioinformatics resources and tools for phage display**. *Molecules* 2011, **16**(1):694-709.
27. Somers K, Geusens P, Elewaut D, De Keyser F, Rummens JL, Coenen M, Blom M, Stinissen P, Somers V: **Novel autoantibody markers for early and seronegative rheumatoid arthritis**. *Journal of autoimmunity* 2011, **36**(1):33-46.
28. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD *et al*: **2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative**. *Annals of the rheumatic diseases* 2010, **69**(9):1580-1588.
29. Brand DD, Latham KA, Rosloniec EF: **Collagen-induced arthritis**. *Nature protocols* 2007, **2**(5):1269-1275.
30. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding**. *Analytical biochemistry* 1976, **72**:248-254.
31. O'Valle F, Peregrina M, Crespo-Lora V, Galindo-Moreno P, Roman M, Padial-Molina M, Mesa F, Aneiros-Fernandez J, Aguilar D, Gonzalez-Rey E *et al*: **Osteoarticular Expression of Musashi-1 in an Experimental Model of Arthritis**. *BioMed Research International* 2015, **Article ID 681456, in press**.
32. Barbas CF BD, Scott JK, Silverman GJ **Phage display: a laboratory manual**. NY: Cold Spring Harbor Laboratory Press; 2001.
33. Stothard P: **The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences**. *BioTechniques* 2000, **28**(6):1102, 1104.

34. Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, Duvaud S, Flegel V, Fortier A, Gasteiger E *et al*: **ExPASy: SIB bioinformatics resource portal**. *Nucleic acids research* 2012, **40**(Web Server issue):W597-603.
35. Mayrose I, Penn O, Erez E, Rubinstein ND, Shlomi T, Freund NT, Bublil EM, Ruppin E, Sharan R, Gershoni JM *et al*: **Pepitope: epitope mapping from affinity-selected peptides**. *Bioinformatics* 2007, **23**(23):3244-3246.
36. Kolaskar AS, Tongaonkar PC: **A semi-empirical method for prediction of antigenic determinants on protein antigens**. *FEBS letters* 1990, **276**(1-2):172-174.
37. Camargo M, Intasqui Lopes P, Del Giudice PT, Carvalho VM, Cardozo KH, Andreoni C, Fraietta R, Bertolla RP: **Unbiased label-free quantitative proteomic profiling and enriched proteomic pathways in seminal plasma of adult men before and after varicocelectomy**. *Human reproduction* 2013, **28**(1):33-46.
38. Casey JL, Coley AM, Street G, Parisi K, Devine PL, Foley M: **Peptide mimotopes selected from a random peptide library for diagnosis of Epstein-Barr virus infection**. *Journal of clinical microbiology* 2006, **44**(3):764-771.
39. Williams RO: **Collagen-induced arthritis in mice: a major role for tumor necrosis factor-alpha**. *Methods in molecular biology* 2007, **361**:265-284.
40. Corthay A, Hansson AS, Holmdahl R: **T lymphocytes are not required for the spontaneous development of entheseal ossification leading to marginal ankylosis in the DBA/1 mouse**. *Arthritis and rheumatism* 2000, **43**(4):844-851.
41. Eros G, Ibrahim S, Siebert N, Boros M, Vollmar B: **Oral phosphatidylcholine pretreatment alleviates the signs of experimental rheumatoid arthritis**. *Arthritis research & therapy* 2009, **11**(2):R43.
42. Chiba A, Mizuno M, Tomi C, Tajima R, Alloza I, di Penta A, Yamamura T, Vandenbroeck K, Miyake S: **A 4-trifluoromethyl analogue of celecoxib inhibits arthritis by suppressing innate immune cell activation**. *Arthritis research & therapy* 2012, **14**(1):R9.

43. Hong SH, Kwone JT, Lee JH, Lee S, Lee AY, Cho WY, Bat-Erdene M, Choi BD, Cho MH: **Ascidian tunicate extracts attenuate rheumatoid arthritis in a collagen-induced murine model**. *Natural product communications* 2014, **9**(6):847-851.

44. Mural RJ, Adams MD, Myers EW, Smith HO, Miklos GL, Wides R, Halpern A, Li PW, Sutton GG, Nadeau J *et al*: **A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome**. *Science* 2002, **296**(5573):1661-1671.

45. Weng X, Liao Q, Li K, Li Y, Mi M, Zhong D: **Screening serum biomarker of knee osteoarthritis using a phage display technique**. *Clinical biochemistry* 2012, **45**(4-5):303-308.

46. Costa LE, Lima MI, Chavez-Fumagalli MA, Menezes-Souza D, Martins VT, Duarte MC, Lage PS, Lopes EG, Lage DP, Ribeiro TG *et al*: **Subtractive phage display selection from canine visceral leishmaniasis identifies novel epitopes that mimic Leishmania infantum antigens with potential serodiagnosis applications**. *Clinical and vaccine immunology : CVI* 2014, **21**(1):96-106.

47. Derda R, Tang SK, Li SC, Ng S, Matochko W, Jafari MR: **Diversity of phage-displayed libraries of peptides during panning and amplification**. *Molecules* 2011, **16**(2):1776-1803.

48. Kuzmicheva GA, Jayanna PK, Sorokulova IB, Petrenko VA: **Diversity and censoring of landscape phage libraries**. *Protein engineering, design & selection : PEDS* 2009, **22**(1):9-18.

49. Rodi DJ, Soares AS, Makowski L: **Quantitative assessment of peptide sequence diversity in M13 combinatorial peptide phage display libraries**. *Journal of molecular biology* 2002, **322**(5):1039-1052.

50. Rodi DJ, Makowski L: **Structure and Function of Macromolecular Assembly**. Japan: Matsushita; 1997.

51. Edwards YH, Tweedie S, Lowe N, Lyons G: **Carbonic anhydrase 3 (CA3), a mesodermal marker**. *Symposia of the Society for Experimental Biology* 1992, **46**:273-283.

52. Robert-Pachot M, Desbos A, Moreira A, Becchi M, Tebib J, Bonnin M, Aitsiselmi T, Bienvenu J, Fabien N: **Carbonic anhydrase III: a new target**

for autoantibodies in autoimmune diseases. *Autoimmunity* 2007, **40**(5):380-389.

53. Liu C, Wei Y, Wang J, Pi L, Huang J, Wang P: **Carbonic anhydrases III and IV autoantibodies in rheumatoid arthritis, systemic lupus erythematosus, diabetes, hypertensive renal disease, and heart failure**. *Clinical & developmental immunology* 2012, **2012**:354594.
54. Srinivasan P, Kumar SP, Karthikeyan M, Jeyakanthan J, Jasrai YT, Pandya HA, Rawal RM, Patel SK: **Epitope-based immunoinformatics and molecular docking studies of nucleocapsid protein and ovarian tumor domain of crimean-congo hemorrhagic Fever virus**. *Frontiers in genetics* 2011, **2**:72.
55. Ranjbar MM, Ghorban K, Alavian SM, Keyvani H, Dadmanesh M, Roayaei Ardakany A, Motedayen MH, Sazmand A: **GB Virus C/Hepatitis G Virus Envelope Glycoprotein E2: Computational Molecular Features and Immunoinformatics Study**. *Hepatitis monthly* 2013, **13**(12):e15342.
56. Sealey KL, Kirk RS, Walker AJ, Rollinson D, Lawton SP: **Adaptive radiation within the vaccine target tetraspanin-23 across nine Schistosoma species from Africa**. *International journal for parasitology* 2013, **43**(1):95-103.
57. Ng PC, Henikoff S: **Predicting the effects of amino acid substitutions on protein function**. *Annual review of genomics and human genetics* 2006, **7**:61-80.
58. Forslind K, Ahlmen M, Eberhardt K, Hafstrom I, Svensson B, Group BS: **Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP)**. *Annals of the rheumatic diseases* 2004, **63**(9):1090-1095.
59. Herold M, Boeser V, Russe E, Klotz W: **Anti-CCP: history and its usefulness**. *Clinical & developmental immunology* 2005, **12**(2):131-135.
60. Wagner E, Skoumal M, Bayer PM, Klaushofer K: **Antibody against mutated citrullinated vimentin: a new sensitive marker in the diagnosis of rheumatoid arthritis**. *Rheumatology international* 2009, **29**(11):1315-1321.

61. Mutlu N, Bicakcigil M, Tasan DA, Kaya A, Yavuz S, Ozden AI: **Comparative performance analysis of 4 different anti-citrullinated protein assays in the diagnosis of rheumatoid arthritis**. *The Journal of rheumatology* 2009, **36**(3):491-500.

62. Fernandez-Suarez A, Reneses S, Wichmann I, Criado R, Nunez A: **Efficacy of three ELISA measurements of anti-cyclic citrullinated peptide antibodies in the early diagnosis of rheumatoid arthritis**. *Clinical chemistry and laboratory medicine : CCLM / FESCC* 2005, **43**(11):1234-1239.

63. Kudo-Tanaka E, Ohshima S, Ishii M, Mima T, Matsushita M, Azuma N, Harada Y, Katada Y, Ikeue H, Umeshita-Sasai M *et al*: **Autoantibodies to cyclic citrullinated peptide 2 (CCP2) are superior to other potential diagnostic biomarkers for predicting rheumatoid arthritis in early undifferentiated arthritis**. *Clinical rheumatology* 2007, **26**(10):1627-1633.

64. van Gaalen FA, Visser H, Huizinga TW: **A comparison of the diagnostic accuracy and prognostic value of the first and second anti-cyclic citrullinated peptides (CCP1 and CCP2) autoantibody tests for rheumatoid arthritis**. *Annals of the rheumatic diseases* 2005, **64**(10):1510-1512.

65. Kim S, Kim JH, Lee JH, Kim HS: **Evaluation of three automated enzyme immunoassays for detection of anti-cyclic citrullinated peptide antibodies in qualitative and quantitative aspects**. *Rheumatology* 2010, **49**(3):450-457.

66. Fariba Binesh, Hossein Soleimani Salehabadi, Nasim Behniafard, Kian Ranginkaman, Behniafard N: **A comparative assessment of the diagnostic value of anti-cyclic citrullinated peptide antibodies and rheumatoid factor in rheumatoid arthritis**. *Journal of Clinical & Experimental Pathology* 2014, **4**(2).

67. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, Saigo K, Morinobu A, Koshiba M, Kuntz KM *et al*: **Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and**

rheumatoid factor for rheumatoid arthritis. *Annals of internal medicine* 2007, **146**(11):797-808.

68. Nell VP, Machold KP, Stamm TA, Eberl G, Heinzl H, Uffmann M, Smolen JS, Steiner G: **Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis**. *Annals of the rheumatic diseases* 2005, **64**(12):1731-1736.
69. Visser K, Goekoop-Ruiterman YP, de Vries-Bouwstra JK, Ronday HK, Seys PE, Kerstens PJ, Huizinga TW, Dijkmans BA, Allaart CF: **A matrix risk model for the prediction of rapid radiographic progression in patients with rheumatoid arthritis receiving different dynamic treatment strategies: post hoc analyses from the BeSt study**. *Annals of the rheumatic diseases* 2010, **69**(7):1333-1337.
70. Mikuls TR, O'Dell JR, Stoner JA, Parrish LA, Arend WP, Norris JM, Holers VM: **Association of rheumatoid arthritis treatment response and disease duration with declines in serum levels of IgM rheumatoid factor and anti-cyclic citrullinated peptide antibody**. *Arthritis and rheumatism* 2004, **50**(12):3776-3782.
71. Vermeer M, Kuper HH, Hoekstra M, Haagsma CJ, Posthumus MD, Brus HL, van Riel PL, van de Laar MA: **Implementation of a treat-to-target strategy in very early rheumatoid arthritis: results of the Dutch Rheumatoid Arthritis Monitoring remission induction cohort study**. *Arthritis and rheumatism* 2011, **63:**2865-72.

CAPÍTULO III

Anti-type II collagen antibodies detection and avidity in patients with oligoarticular and polyarticular forms of juvenile idiopathic arthritis*

*Este capítulo está formatado como Artigo Original de acordo com as normas do periódico *Immunology Letters* (com algumas alterações estruturais para melhor se adequar ao formato da Tese)

Anti-type II collagen antibodies detection and avidity in patients with oligoarticular and polyarticular forms of juvenile idiopathic arthritis

Galber R. Araujo[1,2,*], João E. Fonseca[3,4], Patricia T. Fujimura[1], Jair P. Cunha-Junior[5], Carlos H. M. Silva[6], Ana F. Mourão[3,7], Helena Canhão[3,4], Luiz R. Goulart[1,8], João Gonçalves[2,9], Carlos Ueira-Vieira[1]

[1]Laboratory of Nanobiotecnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, Brazil

[2]iMed – Institute of Medicine, Faculty of Farmacy, University of Lisbon, Lisbon, Portugal

[3]Rheumatology Research Unit, Institute of Molecular Medicine, Lisbon, Portugal

[4]Rheumatology Department, Lisbon Academic Medical Center, Lisbon, Portugal

[5]Laboratory of Immunotecnology and Imunochemistry, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Brazil

[6]Pediatric Rheumatology Department, Clinical Hospital of the Federal University of Uberlândia, Uberlândia, Brazil

[7]Rheumatology Department, Egas Moniz Hospital, Lisbon, Portugal

[8]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA

[9]IMM – Institute of Molecular Medicine, Faculty of Medicine of the University of Lisbon, Lisbon, Portugal

* Corresponding author. Tel: +55 34 3218 2478; Fax: +55 34 3218 2203

E-mail address: galber.araujo@gmail.com (GRA)

ABSTRACT

Juvenile idiopathic arthritis (JIA) refers to a heterogeneous group of illnesses that have in common the occurrence of chronic joint inflammation in children younger than 16 years of age. The diagnosis is made only on clinical assessment. The identification of antibody markers could improve the early diagnosis, optimizing the clinical management of patients. Type II collagen is one potential autoantigen that has been implicated in the process of arthritis development. The aims of our study were to investigate the occurrence of anti-type II collagen antibodies and also to determine the avidity of the antibody-antigen binding. Ninety-six patients with oligoarticular or polyarticular JIA, 13 patients with ankylosing spondylitis (AS) and 61 healthy controls (HC) were tested for anti-type II collagen antibodies by ELISA and avidity ELISA. Sensitivity and specificity were determined by the receiver operating characteristic (ROC) curve analysis. Forty-two JIA patients (44%) were positive for antibodies against type II collagen. Its detection was significantly higher in JIA patients than in AS patients ($p = 0.006$) and HCs ($p < 0.0001$). Furthermore, anti-type II collagen antibody detection was significantly more frequent in patients with JIA of ≤ 6 months duration ($p = 0.0007$). Antibodies displaying high avidity to type II collagen were associated with disease activity ($p = 0.004$). This study demonstrates that antibodies against type II collagen are present in the serum of patients with oligoarticular and polyarticular JIA, being its presence more prevalent in patients with early disease. It also demonstrates that JIA patients with active disease present antibodies with high avidity against type II collagen.

Keywords: Juvenile idiopathic arthritis; type II collagen; serodiagnosis

1. Introduction

Juvenile idiopathic arthritis (JIA), the most common chronic inflammatory rheumatic disorder of childhood, is characterized by the onset of chronic arthritis of unknown etiology in children younger than 16 years of age [1]. According to the International League of Associations for Rheumatology criteria, JIA comprises seven subtypes, being oligoarticular and polyarticular the most common [2]. Arthritis that affects four or fewer joints after the first six months of the disease is classified as persistent oligoarticular JIA. Although sometimes thought of as a benign condition, persistent oligoarticular JIA may, in fact, lead to a wide spectrum of outcomes, ranging from complete remission after discontinuation of medication to development of severe damage to affected joints. The extended oligoarticular JIA subtype has a worse prognosis and includes patients who have five or more joints affected after the first 6 months of disease [3]. Arthritis that affects five or more joints during the first 6 months of the disease is called polyarticular JIA, which is classified as rheumatoid factor-negative (RF^-) or rheumatoid factor-positive (RF^+). JIA also encompasses juvenile psoriatic arthritis, enthesis-related arthritis and systemic arthritis. All JIA subtypes are of unknown causes. Diagnosis is based on the combined evaluation of medical history, clinical presentation and, to some extent, laboratory abnormalities [4,5].

Type II collagen (CII) is the predominant collagen type in joint cartilage and anti-CII antibodies have been reported to be present in serum, synovial fluid and eluted from cartilage explants of Rheumatoid Arthritis (RA) patients [6,7]. Studies have shown that antibodies from patients with RA react to various species of CII such as chicken, bovine, porcine and humans [6,8,9]. However, few studies have evaluated the presence of anti-CII antibodies in JIA [10-13]. Avidity ELISA has been used for measuring antibodies-antigen interaction in many diseases, including RA, in which different profiles could be detected with or without washing with denaturing agents [6,14-16]. There are no published studies evaluating the avidity of anti-CII antibodies in JIA.

In the present study we investigated whether antibodies present in serum of children with oligoarticular and polyarticular JIA reacts with type II collagen. In addition, we measured the level of avidity between type II collagen and IgG antibodies by using the avidity ELISA assay.

2. Materials and Methods

2.1 Patients

For this study, serum samples were obtained from Brazilian and Portuguese donors. A total of 96 patients (72 female and 24 male, mean age 13.6 ± 6.8 years) who fulfilled the diagnostic criteria for oligoarticular and polyarticular JIA, according to the International League of Associations for Rheumatology [2], 13 patients with ankylosing spondylitis (AS) were used as an inflammatory rheumatic disease control (2 female and 11 male, mean age 49.3 ± 7.9 years) and 61 Healthy Controls (HC) (41 female and 20 male, mean age 11.7 ± 4 years), were enrolled in this study. Twenty-seven (27.8%) patients had samples collected in the early phase of the disease (≤ 6 months duration). Seventy-one (73.2%) patients had active disease at the time of sample collection. The criteria adopted to classify active disease were that defined by Consolaro and colleagues [17] and include 4 measures: positivity for CRP or ESR into a continuous measure of inflammation; presence of swollen joints; a physician's global assessment of disease activity, and a parent/patient global assessment of well-being.

Serum samples collected from AS patients were used as a rheumatic disease control group and healthy blood donors were used as the HC group. The study protocol involving Brazilian patients was approved by the ethics committee of the Federal University of Uberlândia whereas the study protocol involving Portuguese patients was approved by the ethics committees of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria, Lisbon Academic Medical Centre. The Portuguese samples were obtained from the Biobanco-IMM, Lisbon Academic Medical Centre. After approval of the consent procedure by the ethics committee, written informed consent was obtained from every parent of each child included in this study. The school-aged children provided their verbal and written informed consent to participate in this study, while children under 6 years of age only provided their verbal informed consent. After signed all informed consent were scanned and the originals were filed. The study was conducted in accordance with the Declaration of Helsinki.

2.2 Blood tests for inflammation detection

C-reactive protein (CRP) detection was determined by latex agglutination test (LAT) and a value of ≥ 0.8 mg/dl was considered elevated. Latex fixation test was utilized for the detection of IgM RF positivity and erythrocyte sedimentation rate (ESR) was determined by modified Westergren technique and considered elevated at ≥15 mm/hr.

2.3 ELISA and avidity ELISA

For antibody analysis we used serum samples stored at -80, collected between 2011-2014. The best serum dilution, CII concentration, coating/blocking/washing buffers and anti-Human IgG were determined after several tests. The best ELISA condition for CII measurement was chosen based on the difference of reactivity between patients with JIA and HCs. ELISA and avidity ELISA were performed simultaneously. The avidity of CII-specific IgG antibodies was determined as previously described [18], with some modifications. Briefly, 96-well microtiter plates (NUNC MaxiSorp) were coated overnight at 4ºC with bovine CII 1.5µg/ml (Becton Dickinson Biosciences, San Jose, CA) previously diluted in 0.06 M carbonate buffer (pH 9.6). To verify the presence of cross-reaction between antibodies and the ELISA plate, each serum sample was tested alone as blank control (without CII). The microplates were blocked with PBS supplemented with 3% of Bovine Serum Albumin (BSA) for 1 h at 37ºC. Serum samples diluted at 1:100 in PBS-BSA 1% plus 0.05% Tween 20 were added in duplicate on separate plates. After incubation for 1 h at 37ºC, the plates were washed with PBS Tween 0.05% and then subjected to differential washing as follows: one plate was incubated with 6 M urea solution diluted in PBS for 10 min, while the other plate was incubated with PBS Tween 0.05% for 10 min. Anti-CII antibodies were detected using horseradish peroxidase-conjugated anti-IgG (Sigma Chemical Co., St. Louis, Mo.) diluted 1:1000 and incubated for 1 h at 37°C. The reaction was revealed with a substrate solution consisting of ortho-phenylenediamine (Sigma Chemical Co.) at 1 mg/ml in 0.01 M citrate-phosphate buffer (pH 5.0) and 0.03% H_2O_2. After incubation for 15 min at room temperature, the reaction was stopped with 2 N H_2SO_4 and read at 492 nm.

For ELISA analysis, the final optical density (OD) was normalized by the ratio of OD readings for each JIA and AS sample divided by the mean of OD value obtained by the HC group. Receiver operating characteristic (ROC) curve was constructed comparing the ELISA results from JIA with HC group. Based on the ROC curve, a cut-off point was determined as the value corresponding to the highest sensitivity without lowering the specificity. Differences in anti-CII antibodies reactivity between JIA patients and control groups are expressed in Reactivity index (RI), which was calculated based on the cut-off point (RI = normalized OD of each sample/cut-off). Samples with RI > 1.49 (cut-off) were considered positive.

The criteria adopted to classify the avidity index (AI) were defined by Marcolino and colleagues [18]. AIs were calculated as the ratio between the absorbance (Abs) obtained for the plate washed with urea (U^+) and the plate without urea (U^-), and was expressed as a percentage: AI (%) = $Abs(U^+)/Abs(U^-) \times 100$. Avidity index was arbitrarily defined as: less than 30%, which was considered low avidity, between 30 and 60%, classified as average avidity and higher than 60%, corresponding to high avidity. Data were presented as a mean value ± standard deviation.

2.4 Statistical Analysis

Unpaired *t* test with Welch's correction was used to evaluate the differences among JIA vs HC and JIA vs AS groups. Kruskall-Wallis test was used when three or more groups were compared. ELISA data were normalized based on the overall average absorbance obtained in the detection of anti-CII antibodies in the HC group. Cut-off that allowed best sensitivity and specificity was determined using the ROC curve. The area under the curve (AUC) was also determined. Correlations were assessed using Pearson's correlation (r). A value of $p < 0.05$ was considered statistically significant. Data were analyzed by using the GraphPad software package 5.0 (GraphPad Software Inc., San Diego, USA).

3. Results

3.1 Demographic characteristics

The studied population is presented in Table 1. At the time of samples collection, 73 JIA patients were receiving treatment. Thirty-nine (40.6%) were

receiving treatment with non-steroidal anti-inflammatory drugs, 46 (47.9%) were on treatment with disease modifying anti-rheumatic drugs and 13 (13.5%) were receiving treatment with biologic agents. Nine (9.4%) patients were untreated because it was their first visit to the rheumatology clinic at the time of sample collection or because they were in disease remission. Fifteen (15.6%) patients were RF positive (mean concentration of 91 ± 260 IU/ml). A total of 30 (31.2%) patients had raised CRP (mean concentration of 1.4 ± 1.3 mg/dl) and 65 (67.7%) had raised ESR (mean concentration 20.2 ± 10.7 mm/hr). JIA patients had mean disease duration of 10 ± 7 years.

Table 1. Demographic and laboratory features of the studied population.

	Juvenile idiopathic arthritis = 96				*P* value		
	Persistent oligoarticular	Extended oligoarticular	Polyarticular [h]RF-	Polyarticular RF+	(Between JIA subtypes)	Ankylosing spondylitis	Healthy children
n[a] (B[b]/P[c])	41 (21/20)	15 (8/7)	25 (5/20)	15 (6/9)		13 (0/13)	61 (38/23)
Gender (F/M)[d]	31/10	10/5	21/4	11/4		2/11	41/20
Mean age ± SD[e]	10.5 ± 6.8	12.8 ± 8	14.1 ± 12	22.7 ± 14	0.0038	49.3 ± 7.9	11.7 ± 4
Mean disease duration (years) ± SD	6.2 ± 5.6	9.1 ± 5.5	10.1 ± 8.1	16 ± 14.2	NS[i]	12 ± 9.1	
CRP[f] ± SD (mg/dL)	1.4 ± 1.8	1.1 ± 0.9	0.9 ± 1.2	2.2 ± 1.3	NS	2.7 ± 0.8	
Positive/Negative	9/32	7/8	6/19	8/7			
ESR[g] ± SD (mm/hr)	20.1 ± 18	19.5 ± 10.7	20.8 ± 14.8	19.6 ± 12.7	NS	Missing	
Positive/Negative	30/11	10/5	14/9	11/4			

[a]*n*: number of subjects; [b]B: Brazilian population; [c]P: Portuguese population; [d]F: female; M: male; [e]SD: standard deviation; [f]CRP: C-reactive protein; [g]ESR: erythrocyte sedimentation rate; [h]RF: rheumatoid factor; [i]NS: not significant. $p < 0.05$ is statistically significant. Variables are expressed as means ± SD. Differences among groups were assessed by Kruskal-Wallis test.

3.2 Immunoreactivity of collagen type II against IgG from JIA patients and controls

Anti-CII antibodies levels were significantly higher in JIA than in AS patients (p = 0.006) and HC group (p < 0.0001) (Fig. 1A and 1B). We found that 42 (44%) JIA patients were positive to CII in ELISA (RI = 1.920 ± 0.3; AUC = 0.856; p < 0.0001). Although we observed differences in positivity across JIA subtypes, no significant differences were observed in the levels of anti-CII reactivity. The performance of the test was evaluated for sensitivity and specificity and found to be 45.3% and 93.4%, respectively. On the other hand, we found that 2 AS patients (15%) and 4 HCs (6.5%) were also anti-CII positives. These results are summarized in Table 2.

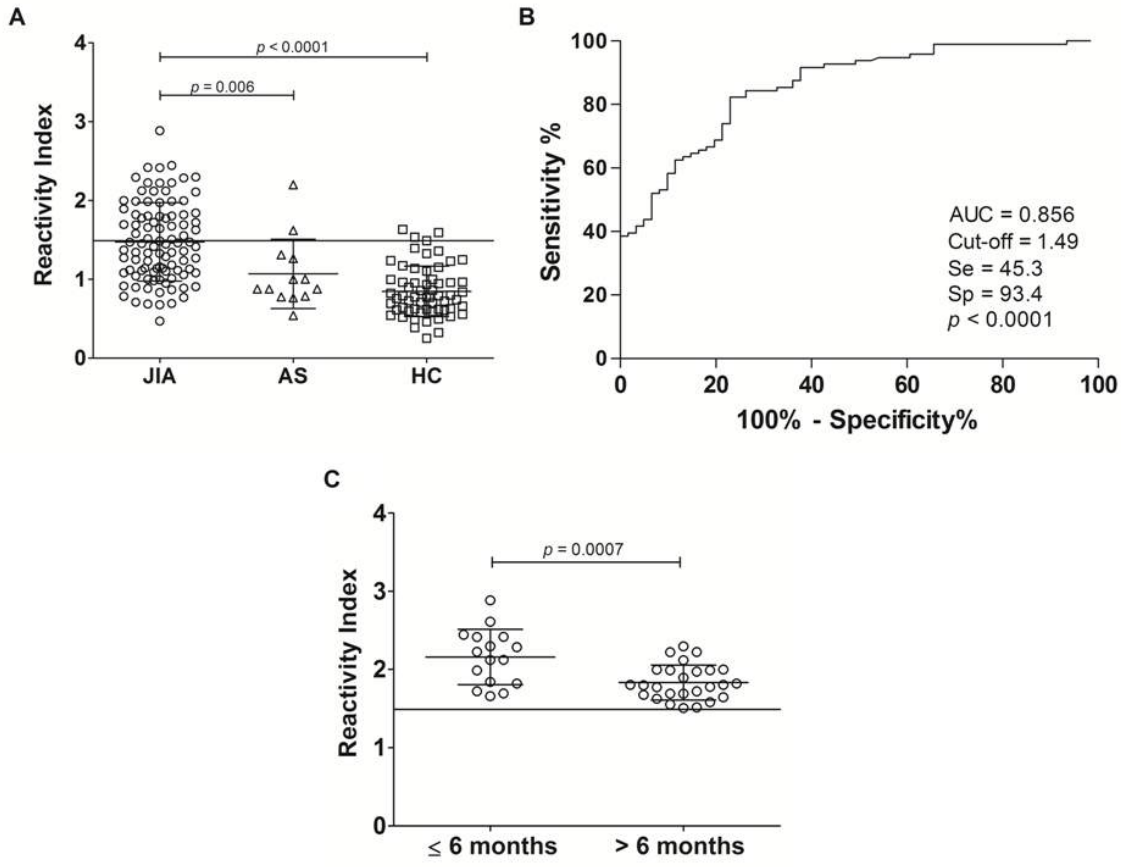


Fig. 1. Anti-collagen type II antibodies detection by ELISA. (A) Detection of anti-CII antibodies in serum samples from JIA patients (96), AS patients (13) and healthy children (61) by ELISA and the respective ROC curve (B). The area under the curve (AUC), cut-off, sensitivity (se), specificity (sp) and corresponding *P*-value are

indicated inside the graph. (C) Anti-CII antibody levels in patients with early and established JIA. The horizontal bar indicates the cut-off (1.49). Reactivity index = normalized OD of each sample/cut-off.

Table 2. Positivity for anti-collagen type II antibodies in the studied population.

	Juvenile idiopathic arthritis						
	Persistent Oligoarticular ***n*** **= 41**	**Extended Oligoaritular** ***n*** **= 15**	**Polyarticular RF⁻** ***n*** **= 25**	**Polyarticular RF⁺** ***n*** **= 15**	***P* Value**	**Ankylosing spondylitis** ***n*** **= 13**	**Healthy children** ***n*** **= 61**
Reactivity Index	1.35 ± 0.5	1.53 ± 0.3	1.6 ± 0.5	1.6 ± 0.4	NS*	1.1 ± 0.4	0.85 ± 0.3
Positivity *n* (%)	16 (39%)	7 (46.6%)	10 (40%)	9 (60%)		2 (15%)	4 (6.5%)

*No Significant. $p < 0.05$ is statistically significant. Variables are expressed as means ± SD. Differences among JIA subtypes were assessed by Kruskal-Wallis test.

We did not observe significant differences in reactivity to CII in JIA patients across gender, ethnicity, number of joints affected or therapy. There was no statistically significant difference between the Portuguese and the Brazilian population. JIA patients with ≤ 6 months of disease duration that were positives for anti-CII antibodies (n = 16) presented levels of anti-CII antibodies significantly higher ($p = 0.0007$) than patients with established disease (n = 26) (Fig. 1C). Of interest, 32 (76%) of the anti-CII positive patients had active disease. Anti-CII antibodies were directly correlated with RF (Pearson $r = 0.7207$, $p < 0.005$). No correlations were found when levels of anti-CII antibodies were analyzed separately for the values of CRP, ESR and the number of inflamed joints. However, a 13-year-old girl with polyarticular RF^+, who presented inflammation in more than 5 joints, high CRP concentration (2.8 mg/dl), increased ESR (38 mm/hr), and very high levels of rheumatoid factor (1118.80 Ul/ml), presented the highest levels of anti-CII antibodies in ELISA (RI = 2.9).

3.3 Avidity of anti-CII antibodies and controls

The levels of anti-CII antibodies detected in the serum from different subtypes of JIA without and after treatment with 6 M urea are shown in Fig. 2.

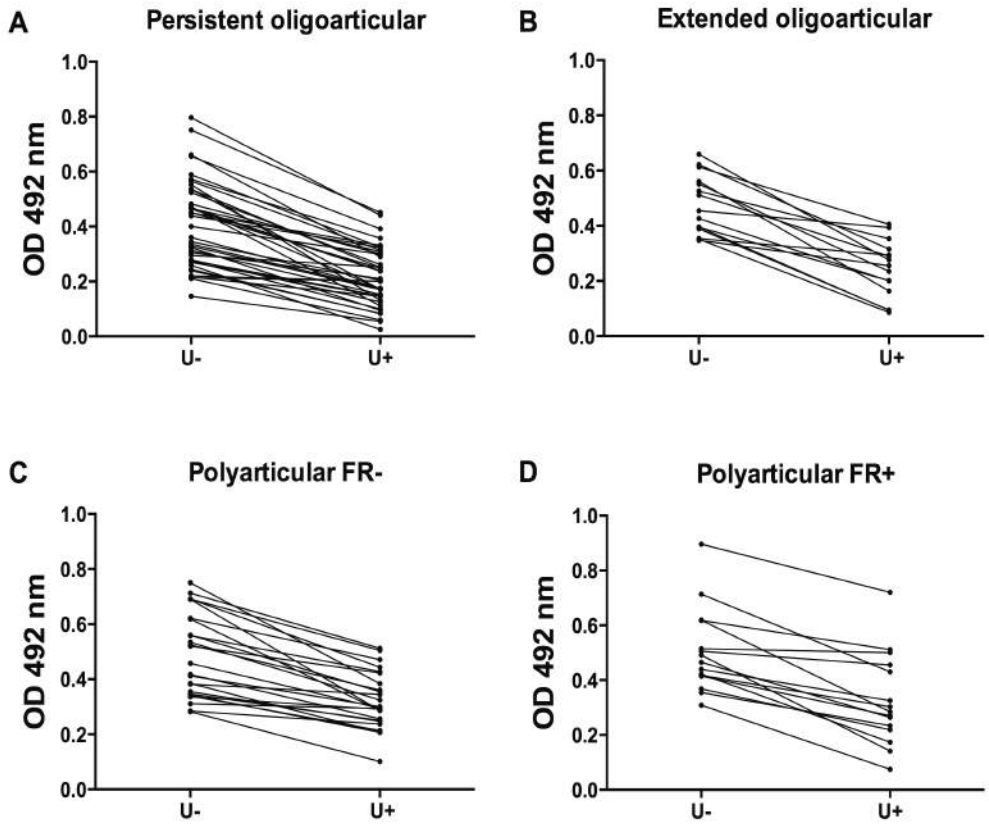


Fig. 2. Anti-CII antibody levels detected in JIA before and after treatment with 6 M urea. Optical density differences before (U-) and after (U+) treatment with urea are shown for each patient separately for each JIA subtype (A, B, C, D). The paired U- and U+ samples from individual patients are identified by a line between points.

In the serum samples from JIA patients, AIs ranged from 9.6% to 97% (mean of 52.2% ± 18) (Fig. 3A). Forty-six (47.9%) samples presented antibodies with high avidity to CII (mean of 73.9% ± 10.4), 39 (40.6%) samples presented antibodies with medium avidity (mean of 47.4% ± 8.1) and 11 (11.4%) samples presented antibodies with low avidity (mean of 24.1% ± 5.3). Serum samples from AS patients presented AIs ranging from 20.3% to 52% (mean of 36.2% ± 7.9), with most patients displaying antibodies with medium avidity to CII. Serum from HC presented AIs ranging from 7% to 47.1% (mean of 26% ± 8.5), with most of patients displaying antibodies with low avidity to CII. When JIA patients were

subdivided according to different populations, gender, ethnicity, treatment or disease duration, no significant difference was observed in the avidity index in any disease subtype. In the group displaying antibodies with high avidity against CII, we observed that patients with active disease (n = 32; mean of 76.5% ± 11) at the time of sample collection presented AIs significantly higher in comparison with patients in inactive/remission disease (n = 14; mean of 68% ± 5.6) (p = 0.004) (Fig. 3B). Interestingly, polyarticular JIA patients presented AIs significantly higher than other JIA subtypes (p = 0.006). We did not observe significant association between high avidity antibodies and levels of RF for polyarticular JIA patients. The avidity of antibodies toward CII was significantly higher in JIA patients when compared with AS (p < 0.0001) or HC group (p < 0.0001).

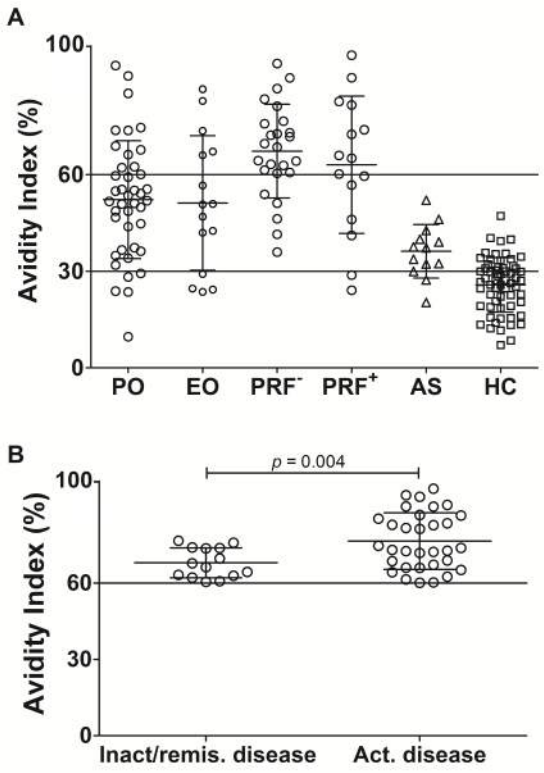


Fig. 3. Avidity profile of anti-collagen type II antibodies. (A) Avidity profiles of anti-CII antibodies observed in 96 patients with oligoarticular and polyarticular forms of JIA, 13 patients with AS and 61 HCs. (B) Antibodies displaying high avidity to type II collagen in patients with inactive and active JIA. The Avidity Index values were defined as: < 30% = low avidity; between 30 and 60% = medium avidity; > 60% = high avidity. Avidity Indexes are indicated as lines.

4 Discussion

Most of what we know about autoimmune response to CII comes from experimental disease models. Particularly, from the collagen-induced arthritis (CIA) experimental models, obtained by immunization with autologous or heterologous type II collagen with adjuvant [19]. Several studies have attempted to identify antibodies to CII in RA [6-9,20-22], however, only a small number of published studies aimed to determine the prevalence of anti-CII antibodies in JIA patients [10-13]. The current work reports the occurrence and avidity of anti-type II collagen antibodies in patients with JIA compared with healthy individuals.

Different compositions of type II collagen have been used as the antigen to antibodies detection in RA and JIA in its native or denatured forms. The vast majority of published studies about the occurrence of anti-CII in JIA and RA, aimed to determine the prevalence of antibodies to citrullinated CII [12,23,24]. Antibodies to native citrullinated CII have already been detected in 78.5% of RA [24] and 24% of JIA patients [12]. Antibodies to native human CII were detected in 20% [25] and 70% of RA [11], 47% [11] of JIA patients and recently, in 3.1% [13]. Antibodies to native bovine CII (the same we have used in our study) were detected in 31.5% of RA patients [6]. Based on the most adequate cut-off value determined by the ROC curve, we found that 44% of our JIA patients were positives to anti-CII antibodies. The positivity of anti-CII antibodies in the AS population (15%) was lower than the results reported in another study [26], which showed positivity in 33.3% of the patients. Although anti-CII antibodies were present in the AS group its detection was much higher in JIA patients. The differences in the prevalence of anti-CII in the several published studies may be due to the different species of CII used as the antigen to antibodies detection, since studies that aimed to analyze CII specificity revealed that autoantibodies are generally associated with specific arthritogenic epitopes present in the CII molecules from different species [27,28]. The different methodologies of data analysis (normalization and cut-off determination), the number of patients in which these anti-CII antibodies have been measured, or even patient's disease activity [6] could also have contributed to these heterogeneous results.

Our results also showed increased levels of anti-CII antibodies in patients with early JIA, with the vast majority of individuals presenting active disease at the

time of sample collection. These data are consistent with previous findings reporting higher frequencies of anti-CII antibodies in early RA [29-31] and recently, in JIA patients [13]. Similar results were also observed in CIA models after arthritis induction by immunization with CII, where the levels of circulating anti-CII antibodies in MRL/l mice were as well highest at 6 months, thus immediately after the development of arthritis [32]. Anti-CII appearing early in the course of the disease suggests that these autoantibodies might play a role in JIA physiopathology. However, the clinical relevance of its detection in early JIA is still an open question. We hypothesize that in patients with early JIA, high levels of anti-CII antibodies might induce an acute inflammation mediated by surface-bound immune complexes (IC) containing anti-CII, as has already been described in RA [9]. Purified CII-specific monoclonal antibodies were shown to induce an acute form of arthritis in mice [33], which demonstrates that anti-CII autoantibodies are indeed directly pathogenic *in vivo*. These anti-CII antibodies appearing around clinical onset suggests that these autoantibodies play an early role in the process of disease development. If it proves to be true, the detection of these antibodies could lead to faster diagnosis and treatment. Our results also showed a high percentage of anti-CII positive patients presenting active disease (76%). Taking into account what has already been reported [34-36], we believe that circulating ICs containing anti-CII can activate the complement system and stimulate the production of cytokines of pathogenic importance in the inflammatory process, such as interleukin-1β (IL-1β), IL-6, IL-10 and tumor necrosis factor α (TNF-α). This event could perpetuate joint inflammation and consequently maintain the disease activity. This hypothesis is corroborated by Mullazehi et al [34] who reported that in RA patients the production of the cytokines TNF-α, IL-1β and IL-8 decreased after the specific blockade of ICs containing anti-CII.

Our results also showed a high percentage of anti-CII antibody positive samples in the group with polyarticular RF^+ and also a direct correlation between anti-CII antibodies and RF. This is in line with the fact that this subtype corresponds basically to a juvenile RA onset, with a more persistent and severe course of the disease as compared to other JIA subtypes [37]. Furthermore, we reported a patient with polyarticular onset displaying high levels of RF, CRP and ESR who presented the highest level of anti-CII antibodies. Although when

analyzed separately no correlation between levels of anti-CII and values of CRP and ESR was found, these findings are in agreement with what has already been reported in RA patients [7], where levels of anti-CII antibodies were higher in patients with high CRP and ESR, and partially, with another study [13] involving JIA patients, where anti-CII antibodies was associated with high levels of CRP.

In the present study, we calculated the AI of specific anti-CII antibodies, determined from the avidity ELISA. A brief 6 M urea treatment allowed the dissociation of the low-avidity interactions, retaining the antibodies that bind with higher avidity to CII. This is the first study investigating the nature of anti-CII antibodies avidity in patients with JIA. We have shown that levels of antibodies displaying high avidity to CII were significantly higher in patients with active disease and in polyarticular patients. The driving force in the antibody binding and progressive destruction of cartilage is still obscure. However, it has been demonstrated that anti-CII antibodies form immune complexes with CII joint cartilage that, when not effectively removed, can activate complement and contribute to chronic inflammation [36]. Treatment-resistant JIA results in joint destruction, especially in patients with polyarticular subtype, which have the poorest prognosis with a remission rate of only 15%. More than 50% of JIA patients do not achieve remission despite treatment and require further rheumatological care as adults [38]. A possible explanation for the high occurrence of antibodies binding with high avidity against CII in samples from patients with active JIA could be that IC's production is not being blocked properly, resulting in active disease. In fact, it has been reported that the blocking of the complement system resulted in decrease of inflammatory cytokine, showing that complement plays a role in arthritis [36]. If this proves to be true, a target therapy based on this mechanism could be highly promising for both RA and JIA.

In conclusion, the present study showed that anti-CII antibodies are present in patients with oligoarticular and polyarticular forms of JIA. Consistent with RA data, high levels of anti-CII antibodies were detected in patients with JIA of ≤ 6 months duration, suggesting that anti-CII antibodies in JIA patients are associated with the early inflammatory process. Patients with active disease had more frequently high avidity antibodies against CII. Our data support the hypothesis that anti CII antibodies may play a role in the physiopathology of JIA.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) funded by the Ministry of Education of Brazil (2027/13-5), and by the Universidade Federal de Uberlândia.

References

[1] Ravelli A, Martini A. Juvenile idiopathic arthritis. Lancet 2007;369:767-778.

[2] Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. The Journal of rheumatology 2004;31:390-392.

[3] Huemer C, Malleson PN, Cabral DA, Huemer M, Falger J, Zidek T, et al. Patterns of joint involvement at onset differentiate oligoarticular juvenile psoriatic arthritis from pauciarticular juvenile rheumatoid arthritis. The Journal of rheumatology 2002;29:1531-1535.

[4] Dannecker GE, Quartier P. Juvenile idiopathic arthritis: classification, clinical presentation and current treatments. Hormone research 2009;72 Suppl 1:4-12.

[5] van Rossum M, van Soesbergen R, de Kort S, ten Cate R, Zwinderman AH, de Jong B, et al. Anti-cyclic citrullinated peptide (anti-CCP) antibodies in children with juvenile idiopathic arthritis. The Journal of rheumatology 2003;30:825-828.

[6] Clague RB, Morgan K, Reynolds I, Williams HJ. The prevalence of serum IgG antibodies to type II collagen in American patients with rheumatoid arthritis. British journal of rheumatology 1994;33:336-338.

[7] Kim WU, Yoo WH, Park W, Kang YM, Kim SI, Park JH, et al. IgG antibodies to type II collagen reflect inflammatory activity in patients with rheumatoid arthritis. The Journal of rheumatology 2000;27:575-581.

[8] Terato K, DeArmey DA, Ye XJ, Griffiths MM, Cremer MA. The mechanism of autoantibody formation to cartilage in rheumatoid arthritis: possible cross-reaction of antibodies to dietary collagens with autologous type II collagen. Clinical immunology and immunopathology 1996;79:142-154.

[9] Mullazehi M, Mathsson L, Lampa J, Ronnelid J. High anti-collagen type-II antibody levels and induction of proinflammatory cytokines by anti-collagen antibody-containing immune complexes in vitro characterise a distinct rheumatoid arthritis phenotype associated with acute inflammation at the time of disease onset. Annals of the rheumatic diseases 2007;66:537-541.

[10] B. LC, L. JL, A. HW, B. LH. IgG anticollagen antibodies in sera of patients with childhood rheumatic diseases [abstract]. Arthritis and rheumatism 1980;23:S56.

[11] Rosenberg AM, Hunt DW, Petty RE. Antibodies to native and denatured type II collagen in children with rheumatic diseases. The Journal of rheumatology 1984;11:425-431.

[12] Gilliam BE, Chauhan AK, Moore TL. Evaluation of anti-citrullinated type II collagen and anti-citrullinated vimentin antibodies in patients with juvenile idiopathic arthritis. Pediatric rheumatology online journal 2013;11:31.

[13] Berntson L, Nordal E, Fasth A, Aalto K, Herlin T, Nielsen S, et al. Anti-type II collagen antibodies, anti-CCP, IgA RF and IgM RF are associated with joint damage, assessed eight years after onset of juvenile idiopathic arthritis (JIA). Pediatric rheumatology online journal 2014;12:22.

[14] Remington JS, Thulliez P, Montoya JG. Recent developments for diagnosis of toxoplasmosis. Journal of clinical microbiology 2004;42:941-945.

[15] Newkirk MM, Fournier MJ, Shiroky J. Rheumatoid factor avidity in patients with rheumatoid arthritis: identification of pathogenic RFs which correlate with disease parameters and with the gal(0) glycoform of IgG. Journal of clinical immunology 1995;15:250-257.

[16] Penkova R, Goshev I, Gorinstein S, Nedkov P. Stability of collagen during denaturation. Journal of protein chemistry 1999;18:397-401.

[17] Consolaro A, Ruperto N, Bazso A, Pistorio A, Magni-Manzoni S, Filocamo G, et al. Development and validation of a composite disease activity score for juvenile idiopathic arthritis. Arthritis Rheum 2009;61:658-666.

[18] Marcolino PT, Silva DA, Leser PG, Camargo ME, Mineo JR. Molecular markers in acute and chronic phases of human toxoplasmosis: determination of immunoglobulin G avidity by Western blotting. Clinical and diagnostic laboratory immunology 2000;7:384-389.

[19] Williams RO. Collagen-induced arthritis as a model for rheumatoid arthritis. Methods in molecular medicine 2004;98:207-216.

[20] Morgan K, Clague RB, Collins I, Ayad S, Phinn SD, Holt PJ. Incidence of antibodies to native and denatured cartilage collagens (types II, IX, and XI) and to type I collagen in rheumatoid arthritis. Annals of the rheumatic diseases 1987;46:902-907.

[21] Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. Antibodies to type II collagen in early rheumatoid arthritis. Correlation with disease progression. Arthritis Rheum 1996;39:1720-1727.

[22] Mullazehi M, Wick MC, Klareskog L, van Vollenhoven R, Ronnelid J. Anti-type II collagen antibodies are associated with early radiographic destruction in rheumatoid arthritis. Arthritis research & therapy 2012;14:R100.

[23] Suwannalai P, van de Stadt LA, Radner H, Steiner G, El-Gabalawy HS, Zijde CM, et al. Avidity maturation of anti-citrullinated protein antibodies in rheumatoid arthritis. Arthritis Rheum 2012;64:1323-1328.

[24] Yoshida M, Tsuji M, Kurosaka D, Kurosaka D, Yasuda J, Ito Y, et al. Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. Modern rheumatology / the Japan Rheumatism Association 2006;16:276-281.

[25] Rowley MJ, Mackay IR, Brand CA, Bateman JF, Chan D. Epitope specificity of antibodies to type II collagen in rheumatoid arthritis and systemic lupus erythematosus. Rheumatology international 1992;12:65-69.

[26] Choi EK, Gatenby PA, McGill NW, Bateman JF, Cole WG, York JR. Autoantibodies to type II collagen: occurrence in rheumatoid arthritis, other arthritides, autoimmune connective tissue diseases, and chronic inflammatory syndromes. Annals of the rheumatic diseases 1988;47:313-322.

[27] Terato K, Shimozuru Y, Katayama K, Takemitsu Y, Yamashita I, Miyatsu M, et al. Specificity of antibodies to type II collagen in rheumatoid arthritis. Arthritis Rheum 1990;33:1493-1500.

[28] Buckee C, Morgan K, Ayad S, Collins I, Clague RB, Holt PJ. Diversity of antibodies to type II collagen in patients with rheumatoid arthritis: detection by binding to alpha-chains and to cyanogen bromide peptides. British journal of rheumatology 1990;29:254-258.

[29] Cook AD, Rowley MJ, Stockman A, Muirden KD, Mackay IR. Specificity of antibodies to type II collagen in early rheumatoid arthritis. The Journal of rheumatology 1994;21:1186-1191.

[30] Pereira RS, Black CM, Duance VC, Jones VE, Jacoby RK, Welsh KI. Disappearing collagen antibodies in rheumatoid arthritis. Lancet 1985;2:501-502.

[31] Fujii K, Tsuji M, Kitamura A, Murota K. The diagnostic significance of anti-type II collagen antibody assay in rheumatoid arthritis. International orthopaedics 1992;16:272-276.

[32] Tarkowski A, Holmdahl R, Rubin K, Klareskog L, Nilsson LA, Gunnarsson K. Patterns of autoreactivity to collagen type II in autoimmune MRL/l mice. Clinical and experimental immunology 1986;63:441-449.

[33] Nandakumar KS, Svensson L, Holmdahl R. Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. The American journal of pathology 2003;163:1827-1837.

[34] Mullazehi M, Mathsson L, Lampa J, Ronnelid J. Surface-bound anti-type II collagen-containing immune complexes induce production of tumor necrosis factor alpha, interleukin-1beta, and interleukin-8 from peripheral blood monocytes via Fc gamma receptor IIA: a potential pathophysiologic mechanism for humoral anti-type II collagen immunity in arthritis. Arthritis Rheum 2006;54:1759-1771.

[35] Ronnelid J, Tejde A, Mathsson L, Nilsson-Ekdahl K, Nilsson B. Immune complexes from SLE sera induce IL10 production from normal peripheral blood mononuclear cells by an FcgammaRII dependent mechanism:

implications for a possible vicious cycle maintaining B cell hyperactivity in SLE. Annals of the rheumatic diseases 2003;62:37-42.

[36] Mathsson L, Tejde A, Carlson K, Hoglund M, Nilsson B, Nilsson-Ekdahl K, et al. Cryoglobulin-induced cytokine production via FcgammaRIIa: inverse effects of complement blockade on the production of TNF-alpha and IL-10. Implications for the growth of malignant B-cell clones. British journal of haematology 2005;129:830-838.

[37] Schneider R, Passo MH. Juvenile rheumatoid arthritis. Rheumatic diseases clinics of North America 2002;28:503-530.

[38] Minden K, Kiessling U, Listing J, Niewerth M, Doring E, Meincke J, et al. Prognosis of patients with juvenile chronic arthritis and juvenile spondyloarthropathy. The Journal of rheumatology 2000;27:2256-2263.

CAPÍTULO IV

A highly reactive mimotope target by autoantibodies of patients with juvenile idiopathic arthritis and rheumatoid arthritis and its potential application in a sensor platform*

*Este capítulo está formatado como *Brief report* de acordo com as normas do periódico *Arthritis & Rheumatology* (com algumas alterações estruturais para melhor se adequar ao formato da Tese)

A highly reactive mimotope target by autoantibodies of patients with juvenile idiopathic arthritis and rheumatoid arthritis and its potential application in a sensor platform

Galber R. Araujo,[1] Patricia T. Fujimura,[1] Emília R. Vaz,[1] Tamiris A. Silva,[1] Vinícius R. Rodovalho,[2] Ana Graci B. Madurro,[2] João M. Madurro,[2] João E. Fonseca,[3] Carlos H. M. Silva,[4] Paula S. Santos,[1] Ana F. Mourão,[3] Helena Canhão,[3] Luiz R. Goulart,[1,5] João Gonçalves,[6] Carlos Ueira-Vieira[1]

[1]Galber R. Araujo, MSc, Patricia T. Fujimura, PhD, Emília R. Vaz, MSc, Tamiris A. Silva, MSc, Paula S. Santos, PhD, Luiz R. Goulart, PhD, Carlos Ueira-Vieira, PhD: Laboratório de Nanobiotecnologia, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, Brazil

[2]Vinícius R. Rodovalho, BS, Ana Graci B. Madurro, PhD, João M. Madurro, PhD: Laboratório de Filmes Poliméricos e Nanotecnologia, Instituto de Química, Universidade Federal de Uberlândia, Uberlândia, Brazil

[3]João E. Fonseca, MD, PhD, Ana F. Mourão, MD, PhD, Helena Canhão, MD, PhD: Unidade de Investigação em Reumatologia, Instituto de Medicina Molecular, Lisboa, Portugal

[4]Carlos H. M. Silva, MD, PhD: Departamento de Reumatologia Pediatrica, Hospital de Clínicas, Universidade Federal de Uberlândia, Uberlândia, Brazil

[5]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA

[6]João Gonçalve, PhD: Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

Address correspondence to Galber R. Araujo, MSc, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Av. Pará 1720, Campus Umuarama, 2E-248, CEP 38400-902, Uberlândia, MG, Brazil.
E-mail: galber.araujo@gmail.com

Supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Foundation, Ministry of Education of Brazil (CSF SDW-2027/13-5), the

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 301562/2011-3 and 474338/2009-6) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG; APQ 00981-11).

GRA; ERV; CHMS; LRG and CUV are co-inventors of a patent protecting the 38 peptide sequences selected in this study. The remaining authors declare that they have no competing interests.

Abstract

Objective: Juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) diagnosis are based on the clinical presentation, and many blood tests. The aim of this study was to select new epitope-like peptides that could be targeted by circulating autoantibodies of JIA patients, which could be used for JIA and RA diagnosis.

Methods: A phage displayed random peptide library was screened against immobilized IgG from JIA patients. ELISA assay was carried out to test immunoreactivity of selected mimotopes against sera from JIA patients, healthy individuals and patients with other autoimmune diseases. The PRF+1 mimotope that was able to efficiently discriminate JIA patients from controls was chosen to be chemically synthetized. Electrochemical analysis was carried out to test the immobilization of the synthetic peptide onto bioelectrode surface for antibody detection by differential pulse voltammetry.

Results: The PRF+1 synthetic peptide was able to efficiently discriminate patients with JIA and RA from control groups ($p < 0.0001$). Its diagnostic potential was high to both JIA (AUC > 0.84; sensitivity = 61%; specificity = 91%) and RA (AUC > 0.98; sensitivity = 94%; specificity = 93%). The electrochemical sensor proved to be fast, low cost and effective in discriminating sera from JIA and RA patients from healthy controls.

Conclusion: This study demonstrates an epitope-based biomarker for JIA and RA diagnosis that was promptly incorporated onto bioelectrodes for a simple and fast detection in an electrochemical platform. Its high accuracy makes it a promising biomarker for RA.

Juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) are inflammatory rheumatic diseases that share clinical and pathological features, which includes persistent synovitis, progressive joint destruction, and some systemic and extra-articular manifestations. JIA and RA diagnosis up until now is based on the combined evaluation of medical history of the patient, physical manifestation, radiographic imaging, and some serologic markers (1, 2). Thus, good biomarkers are still needed to guide decisions in the diagnosis and clinical management of JIA and RA. Aiming to select novel mimetic peptides with potential to be used as serum biomarkers, we screened a phage displayed random peptide library against circulating IgG purified from JIA patients, and submitted a selected peptide to further validation to check sensitivity, specificity and electrochemical properties.

The selected peptide that showed to be targeted by circulating autoantibodies in patients with JIA and RA was successfully immobilized onto an electrochemical sensor. This new platform could be used with high accuracy as a portable, low cost and fast tool for antibodies detection to aid in diagnosis. Additional studies still need to be performed to identify the protein that this peptide mimics.

Patients and Methods

Ethical considerations. This research was approved by the Research Ethics Committee from Federal University of Uberlândia (CEP-UFU; number 685/09), state of Minas Gerais, Brazil and by the Ethics Committee of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria and the Hospital Garcia de Orta, Lisbon, Portugal. Informed consent form was signed by all subjects before any protocol procedure was carried out.

Samples used for mimotopes selection. A total of 40 JIA patients who fulfilled the diagnostic criteria for persistent oligoarticular (*n* = 21), extended oligoarticular (*n* = 8), polyarticular rheumatoid factor negative (*n* = 5) and polyarticular rheumatoid factor positive (*n* = 6), according to the International League of Associations for Rheumatology (1), 20 healthy children, and 17 patients with other autoimmune diseases were enrolled in this stage. In order to increase specificity of

the selected mimotopes, a subtractive selection step was performed using pooled serum from patients with rheumatic fever (*n* = 5), uveitis (*n* = 3), systemic lupus erythematous (*n* = 4) and Hashimoto's thyroiditis (*n* = 5).

Samples used for synthetic peptide validation. Well-characterized serum samples were requested from Biobanco-IMM, Lisbon Academic Medical Center (Lisbon, Portugal). A total of 57 JIA patients who fulfilled the diagnostic criteria for persistent oligoarticular (*n* = 21), extended oligoarticular (*n* = 7), polyarticular rheumatoid factor negative (*n* = 20) and polyarticular rheumatoid factor positive (*n* = 9), 51 healthy children, 23 systemic lupus erythematous, 103 RA patients who fulfilled the RA diagnostic criteria (revised American Rheumatism Association (ARA) criteria, 1987) (3) and also the new revised criteria EULAR/ACR (2), and 43 healthy adults were enrolled in this stage.

Laboratory and clinical features. At the time of sample collection, personal and clinical data as disease duration, current medication, presence of RF, CRP and ESR, tender and swollen joint counts were obtained for each patient used in the study. Demographic and laboratory characteristics of the studied subjects are listed in Table 1.

Table 1. Demographic and laboratory characteristics of the different groups included in the study.

	Juvenile idiopathic arthritis = 97*										
	PO	EO	P RF$^-$	P RF$^+$	Rhe F	UV	SLE	HT	HC1	RA	HC2
n	42 (21/42) **	15 (8/15)	25 (5/25)	15 (6/15)	5 (5/5)	3 (3/3)	23 (4/23)	5 (5/50	71 (20/51)	103 (0/103)	43 (0/43)
Gender (F/M)	31/11	10/5	21/4	11/4	2/3	2/1	13/10	0/5	46/25	85/18	31/12
Mean age ± SD	11 ± 7	12.8 ± 8	14.1 ± 12	22.7 ± 14	9.6 ± 3.1	8 ± 4	14.3 ± 5.7	23.8 ± 2.3	12.9 ± 7.7	39.6 ± 17	53 ± 12.4
RF positives	0	0	0	15	***	***	***	***	N/A	55	N/A
Mean disease duration (years ± SD)	6.2 ± 5.6	9.1 ± 5.5	10 ± 8.1	16 ± 14.2	2.4 ± 0.3	1.3 ± 0.9	6.9 ± 3	4.7 ± 2.3	N/A	10.4 ± 20	N/A
CRP ± SD (mg/dL)	1.4 ± 1.8	1.1 ± 0.9	0.9 ± 1.2	2.2 ± 1.3	***	***	3.1 ± 1.1	***	N/A	1.4 ± 2.3	N/A
ESR ± SD (mm/hr)	20.1 ± 18	19 ± 10.7	21 ± 14.8	20 ± 13	***	***	***	***	N/A	34.2 ± 43	N/A

PO, persistent oligoarticular; EO, extended oligoarticular; PRF-, polyarticular rheumatoid factor negative; PRF+, polyarticular rheumatoid factor positive; Rhe F, rheumatic fever; UV, uveitis; SLE, systemic lupus erythematosus; HT, Hashimoto's thyroiditis; HC1, healthy children; RA, rheumatoid arthritis; HC2, healthy adults; *n*, number of subjects; F, female; M, male; SD, standard deviation; RF, rheumatoid factor; N/A, not applicable; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate. *except for one patient, all JIA patients were tested in a previous publication (4). **Total of samples used for mimotopes selection/total of samples used for synthetic peptide validation. ***missing data. Variables are expressed as means ± SD.

Immunoglobulin G purification and coupling. Immunoglobulins G (IgG) from pooled sera of different JIA subtypes; healthy children; and other autoimmune diseases were covalently bound to protein G-coupled magnetic beads via their Fc-region, according to the manufacturer's recommendations (Dynabeads® Protein G, Invitrogen).

Selection of peptides specifically binding to IgG purified from JIA patients. For mimotopes selection we used a Ph.D.-C7C Phage Display (PD) Peptide Library Kit (New England Biolabs). This library is based on a 7-mer random peptide combinatorial library fused to the pIII capsid of the M13 phage. For screening the PD library to select JIA mimotopes, we used a subtractive step against purified IgGs from healthy children and patients with other autoimmune diseases, followed by a positive selection against purified IgGs from JIA subtypes. The biopanning procedure was performed as described elsewhere with some modifications (5). Briefly, 1×10^{11} phages particles of the PD library was diluted in 190 µl of TBS-T 0.1% (137 mM NaCl, 5.1 mM KCl, 1.35 mM $CaCl_2$, 1.05 mM $MgCl_2$, 1.4 mM Na_2HPO_4, 24.8 mM Tris, pH 7.5 plus 0.1% Tween-20). A volume of 100 µl of the diluted PD library was transferred to a microtube containing 20 µl of beads coupled to IgGs purified from serum of healthy children, and incubated for 30 min at room temperature (RT). The microtube was placed on a Magnetic Particle Concentrator (Dynal MPC™ - Invitrogen), and the supernatant containing non-bound phages was transferred to a microtube containing 20 µl of beads

coupled to IgGs purified from serum of RF patients, and incubated for 30 min at RT. The same procedure was performed for each autoimmune disease. After subtractive step, the non-bound phages were collected and divided in four microtubes (55 µl each) containing 20 µl of beads coupled to IgGs purified from serum of four JIA subtypes and incubated for 30 min at RT. Non-bound phages were removed after 10 washes with TBS-T 0.1% in the first round, and TBS-T 0.5% in the two subsequent rounds. Between each round of selection, bound-phages were eluted with 100 µl of 0.2 M glycine-HCl buffer (pH 2.2), neutralized with 75 µl of Tris (pH 9) and 1 µl of the eluted phages was subjected to titration. To enrich the phage clones selected, the eluted phages were amplified in *Escherichia coli* ER2738 (New England Biolabs) between each round. The mimotopes selection was performed separately for each JIA subtype due to the fact that our initial strategy was to identify biomarkers able to differentiate each subtype of the disease.

DNA sequencing of selected phage clones. After the third round of biopanning, 48 individual phage clones of each JIA subtype (total of 192 phage clones) were randomly selected and amplified for DNA sequencing. Phages single-stranded DNA were isolated by iodide buffer extraction procedure (Instruction Manual Ph.D.-C7C Phage Display Peptide Library Kit) and analyzed with MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer using 200 ng of primer -96 gIII (5'-OH CCC TCA TAG TTA GCG TAA CG-3'; New England Biolabs).

Peptide synthesis. The PFR+1 peptide (SSWLPRG) that presented highest specificity in the phage-ELISA was chemically synthesized by GenScript USA Inc. (Piscataway, NJ, USA), and submitted for further analysis in a larger cohort. The peptide was constructed with 14 residues (ACSSWLPRGCGGGS) with amidation of the C-Terminal region.

Synthetic peptide detection by ELISA. ELISA assay was carried out to test the reactivity and binding specificity of the PRF+1 synthetic peptide against individual sera from JIA patients, healthy children, patients with other autoimmune diseases

and healthy adults. Briefly, a ninety-six-well Maxisorp microtiter plate (NUNC, NY, USA) was coated with the synthetic PRF+1 peptide at 3 µg/ml diluted in carbonate buffer (0.1 M $NaHCO_3$, pH 8.6), and incubated overnight at 4°C. Peptide-coated wells were emptied and washed once with PBS, and then blocked with 3% BSA in PBS at 37°C for 1h. After blocking, a total of 100 µL of the blocking buffer solution containing sera from the different groups diluted at 1:100 was added to each well followed by incubation for 1h at 37°C under gentle agitation. After four washes with PBS-T 0.05%, HRP-conjugated rabbit anti-human IgG (Roche Applied Science) diluted at 1:5000 in the blocking buffer was added to each well followed by incubation for 1h at 37°C under gentle agitation. ELISA plate was washed three times with PBS-T 0.05%, and the reaction was revealed with OPD SigmaFast™ (Sigma-Aldrich) and read at 492 nm. All samples were tested in duplicates. Each serum sample was tested alone (without PFR+1 peptide) as negative control.
The final OD was adjusted by subtracting the ratio of OD readings obtained for each serum sample to the ratio of OD readings obtained by the negative control. The optimum point of reaction for anti-PFR+1 antibodies detection was determined using the receiver operating characteristic (ROC) curve, where a cut-off point was determined as the value of the parameter corresponding to the highest possible sensitivity without losing specificity. To calculate the ROC curves, sensitivity and specificity, we considered the control groups as a single group. The overall average achieved by control groups was used for data normalization.

Electrochemical analysis. To test the immobilization of the PFR+1 peptide onto bioeletrodes surface for antibody detection, electrochemical analysis was carried out. A 7 mm^2 ItalSens IS-Au gold screen-printed electrode was pretreated with cyclic voltammetry in sulfuric acid 0.5 mol L^{-1} and the PFR+1 peptide solution (1 µg mL^{-1}) was applied onto the electrode surface. The surface was blocked with PBS-BSA 3% and separately incubated with serum samples from patients with JIA, RA or healthy controls. After each step, the system was incubated at 4°C for 40 min and then it was rinsed with 0.1 mol L^{-1} phosphate buffer. All measures were performed though differential pulse voltammetry. The electrolyte was phosphate buffer 0.1 mol L^{-1} to detect the immobilization of the peptide and 5 mmol L^{-1}

potassium ferrocyanide ($K_4[Fe(CN)_6]$) solution containing 0.1 $mol.L^{-1}$ KCl to evaluate the serum samples.

Statistical analysis. Unpaired *t* test with Welch's correction was used to determine significant differences in reactivity between groups. Sensitivity and specificity parameters were calculated based on the receiver operating characteristic (ROC) curve analysis. To estimate the positive predictive accuracy, the area under the curve (AUC) was also determined. All statistical analyses were performed using GraphPad Prism 5.0 software. *P* values less than 0.05 were considered statistically significant.

Results

Mimotopes selected by phage display and PRF+1 synthetic peptide validation by ELISA. A total of 38 selected mimotopes presented different amino acid sequences (Supplementary Table 1) and was tested for immunoreactivity evaluation by phage-ELISA (Supplementary Figure 1). Although most mimotopes was able to discriminate JIA patients from healthy children and patients with other inflammatory diseases, the PRF+1 mimotope presented highest specificity (Supplementary Figure 1E) and for this reason, was chosen to be chemically synthetized for further evaluation. The other mimotopes selected will be explored in forthcoming studies.

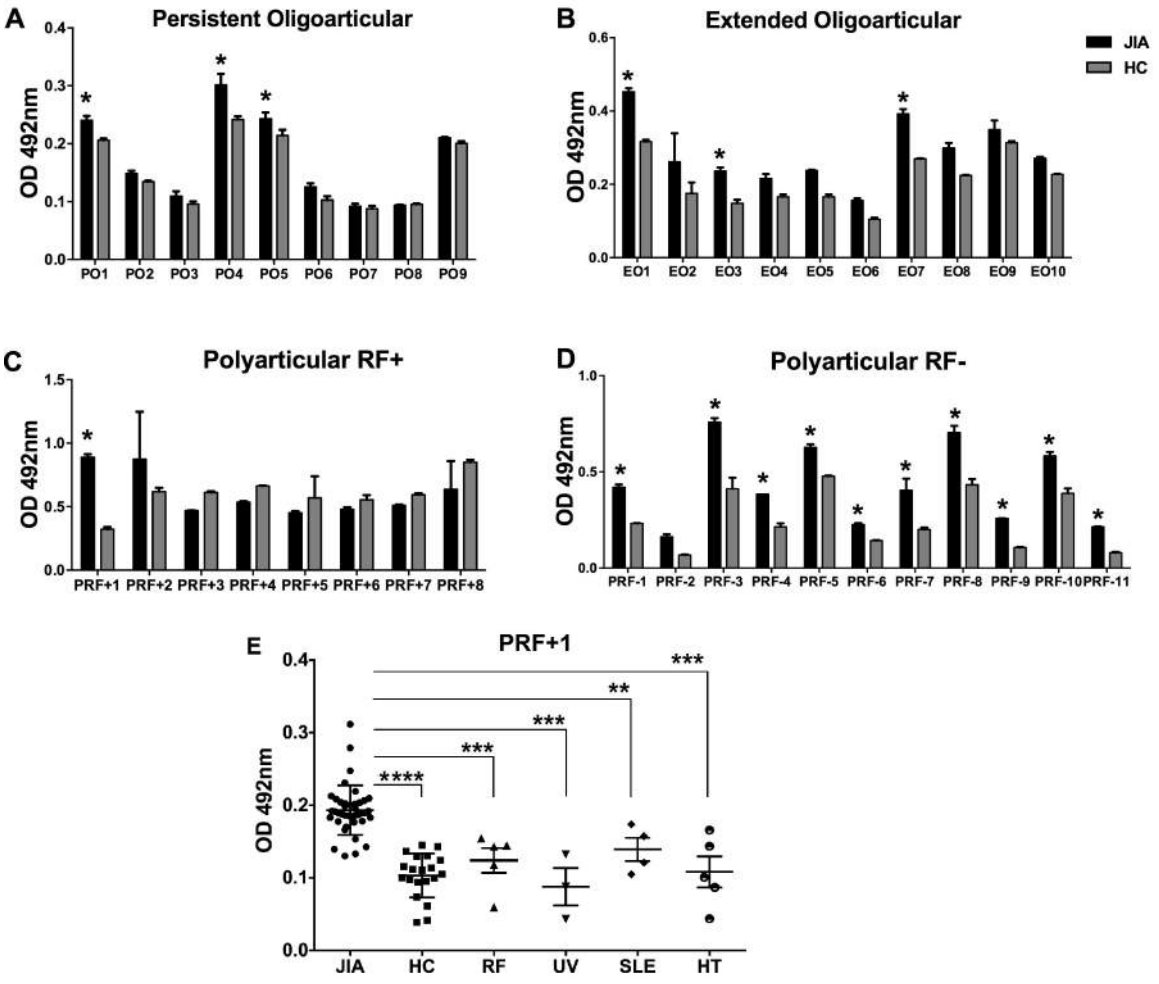
Supplementary Table1. Sequence and frequency of the peptides selected.

Mimotopes ID	Amino acids sequence	Frequency (%)
PO1	PGLATNF	1
PO2	SPFWWSD	25
PO3	SPFWWTD	36
PO4	PNPFVLD	1
PO5	LGSVQHT	1
PO6	SPFWWSL	1
PO7	FSPFFWN	1
PO8	WILFFLD	1
PO9	NSPFQLF	1
EO1	SPFFLTP	1

EO2	WNPFLLD	1
EO3	QSPFHLF	1
EO4	SPFWWHQ	3
EO5	NPGWLGS	1
EO6	SAVIKSS	1
EO7	SSHLLSE	1
EO8	LPSRSQT	1
EO9	NNPFQLW	1
EO10	SSFWWTD	1
PRF+1	SSWLPRG	1
PRF+2	WSPFLAP	1
PRF+3	FDPFGWS	2
PRF+4	SPFDWWF	1
PRF+5	NPFFLTA	1
PRF+6	SPNPPLH	1
PRF+7	SSPFFLW	1
PRF+8	SPFHLLP	1
PRF-1	NPFSLLA	1
PRF-2	YFTAPPD	1
PRF-3	DSPFRLW	1
PRF-4	TRPTASH	1
PRF-5	SESPGIA	1
PRF-6	FSPFFAP	1
PRF-7	SPFWLAA	1
PRF-8	SPFFLGP	1
PRF-9	TPFWFLD	1
PRF-10	PAPSRSQ	1
PRF-11	RFGGLIA	1
Total		100

To confirm the ability of the PRF+1 synthetic peptide to discriminate JIA patients from HCs and patients with other inflammatory diseases, ELISA assay was carried out. Based on the determined cut-off point, 57 out of 97 (59%) JIA patients were positive for anti-PRF+1 antibodies. The PRF+1 peptide was able to efficiently discriminate JIA patients from healthy children ($P < 0.0001$), rheumatic fever ($p < 0.0001$), uveitis ($P < 0.0001$), systemic lupus erythematous ($P < 0.0001$) and Hashimoto's thyroiditis ($P < 0.0001$) (Figure 1A). The ROC curve constructed for the anti-PRF+1 antibodies detection in samples from JIA patients was

significant (AUC = 0.8478; *P* < 0.0001) and based on the cut-off value determined presented sensitivity of 61% and specificity of 91% (Figure 1B).



Supplementary Figure 1. Mimotopes selected by phage display and its immunoreactivities. A total of 38 mimotopes was selected against IgG purified from JIA patients. Phage-ELISA assay was first performed to test the immunoreactivity and binding specificity of the selected mimotopes against pooled sera from different subtypes of JIA patients and healthy children (A, B, C, D). Mimotopes that presented significantly higher absorbance when compared to the healthy control were individually tested with sera from 40 patients with JIA, 5 with RF, 3 with UV, 4 with SLE, 5 patients with HT and 20 healthy children. Each pooled serum sample was tested against wild M13 phage (without displaying any peptide on its surface) as negative control. For data adjustment, the final optimal density (OD) was adjusted by subtracting the ratio of OD readings obtained by the

tested mimotopes to the OD readings obtained by the wild-type M13 phage. None of the mimotopes selected was able to discriminate the subtype to which it was obtained from other subtypes of the disease. (E) The PRF+1 mimotope presented highest specificity in discriminating JIA patients from controls and was chosen to be chemically synthetized for further analysis and validation.

When tested for RA detection, PRF+1 peptide was also able to discriminate RA patients from healthy adults. A total of 97 out of 103 (94%) RA patients were positive for anti-PRF+1 antibodies. The PRF+1 peptide was able to efficiently discriminate RA patients from healthy adults ($P < 0.0001$) (Figure 1C). The ROC curve constructed for anti-PRF+1 antibodies detection in samples from RA patients was significant (AUC = 0.9836; $P < 0.0001$)) and based on the cut-off value determined presented sensitivity of 94% and specificity of 93% (Figure 1D).

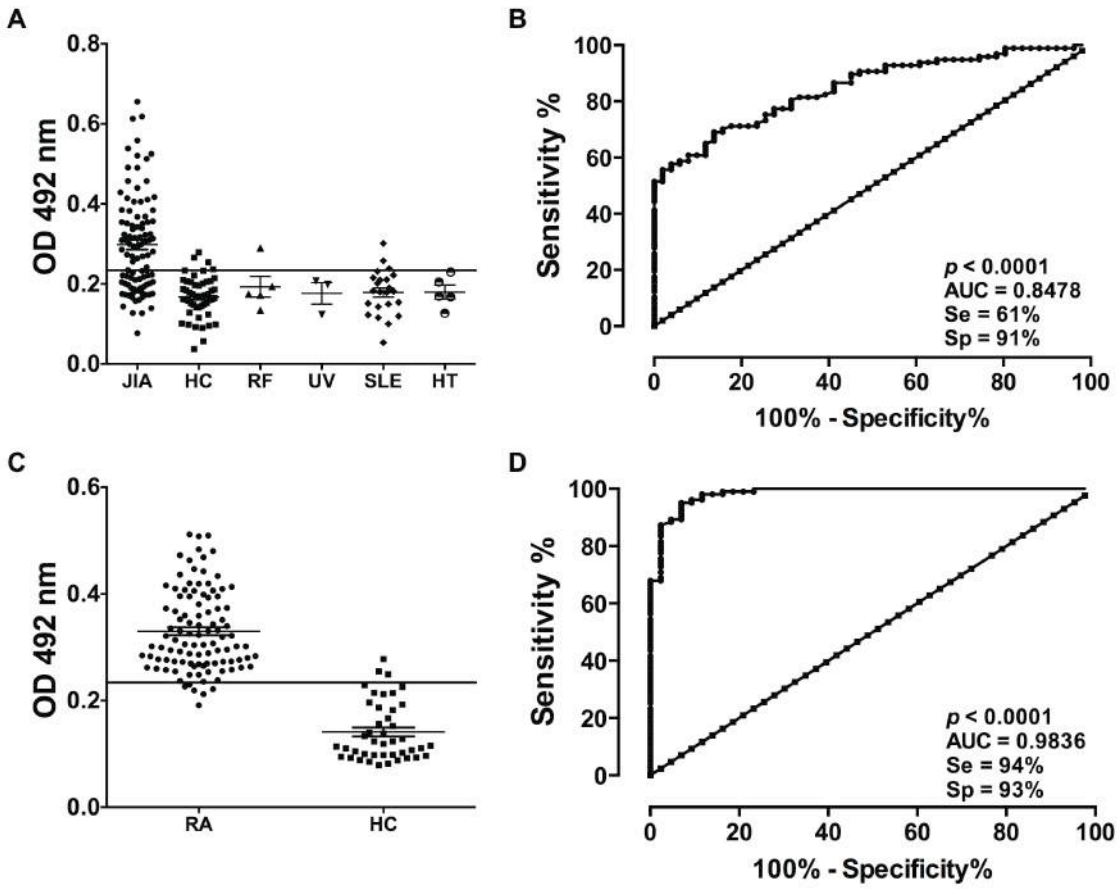


Figure 1. Anti-PFR+1 antibody detection by ELISA. Immunoglobulin G antibodies detection in serum samples from patients with JIA (n = 97), rheumatic fever (n= 5),

uveitis (*n* = 3), systemic lupus erythematosus (*n* = 23), Hashimoto's thyroiditis (*n* = 5) and healthy children (*n* = 51); rheumatoid arthritis (*n* = 103) and healthy adults (*n* = 43) by ELISA assay using the synthetic PRF+1 peptide as the antigen. (A) Anti-PFR+1 antibodies detected in JIA patients and controls and the respective ROC curve (B). (C) Anti-PFR+1 antibodies detected in RA patients and controls and the respective ROC curve (D).

Correlation between anti-PRF+1 antibodies levels and studied variables. Antibodies against PRF+1 peptide in JIA patients were directly correlated with the number of swollen joints (Pearson *r* = 0.4626, *P* = 0.0005). On the other hand, no correlation was found between anti-PRF+1 antibodies levels and any variable analyzed to RA patients.

Anti-PRF+1 antibodies detection by electrochemical analysis. The differential pulse voltammogram in phosphate buffer of the bare gold electrode (Figure 1A) was significantly different from the base line in the gold electrode with the immobilized peptide and between groups (Figure 1B). The proposed bioelectrode was able to efficiently discriminate healthy controls from patients with JIA (*P* = 0.0049) and RA (*P* < 0.0001) (Figure 1C).

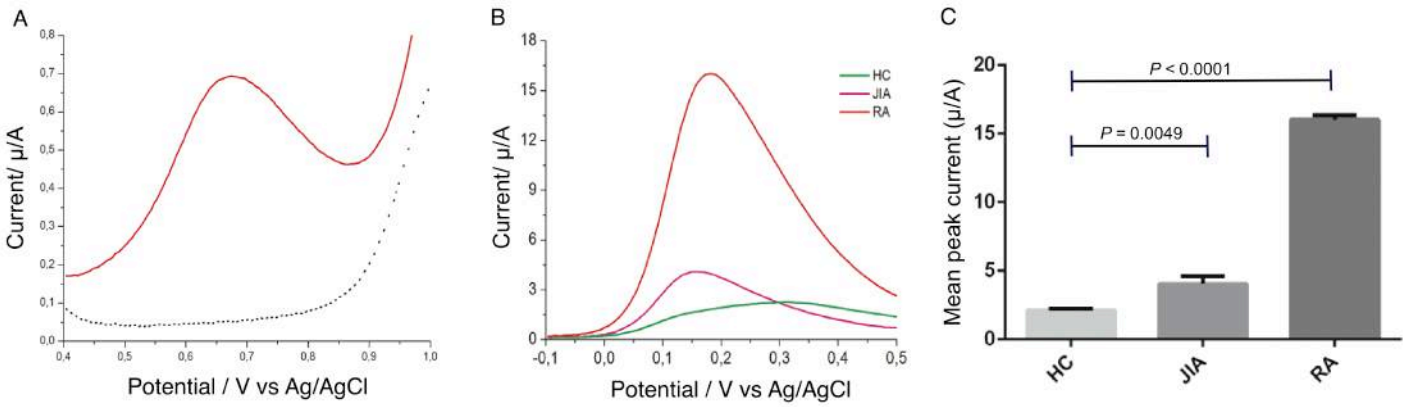


Figure 2. Electrochemical analysis. (A) Differential pulse voltammograms in phosphate buffer 0.1 mol L^{-1} of bare gold electrode (dashed line) and gold with immobilized peptide (red line). (B) Differential pulse voltammograms of gold electrodes modified with synthetic PFR+1 peptide after applying serum from

patient with JIA, RA and healthy individuals. The voltammetry was performed in 5 mmol L^{-1} $K_4[Fe(CN)_6]$ solution containing 0.1 mol.L^{-1} KCl. (C) Bar plot of mean peak current showing that the biosensor was able to significantly differentiate patients with JIA and RA from healthy controls.

Discussion

To date, there are no specific laboratory tests for confirming JIA and RA diagnosis. This study describes the successful strategy employed to select peptides with high specificity that could be used as serum biomarkers to aid in JIA and RA diagnosis. Different serum biomarkers have been reported to JIA (4, 6, 7) and RA (8-10) patients. However, up to this date, only rheumatoid factor and antibodies against cyclic citrullinated peptides (CCP) have been incorporated into the clinical routine (11). The aim of this work was to select by phage displayed random peptide library mimotopes that could be potentially recognized by IgG antibodies present in JIA and RA patients. This approach enabled the selection of a short peptide, named as PRF+1, that is target by autoantibodies in patients with JIA and RA with high specificity and sensitivity.

Phage display libraries contain random peptides presenting either continuous or discontinuous mimotopes, which are antibody-binding sites that mimic essential properties of epitopes but do not necessarily present the same amino acids sequence (12). Due to this advantage, we have chosen this method to select new peptides against IgG antibodies that could mimic epitopes present in JIA and RA patients. Our strategy included a subtractive panning against purified IgGs from different inflammatory diseases and healthy subjects, which is strongly recommended to limit the recovery of target-unrelated peptides (13), followed by a positive panning against purified IgG from JIA patients. This strategy led us to select mimotopes with 38 different amino acid sequences, suggesting that these are immunodominant epitopes involved in the humoral response in patients with JIA.

We found that anti-PRF+1 antibodies were present in 59% of JIA and 94% of RA patients, presenting AUC of 0.84 and 0.98, sensitivities of 61% and 94%, and specificities of 91% and 93%, respectively. These results represent a very good accuracy for diagnostic tests compared to anti-CPP antibodies and RF

detection, the two most useful serum biomarkers for JIA and RA diagnosis (14, 15). The variable potentially associated with anti-PRF+1 levels was the number of swollen joints in patients with JIA, suggesting that this peptide might be linked to disease activity.

Electrochemical analysis demonstrated that the PRF+1 synthetic peptide was successfully immobilized onto the gold electrode surface and after differential pulse voltammograms in phosphate buffer was able to efficiently discriminate healthy controls from JIA and RA patients, and although serum samples contain many interfering substances, none of them have disturbed the detection process. This immunosensor based on the anti-PFR+1 antibodies detection presented to be very sensitive, low cost fast and reproducible.

In conclusion, we have selected a new target for circulating autoantibodies in patients with JIA that presented high specificity and sensitivity to JIA and RA in two different approaches and could be potentially used to aid in diagnosis. Although this peptide is a promising target for antibodies detection, the protein that this peptide mimics still needs to be identified in a forthcoming study.

Acknowledgements. The authors would like to thank Dr. Karla Pereira Fernandes from pediatric rheumatology department of the Clinics Hospital at the Federal University of Uberlândia for her assistance and help in obtaining blood samples from Brazilian children. We would also like to thank all members of the Biobanco-IMM from the Lisbon Academic Medical Center, for providing blood samples and the relevant patient data.

Author contributions. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ueira-Vieira and MSc Araujo had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Araujo, Fujimura, Vaz, T. Silva, Rodovalho, A. Madurro, J. Madurro, Fonseca, C. Silva, Santos, Mourão, Canhão, Goulart, Gonçalves, Ueira-Vieira

Acquisition data. A. Madurro, J. Madurro, C. Silva, Mourão, Canhão, Goulart, Gonçalvez, Ueira-Vieira

Analysis and interpretation of data. Araujo, Fujimura, Vaz, T. Silva, Rodovalho, A. Madurro, Fonseca, Goulart, Ueira-Vieira

References

1. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. The Journal of rheumatology 2004;31(2):390-2.

2. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Annals of the rheumatic diseases 2010;69(9):1580-8.

3. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis and rheumatism 1988;31(3):315-24.

4. Araujo GR, Fonseca JE, Fujimura PT, Cunha-Junior JP, Silva CH, Mourao AF, et al. Anti-type II collagen antibodies detection and avidity in patients with oligoarticular and polyarticular forms of juvenile idiopathic arthritis. Immunology letters 2015;165(1):20-5.

5. Barbas CF, Burton DR, Scott JK, Silverman GJ. Phage display: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 2001.

6. Gibson DS, Qiu J, Mendoza EA, Barker K, Rooney ME, LaBaer J. Circulating and synovial antibody profiling of juvenile arthritis patients by nucleic acid programmable protein arrays. Arthritis research & therapy 2012;14(2):R77.

7. Kamiya Y, Kawada JI, Kawano Y, Torii Y, Kawabe S, Iwata N, et al. Serum microRNAs as Potential Biomarkers of Juvenile Idiopathic Arthritis. Clinical rheumatology 2015.

8. Verheul MK, Fearon U, Trouw LA, Veale DJ. Biomarkers for rheumatoid and psoriatic arthritis Clinical immunology. 2015.

9. Liao H, Wu J, Kuhn E, Chin W, Chang B, Jones MD, et al. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. Arthritis and rheumatism 2004;50(12):3792-803.

10. Robert-Pachot M, Desbos A, Moreira A, Becchi M, Tebib J, Bonnin M, et al. Carbonic anhydrase III: a new target for autoantibodies in autoimmune diseases. Autoimmunity 2007;40(5):380-9.

11. Taylor P, Gartemann J, Hsieh J, Creeden J. A systematic review of serum biomarkers anti-cyclic citrullinated Peptide and rheumatoid factor as tests for rheumatoid arthritis. Autoimmune diseases 2011;2011:815038.

12. Geysen HM, Meloen RH, Barteling SJ. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proceedings of the National Academy of Sciences of the United States of America 1984;81(13):3998-4002.

13. Molek P, Strukelj B, Bratkovic T. Peptide phage display as a tool for drug discovery: targeting membrane receptors. Molecules 2011;16(1):857-87.

14. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Annals of internal medicine 2007;146(11):797-808.

15. Sun J, Zhang Y, Liu L, Liu G. Diagnostic accuracy of combined tests of anti cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis: a meta-analysis. Clinical and experimental rheumatology 2014;32(1):11-21.

ANEXOS

Araujo et al. Arthritis Research & Therapy (2015) 17:168
DOI 10.1186/s13075-015-0685-3

arthritis research&therapy

RESEARCH ARTICLE Open Access

Improved serological detection of rheumatoid arthritis: a highly antigenic mimotope of carbonic anhydrase III selected in a murine model by phage display

Galber Rodrigues Araujo[1,2*], Emília Rezende Vaz[1], Patricia Tiemi Fujimura[1], João Eurico Fonseca[3,4], Lucélia Maria de Lima[1], Helena Canhão[3,4], Gabriela Venturini[5], Karina Helena Morais Cardozo[6], Valdemir Melechco Carvalho[6], Marcelo Henrique Napimoga[7], Luiz Ricardo Goulart[1,8], João Gonçalves[2,9] and Carlos Ueira-Vieira[1]

Abstract

Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects around 1 % of the human population worldwide. RA diagnosis can be difficult as there is no definitive test for its detection. Therefore, the aim of this study was to identify biomarkers that could be used for RA diagnosis.

Methods: Sera from a collagen-induced arthritis mouse model were used to select potential biomarkers for RA diagnosis by phage display technology. In silico and in vitro analyses were performed to characterize and validate the selected peptides. Samples were classified into three groups: RA; two other immune-mediated rheumatic diseases (systemic lupus erythematosus (SLE) and ankylosing spondylitis (AS)); and healthy controls (HC). Enzyme-linked immunosorbent assay (ELISA) was carried out to determine antibody levels, and diagnostic parameters were determined by constructing receiver operating characteristic curves. Mass spectrometry and Western blot were performed to identify the putative autoantigen that was mimicked by a highly reactive mimotope.

Results: After three rounds of selection, 14 clones were obtained and tested for immunoreactivity analysis against sera from RA and HC groups. The phage-fused peptide with the highest immunoreactivity (M12) was synthesized, and was able to efficiently discriminate RA patients from SLE, AS and HCs ($p < 0.0001$) by ELISA. The specificity and sensitivity of anti-M12 antibodies for RA diagnosis were 91 % and 84.3 %, respectively. The M12 peptide was identified as one that mimics a predicted antigenic site of the carbonic anhydrase III (CAIII) protein, a ubiquitous biomarker that has been identified in patients with other diseases.

Conclusion: M12 is the first peptide associated with the CAIII protein that may be used as an antigen for antibody detection to aid in RA diagnosis with high sensitivity and specificity.

* Correspondence: galber.araujo@gmail.com
[1]Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, MG, Brazil
[2]iMed.UL – Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal
Full list of author information is available at the end of the article

BioMed Central

© 2015 Araujo et al. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

Introduction

Rheumatoid arthritis (RA), the most common inflammatory autoimmune disease, affects 0.8 % of the adult population worldwide [1]. RA diagnosis is largely a clinical one, relying, particularly in the early stages, on the history and examination of the patient, with tests (blood or imaging) sometimes helping to confirm the diagnosis [2]. Serological support to diagnosis has, up to now, been restricted to the determination of rheumatoid factor (RF) and anticitrullinated peptide antibodies (ACPAs), where assays using cyclic citrullinated peptides (CCP) as antigen for ACPA detection have gained wide acceptance [3]. RF presents higher sensitivity as compared with antiCCP antibodies for established disease, with a relatively low specificity. In fact, the RF antibody is not specific for RA due to crossreactivity with many other inflammatory diseases, as well as in elderly healthy individuals [4]. ACPAs are considered a valuable serological biomarker for RA [5] and the diagnostic performance of different generations of CCPs (CCP1, CCP2 and CCP3) have been evaluated in many different studies [6–8]. Differences in cut-off values, specificities and sensitivities exist between the three different generations and also between different assays used for antibody detection. However, antiCCP2 showed better performance characteristics with values of sensitivity ranging from 41 % to 92.2 % and specificity ranging from 65 % to 100 % [9]. At present, the detection of antibodies against CCP2 by enzyme-linked immunosorbent assay (ELISA) is the most widely used assay in studies involving ACPAs worldwide. The combination of RF and antiCCP2 assays demonstrate a positive predictive value close to 100 %, which is much higher than the value of either of the tests alone [10]. The presence of RF and antiCCP has been associated with progressive and destructive disease [11, 12]. Seronegativity in both early and established RA remains a major limitation of these two biomarkers, highlighting the need for new complementary markers that could improve diagnostic sensitivity [13]. Because of the low sensitivity or specificity of the current serological tests, the quest for new efficient auxiliary biomarkers in RA is of clinical relevance.

Animal models of arthritis have contributed to the overall knowledge on RA physiopathology and to the identification of important mediators of inflammation. The collagen-induced arthritis (CIA) mouse model has proven to be a valuable experimental model for inflammatory RA studies [14–17]. After immunization with type II collagen (CII), DBA/1 J mice develop a severe polyarthritis mediated by an autoimmune response that shares many features with human RA [18].

With the goal of identifying new clinically useful biomarkers for RA, we have explored the CIA mouse model and phage display (PD) technology to isolate peptides that can mimic RA autoantigens. PD technology has been widely used by our group and others to screen targeting peptides in drug discovery and biomarker selection, and has been highly effective in discovering peptides with affinities to virtually any target [19–24]. Short peptide sequences selected by PD libraries with high affinity to antibodies, receptors or proteins may present potential applications in diagnostics or therapeutic kits and vaccines [25, 26]. Using the cDNA PD library for autoantigen selection, were recently identified novel autoantibodies in early and seronegative RA patients with sensitivities ranging from 2 % to 29 %, and specificities ranging from 95 % to 100 %. These autoantibodies can be found in 44 % to 67 % of ACPA-negative RA patients [27].

This investigation describes the identification of a short peptide selected by PD technology against sera from CIA mice. This short peptide was characterized by in silico and in vitro strategies, and further tested as a potential biomarker for RA diagnosis in comparison to other rheumatic diseases and healthy controls. Its predicted antigenic epitope target was deduced by mass spectrometry (MS) and Western blot analyses.

Methods

Study subjects

For this study, we used serum samples from patients who fulfilled the RA diagnostic criteria according to the 2010 classification [28]. The demographic and laboratory characteristics of all studied subjects are presented in Table 1. Well-characterized serum samples used in this study were requested from Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. Samples from healthy subjects who did not have any arthritis symptoms were used as the healthy control (HC) group. To provide data on assay specificity, samples from patients with other rheumatic diseases (nonRA) were also analyzed. The subjects enrolled in this study were classified into three groups: RA, 172 patients (151 females and 21 males, mean age 53.7 ± 11.2 years); HC, 113 subjects (79 females and 34 males, mean age 58.8 ± 8.7 years); and nonRA, 32 patients with other immune-mediated rheumatic diseases that consisted of 19 systemic lupus erythematosus (SLE) and 13 ankylosing spondylitis (AS) patients. For RA patients, data recorded were age at disease diagnosis, disease duration, medication status, presence of RF and antiCCP, erythrocyte sedimentation rate (ESR), levels of C-reactive protein (CRP), and tender and swollen joint counts obtained at the time of blood sample collection. Blood samples were allowed to clot and then centrifuged at 4,000 rpm for 10 minutes. All sera samples were filtrated with a microcell filter (0.22 μm) to eliminate red blood cell fragments and bacteria, and then frozen at −80 °C immediately until analysis. The parts of the study which involved human subjects was approved by the ethics committees of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria and the Hospital

Table 1 Demographic and laboratory characteristics of the studied population

	RA	HC	SLE	AS
Age (years; mean ± SD)	53.7 ± 11.2	58.8 ± 8.7	55.7 ± 5.7	49.5 ± 7.5
Gender (female/male)	151/21	79/34	16/3	4/9
Caucasian (n (%))	146 (85 %)	104 (92 %)	18 (95 %)	11 (85 %)
Disease duration (years; mean ± SD)	8.1 ± 8.4		9.2 ± 6.5	12 ± 9.1
Rheumatoid factor positive (n)	102 (59.3 %)		NA	NA
CRP (mg/dl; mean ± SD)	2.4 ± 1.1		3.1 ± 0.9	2.7 ± 0.8
ESR (mm/hour; mean ± SD)	32 ± 26		33.4 ± 19.5	NA
AntiCCP positive (n)	75 (43.6 %)		NA	NA
Biologic therapy (n in treatment)	62 (36 %)		4 (21 %)	3 (23 %)
NSAIDs (n in treatment)	127 (74 %)		9 (47 %)	5 (38 %)

CRP value ≥0.8 mg/dl is considered elevated. ESR value ≥15 mm/hour is considered elevated. *antiCCP* anticyclic citrullinated peptide antibodies, *AS* ankylosing spondylitis, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *HC* healthy control, *NA* not available, *NSAIDs* nonsteroidal anti-inflammatory drugs, *RA* rheumatoid arthritis, *SD* standard deviation, *SLE* systemic lupus erythematosus

Garcia de Orta, Lisbon, Portugal, and conducted in accordance with the regulations governing clinical trials such as the Declaration of Helsinki (2008). An informed consent form was signed by all subjects enrolled in this study before any protocol procedure was carried out.

CIA mice

A total of twelve male DBA/1 J mice (8–10 weeks old) were housed at the animal facility of the Federal University of Uberlândia (Uberlândia-MG, Brazil) where they fed ad libitum. This study was carried out in strict accordance with the recommendations contained in terms for the use of animals in research and teaching of the Federal University of Uberlândia (Uberlândia, MG, Brazil), in compliance with the National Guidelines, as set forth by the Institutional Animal Care (Law number 11.794, 2008). The study protocol was approved by the Animal Ethics Committee from the Federal University of Uberlândia (UFU) under the number CEUA/UFU 059/10.

Arthritis induction and assessment of CIA mice

Arthritis was induced in the isogenic murine model DBA/1 J (males; 8–10 weeks old) as described previously with some modifications [18]. Briefly, a commercial bovine CII (Becton Sigma-Aldrich, St. Louis, MO, USA) emulsified in complete Freund's adjuvant (CFA) (Sigma-Aldrich) was used for mice immunization. DBA/1 J mice (n = 9) were immunized by intradermal injections at the base of the tail with 100 μg bovine CII. For the control group, mice (n = 3) were immunized with phosphate-buffered saline (PBS) in CFA. Mice were boosted at day 30 with 50 μg bovine CII in CFA. After immunization, mice were examined twice a week when paw edema was measured using manual calipers and arthritis signs were visually scored by evaluating joint inflammation, using an established scoring system from 0 to 4 [29], where 0 = no evidence of erythema and swelling, 1 = erythema and mild swelling confined to the tarsals or ankle joints, 2 = erythema and mild swelling extending from the ankle to the tarsals, 3 = erythema and moderate swelling extending from the ankle to metatarsal joints, 4 = erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb. Around 60 days after immunization, mice presenting acute arthritis (score 4) were sacrificed under anesthesia. Blood (for sera extraction) and inflamed joints were then extracted for peptide selection through PD technology.

Total protein extraction from inflamed joints of CIA mice

Inflamed joints were removed from CIA mice, macerated with liquid nitrogen and suspended in extraction buffer (20 mM Tris–HCl pH 7.2, 10 mM EDTA, 2 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM Benzamidine, 1 mM PMSF). The resulting material was transferred to a microtube, and centrifuged at 20,000 × *g* for 30 minutes at 4 °C. The supernatant was collected and the concentration of the extracted proteins was determined by the Bradford method [30]. As many inflammatory cytokines indicative of RA are expressed in the cartilage, ligament, pannus, articular capsule, and so forth, of CIA mice [31], we used these proteins to dissociate the selected phage clones from target antibodies by competitive elution.

Purification and isolation of immunoglobulin G from sera

Immunoglobulin G (IgG) purification and isolation from sera of mice with acute arthritis and naïve mice was performed with Dynabeads® Protein G (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Briefly, a total of 50 μl magnetic beads were washed twice with TBS-T 0.1 % (Tris-buffered saline: 50 mM Tris–HCl, 150 mM NaCl, pH 7.5 plus 0.1 % Tween 20) and then incubated with 100 μl pooled serum for 1 hour at room temperature. After binding, the bead–IgG complex was

blocked with TBS plus 5 % bovine serum albumin (BSA) at 37 °C for 1 hour, washed three times with TBS-T 0.1 % and then resuspended in 200 μl TBS.

Mimotope selection through phage display

For mimotope (phages expressing peptides on their surface) selection a PhD-12mer phage display peptide library kit (New England Biolabs, Beverly, MA, USA) was screened against IgG purified from CIA mice. This is a combinatorial library of random dodecapeptides fused to the N-terminus of the minor coat protein (pIII) of M13 phages. The library consists of 2.7×10^9 diverse sequences that were amplified once to yield about 50 copies of each peptide sequence.

Based on scientific evidence showing that CIA mice and RA patients share several pathological features, circulating autoantibodies to common targets [29], and also because arthritis can be much easier to monitor in experimental animal models than in humans due to the complexity of the symptoms, we decided to perform the mimotope selection against IgG purified from CIA mice.

The strategy adopted for the mimotope selection consisted of a subtractive step to remove nonspecific phages by pre-incubating the phage peptide library with IgG purified from naïve mice. In order to remove nonspecific phages, a volume of 7×10^8 beads/ml coupled with IgG purified from naïve mice serum was incubated with 1×10^{11} phage particles from the PhD-12 library in 200 μl TBS-T 0.1 % solution for 30 minutes at room temperature. After paramagnetic bead precipitation using the Magnetic Particle Concentrator (Dynal MPC™; Invitrogen), unbound phages were collected and incubated with beads coupled with IgG purified from CIA mice presenting signs of acute arthritis, following incubation for 30 minutes at room temperature. The unbound phages were discarded this time by washing ten times with TBS-T 0.1 %. For competitive elution, the bound phages were incubated for 30 minutes with 10 μg of the total protein extracted from inflamed joints of CIA mice. The eluted phages were amplified in *Escherichia coli* ER2738 strain (New England Biolabs) and purified by PEG-NaCl precipitation. After each of the three rounds of selection, individual bacterial colonies containing amplified phage clones were grown in a microtiter plate and titrated as described elsewhere [32].

DNA extraction and sequencing

Phage DNA was isolated from 1 ml overnight cultures by precipitation with 1/6 volume PEG/NaI (20 % w/w, polyethylene glycol 8000) and iodide buffer (10 mm Tris–HCl (pH 8.0), 1 mm EDTA, and 4 m NaI). Phage DNA was precipitated with absolute ethanol, followed by a wash with 70 % ethanol, and resuspended in 20 μl Milli-Q water. Electrophoresis was performed on 0.8 % agarose gel stained with ethidium bromide solution in order to verify DNA quality. Sequencing reactions were carried out using the DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Pittsburg, PA, USA), with the primer –96 M13 (5'-OH CCC TCA TAG TTA GCG TAA CG-3') following the manufacturer's instructions, and detection was performed in a MegaBace 1000 Genetic Analyzer (Amersham Biosciences, Little Chalfont, UK) automatic capillary sequencer.

Bioinformatic analysis

A tool that can be found on the Sequence Manipulation Suite collection of JavaScript programs [33] was utilized to obtain the reverse complementary sequences of the DNA extracted from phages. Amino acid sequences were deducted by ExPASy Proteomics and Sequence Analysis tool [34, 35]. For sequence similarity analysis, multiple sequence alignment was performed by ClustalW2 online server [36]. Three-dimensional structure prediction was performed using The Pepitope Server [37, 38], and Immune Epitope Database and Analysis Resource [39]. The PyMOL (available at [40]) plugin was used for showing the peptide surface. Antigenicity prediction was carried out using Kolaskar and Tongaonkar antigenicity scale [41, 42].

Immunoreactivity of the selected mimotopes by phage-ELISA

For immunoreactivity measurements of selected mimotopes against sera from RA and HC groups, a phage-ELISA assay was performed. A 96-well Maxisorp™ microtiter plate (NUNC, New York, NY, USA) was coated in triplicate with anti-M13 monoclonal antibody (Amersham Biosciences) diluted (1:100) in carbonate buffer (0.1 M $NaHCO_3$, pH 8.6) overnight at 4 °C. The plate was washed once with TBS-T 0.5 % and blocked for 1 hour at 37 °C with 5 % BSA diluted in TBS. Additionally, the plate was washed twice and incubated with culture supernatant containing amplified phage particles (~ 10^{11} pfu/μl) for 1 hour at 37 °C. The plate was washed three times followed by incubation with serum pools from the RA and HC groups diluted (1:100) in TBS-T 0.5 % plus 5 % BSA for 1 hour at 37 °C. The plate was washed three times more with TBS-T 0.5 % followed by incubation with HRP-conjugated rabbit anti-human IgG (Roche Applied Science, Indianapolis, IN, USA.) diluted (1:5,000) in TBS-T 0.5 % plus 5 % BSA for 1 hour at 37 °C. The ELISA plate was washed three times, revealed with OPD SigmaFast™ (Sigma-Aldrich) and read at 492 nm. The reactivity obtained by the wild-type M13 phage without displaying any peptide (performed for each sample tested) was used for data adjustment, where the final optical density (OD) values obtained for each mimotope were adjusted by subtracting the corresponding OD values obtained by the wild-type M13 phage.

Purification of human anti-M12 antibody and M12 mimotope by immunoprecipitation

For purification of IgG antibodies that bind to the M12 mimotope, 1×10^{11} of M12 phage particles were covalently bound to Dynabeads® (Invitrogen). Thereafter, a volume of 50 µl of the solution containing the bead–M12 complex was separately incubated with 100 µl pooled sera from RA patients and HCs for 1 hour at room temperature under shaking for IgG:M12 binding. After incubation, the complex was washed ten times with TBS-T 0.1 % and the unbound nonspecific IgG present in the supernatant was discarded. The IgG that bound with high affinity to the M12 phage clone were eluted with 100 µl elution buffer (0.2 M Glycine-HCl, pH 2.2 and BSA 1 mg/ml) after incubation for 10 minutes at room temperature, followed by neutralization with 15 µl 1 M Tris–HCl (pH 9.1). The eluted IgG was incubated with 50 µl magnetic beads, as previous described. Subsequently, the solution containing the bead–IgG complex was incubated with 1 µg total protein extracted from inflamed joints of CIA mice for 1 hour at room temperature, under shaking condition for protein binding. The unbound proteins were discarded by washing ten times with TBS-T 0.1 %, followed by elution of the bound proteins with 100 µl 0.8 M acetic acid (pH 2.0). Proteins eluted were dried and submitted for MS analysis.

Western blot analysis

Total proteins extracted from inflamed joints of CIA mice (1 µg) were separated by 10 % SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 1 hour with 3 % BSA in PBS, and then rinsed three times in washing buffer containing PBS-T 0.1 %. Thereafter, the membrane was incubated overnight at 4 °C with IgG purified from sera of RA patients and HCs diluted at 100 µg/ml in PBS plus 3 % BSA, washed three times and incubated for 1 hour with rabbit anti-human IgG conjugated with peroxidase (Roche Applied Science) diluted 1:5,000 in PBS plus 3 % BSA. The subsequent washing steps and detection procedures were performed according to the ECL Plus manual (GE Healthcare).

Mass spectrometry

Protein digestion: dried proteins were suspended in 30 µl 0.2 % RapiGest and vortexed for 5 minutes. Protein digestion was carried out as described elsewhere with a few modifications [43]. Briefly, 10 µl 50 mM ammonium bicarbonate was added to the protein suspension to a final volume of 40 µl. Protein samples were denatured with 25 µl 0.2 % (w:v) RapiGest SF for 15 minutes at 80 °C, reduced with 2.5 µl 100 mM dithiothreitol at 60 °C for 30 minutes, alkylated with 2.5 µl 300 mM iodoacetamide at room temperature, and enzymatically digested at 37 °C overnight with trypsin at a 1:100 (w/w) enzyme to protein ratio. Then, 10 µl 5 % trifluoroacetic acid (TFA) was added to the digestion mixture to hydrolyze the RapiGest, and samples were incubated at 37 °C for 90 minutes. The tryptic peptide solution was then centrifuged at 14,000 rpm for 30 minutes at 6 °C, and the pH of the supernatant was adjusted to 2.6 by the addition of 10 µl 3 % acetonitrile 0.1 % formic acid.

Liquid chromatography/MS analysis: separation of peptides was performed using nanoliquid chromatography employing reverse phase. Peptides were injected into nanoLC through a nanoACQUITY system (Waters, Manchester, UK). Samples were first trapped in a Symmetry C18 5 µm, 180 mm × 20 mm column (Waters) with 0.1 % TFA in 3 % acetonitrile, then peptides were eluted from the trap column to an HSS T3 1.8 µm, 75 µm × 15 cm analytical column (Waters; mobile phase A, water with 0.1 % formic acid and B, 0.1 % formic acid in acetonitrile). Mass spectrometric acquisition was achieved in a Synapt MS Q-TOF mass spectrometer equipped with a NanoLockSpray source in the positive ion mode (Waters). For all measurements, the mass spectrometer was operated in the 'V' mode with a typical resolving power of at least 12,500. Data-independent scanning (MSE) experiments were performed by switching between low (3 eV) and elevated collision energies (15–50 eV) applied to the trap 'T-wave' cell filled with argon. Scan times of 0.8 s were used for low- and high-energy scans from m/z 50 to 2,000.

Protein identification and database analysis: protein identification was performed in Global Server v.2.5 (PLGS, Waters) with mouse UniProtKB Complete Proteome database. Up to one maximum missed cleavage by trypsin was allowed; fixed modification by carbamidomethylation (cysteine) and variable modifications by acetyl N-terminal and oxidation (methionine) were considered. The precursor and fragment ion mass error tolerances were adjusted to 10 and 20 ppm, respectively (default values). The criteria used for a positive protein match were at least three fragment ions per peptide, seven fragment ions per protein, and at least one peptide per protein hit. A false-positive discovery rate was allowed up to 4 %.

Peptide design and synthesis

After bioinformatic analysis of the selected clones, the M12 peptide sequence was designed and chemically synthesized by GenScript USA Inc. (Piscataway, NJ, USA). The peptide was constructed with 17 residues (CNVNSKSPVERITGGGS), with amidation of the C-terminal and conjugation of the BSA to cysteine at the N-terminus, a design strategy to increase sensitivity and decrease crossreactivity on ELISA assay [44].

Anti-M12 antibody detection by ELISA

A specific ELISA test was carried out to determine the M12 synthetic peptide reactivity to sera from patients with RA, SLE, AS and HCs. Ninety-six-well Maxisorp™ microtiter plates (NUNC) were coated with synthetic M12 (1.5 μg/ml) in carbonate buffer (0.1 M $NaHCO_3$, pH 8.6), and incubated overnight at 4 °C. After blocking with 3 % BSA in PBS at 37 °C for 1 hour, 100 μl/well of sera from the different groups were diluted in blocking buffer (1:100) and incubated for 1 hour at 37 °C under gentle agitation. After four washes with PBS-T 0.05 %, HRP-conjugated rabbit anti-human IgG (Roche Applied Science) diluted (1:5,000) in the blocking buffer was incubated for 1 hour at 37 °C under gentle agitation. The ELISA plate was washed three times with PBS-T 0.05 %, revealed with OPD SigmaFast™ (Sigma-Aldrich) and read at 492 nm. All samples were tested in triplicate. The optimum point of reaction for anti-M12 antibody detection was determined using the receiver operating characteristic (ROC) curve, where a cut-off point was determined as the value of the parameter corresponding to the highest possible sensitivity without losing specificity. To calculate the ROC curve, sensitivity and specificity, we considered the control groups as a single group. Each serum sample was tested without M12 as negative control. The final OD values obtained for each RA, HC, SLE or AS samples were adjusted by subtracting the corresponding OD value obtained by the negative control. After data adjustment, OD values obtained for each sample from all groups were divided by the cut-off value for data normalization. The values obtained are expressed as reactivity index (RI), where samples presenting RI ≥1 were considered positives.

Statistical analysis

Unpaired *t* test with Welch's correction was used to evaluate the differences in sera reactivity in ELISA assays among groups for phage clones and the synthetic peptide. Sensitivity and specificity parameters were calculated based on the ROC curve analysis, and Fisher's exact test was used for categorical data. To estimate the positive predictive accuracy, the area under the curve (AUC) was also determined. Pearson's correlation was used for analysis among variables. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). *p* values less than 0.05 were considered statistically significant.

Results

Arthritis induction

Arthritis induction was efficient in CIA mice. A total of 80 % of male DBA/1 J mice developed acute arthritis around 60 days after CII immunization. The severity and incidence of arthritis were assessed as described in Methods. No manifestation of arthritis was observed in mice in the control group treated with PBS in CFA. A schematic workflow illustrating the steps employed in this study is shown in Fig. 1.

Selection of mimotopes by phage display

The enrichment of phages recovered after each round of biopanning was determined by the output to input ratio. The increase from 8×10^4 in the first round to 4×10^5 after three rounds of affinity selection showed a clear enrichment of phage particles (Table 2). These data indicate a successful affinity selection of phages that specifically recognized IgG present in sera of DBA1/J mice with acute arthritis. A total of 37 randomly selected mimotopes were obtained after three rounds of biopanning using a phage displayed 12-mer random peptide library. From the 37 mimotopes selected, 14 presented different sequences. Alignment analysis revealed some consensus sequence between the mimotopes selected (Fig. 2a), indicating that these motifs were positively selected during the biopanning. The selected mimotopes showed different reactivities when tested against human sera by phage-ELISA, and all of them were able to discriminate RA patients from HCs. The reactivity values were very similar for all tested mimotopes, except for M12, which showed the highest values of absorbance (Fig. 2b) and the highest difference compared to the other clones ($p < 0.05$). Due to the higher reactivity compared to the other mimotopes, the common motif shared with another five clones, and the highest ratio of RA:HC, we focused on M12 peptide for further investigation. However, the other mimotopes selected will be explored in future studies.

Western blot analysis

SDS-PAGE fractionated proteins from inflamed joints of CIA mice were transferred to a nitrocellulose membrane. Western blot revealed that purified IgG from RA patients against the M12 mimotope recognized a protein with a molecular weight of approximately 29 kDa (Fig. 3).

Antigen target and epitope prediction

MS analysis identified 15 putative proteins associated with anti-M12 IgG, where 14 proteins presented few peptide matches, with very low scores and sequence coverage and none of them presented a molecular mass close to 29 kDa. Therefore, based on the molecular weight of the protein target by anti-M12 antibodies observed in the Western blot results (29 kDa), the alignment of putative proteins with the M12 peptide sequence, and the sequencing coverage by MS, we have identified the carbonic anhydrase III (CAIII) [Uniprot/Swiss-Prot:P16015] as the protein target that the M12 peptide mimics. The CAIII protein showed 49.62 % of sequencing coverage, including the peptide sequence, which was aligned by

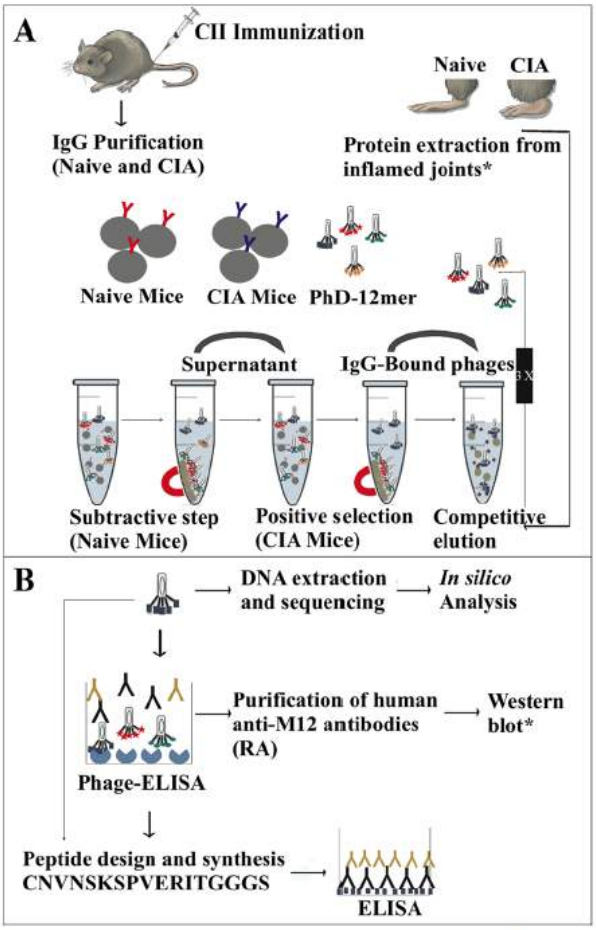


Fig. 1 Schematic workflow illustrating the steps involved in the study. **a** Selection step, and **b** validation step. Arthritis was induced in collagen-induced arthritis (CIA) mice by immunization with type II collagen (CII). Immunoglobulin G (IgG) was purified by protein G beads from serum of mice presenting with acute arthritis and naïve mice. A phage display library was used to select mimotopes against purified IgG from CIA mice (three cycles of biopanning), with a subtractive step against IgG from naïve mice. IgG-bound phages from CIA mice were competitively eluted by incubation with total proteins extracted from inflamed joints. Total proteins extracted from inflamed joints were also used for anti-M12 antibody recognition in Western blot. Immunoreactivity of each selected mimotope was tested by phage exyme-linked immunosorbent assay (ELISA). After DNA extraction and in silico and in vitro analysis, the most reactive mimotope (M12) was identified as a peptide that mimics a predicted antigenic site of the human carbonic anhydrase III protein. Validation of the M12 synthetic peptide as a possible rheumatoid arthritis (RA) autoantigen was carried out by ELISA assay

conserved and semi-conserved amino acid (aa) residues between position 24–35 (KGDNQSPIELHT).

The multiple sequence alignment revealed several homologous sequences between M12 peptide and the CAIII protein sequences from mouse and human. Nine (75 %) conserved or semi-conserved aa residues of the M12 peptide sequence matched a domain of the CAIII protein (Fig. 4a). A three-dimensional structural alignment

Table 2 Enrichment of phage for each round of selection from phage display peptide library

	Number of phage particles		
Round	Input (cpu)	Output (cpu)	Ratio (output/input)
1st	1×10^{11}	8×10^{4}	8×10^{-7}
2nd	1×10^{11}	9×10^{3}	9×10^{-8}
3rd	1×10^{11}	4×10^{5}	4×10^{-6}

cpu phage units

was performed to predict the putative epitope site of the M12 peptide in the CAIII protein structure [PDB:3UYN], which confirmed its surface exposure and corroborated to the possible antibody-binding region in the external sequences of the predicted protein (Fig. 4b). Five (41.6 %) aa residues (SPVET) of the M12 peptide was matched to an antigenic region of the human CAIII protein (Fig. 5).

Evaluation of the M12 peptide as a possible biomarker for RA diagnosis

The M12 synthetic peptide was tested by ELISA with individual sera obtained from 172 RA patients, 113 HCs, 19 SLE patients and 13 AS patients to evaluate its potential as a diagnostic. The cut-off point was determined as the value of the parameter corresponding to the highest sensitivity without lowering the specificity. Of 172 RA patients, 145 (84.3 %) were positive to anti-M12 antibodies. The synthetic molecule was able to efficiently discriminate sera from RA patients and HCs ($p < 0.0001$), SLE ($p < 0.0001$) and AS ($p < 0.0001$) (Fig. 6a). The ROC curve analysis constructed based on the control groups was significant ($p < 0.0001$; AUC = 0.946). Based on the determined cut-off point, M12 synthetic peptide presented a specificity of 91 % and sensitivity of 84.3 % (Fig. 6b). For humoral immune response evaluation, 55 (32 %) serum samples from RA patients were simultaneously positive for RF, antiCCP and anti-M12 antibodies, while 45 (26.2 %) samples were positive only for anti-M12 and 12 (7 %) were negative for the three antibodies (Table 3).

Correlation of anti-M12 humoral response with clinical variables

The weakly associated variables with anti-M12 levels from Gaussian populations (Pearson) analysis were disease duration ($r = 0.1753$) and the use of biological therapy ($r = -0.2934$) (Table 4).

Discussion

In order to identify new biomarkers for the improvement of RA diagnosis, we have used the CIA mouse model and PD technologies to select peptides that could mimic RA autoantigens. This approach allowed us to discover a new peptide with 12 amino acids (M12) that

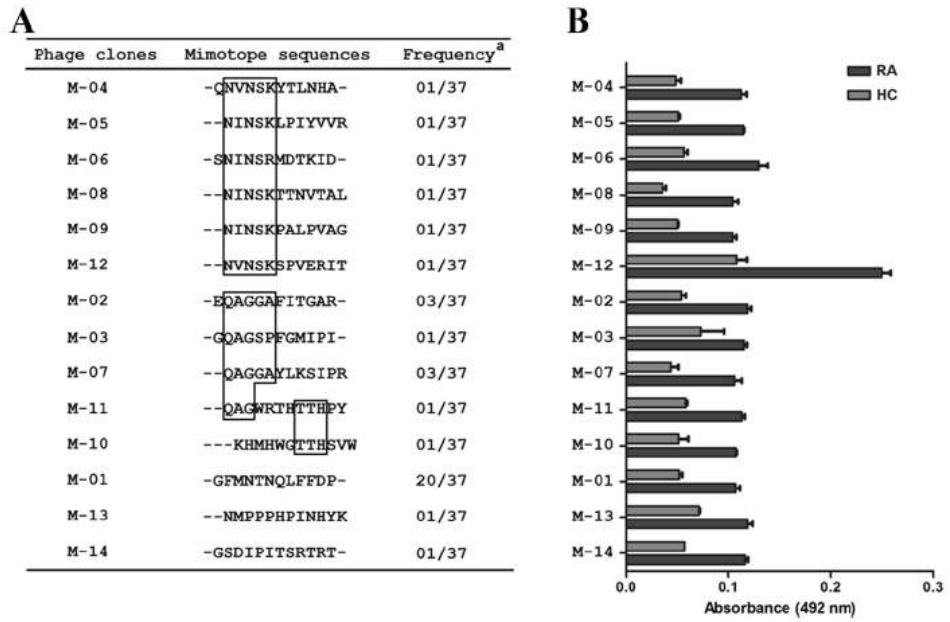


Fig. 2 Perfomance of the mimotopes selected by phage display. **a** Multiple sequence alignment with all selected mimotopes showing their consensus sequences and frequency. **b** Reactivity obtained though the interaction of the mimotopes selected and pooled sera from rheumatoid arthritis (RA) patients and health controls (HC). [a]Frequency is defined as the ratio of the number of phage clones expressing a common peptide sequence to that of the total phage clones obtained in the biopanning

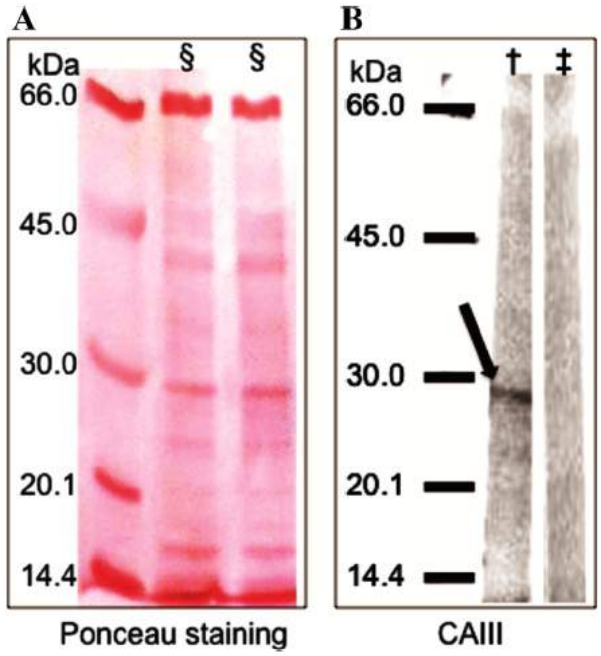


Fig. 3 Western blot analyses. **a** The Ponceau staining shows 1 μg of total proteins extracted from inflamed joints of CIA mice (§) separated by 10 % SDS-PAGE. **b** The same membrane stained with Ponceau was probed with anti-M12 antibodies purified from pooled sera of RA patients (†) and HCs (‡). Anti-M12 antibodies displayed strong reactivity with a protein extracted from inflamed joints of CIA mice presenting with mass of approximately 29 kDa (*arrow*). Carbonic anhydrase III (CAIII) was identified as the protein target that is mimicked by the M12 mimotope. The membrane was cropped to allow differential incubation with anti-M12 antibodies purified from RA patients and HCs

encompasses an antigenic domain of the CAIII protein. The use of the synthetic M12 peptide as an autoantigen in ELISA confirmed the presence of specific anti-M12 antibodies in sera of patients with RA with high specificity and sensitivity.

We have used the DBA1/J mouse as a model for inflammatory RA, where arthritis can be induced in susceptible mouse strains by immunization with CII [45]. DBA/1 J mice are CIA models of particular interest, since this strain is not known to have any immunologic aberrations or other pathologic defects [46]. CIA mice are widely used as an animal model for RA studies [14–17, 47–49]. We have chosen this experimental model due to the fact that genomic similarities between mice and humans are quite high, reaching 98 % [50]; it is much easier to monitor arthritis in mice than in humans, and also it is a unique experimental opportunity to increase our understanding of human arthritis. We confirmed the efficiency of the arthritis induction in our CIA mice strain, when a total of 80 % of DBA/1 J developed signs of arthritis around 60 days after CII immunization. We have used a subtractive PD selection against IgG present in sera of CIA mice that aims to select new RA biomarkers. We have chosen this technology due to its successful applications in biomarker discovery, especially when short random peptides are selected against specific target molecules, such as those selected against IgG present in sera for antigen discovery [51, 52].

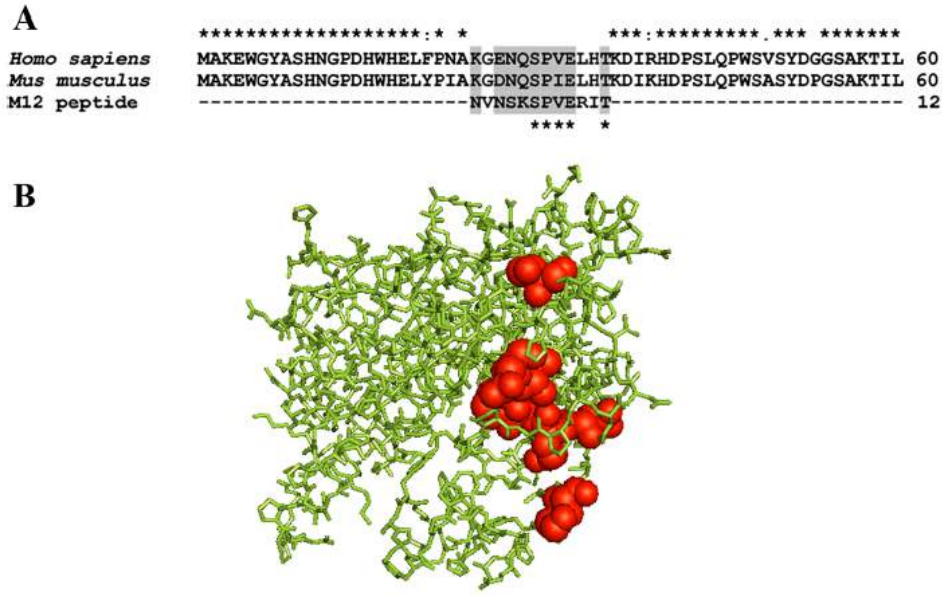


Fig. 4 Peptide localization. **a** Multiple alignments of M12 peptide and deduced amino acid sequences of carbonic anhydrase III from *Homo sapiens* and *Mus musculus*. Conserved (*stars*) and semi-conserved residues (*gray*) are highlighted. Valine (V) amino acid is star marked because it matches a position between M12 peptide and human CAIII protein. **b** Model of the three-dimensional structure predicted in the PyMol server software for the human carbonic anhydrase III with M12 peptide localization

The highly reactive peptide M12 was able to efficiently discriminate RA patients from HCs as well as from patients affected by SLE and AS, two other immune-mediated rheumatic diseases. In fact, the M12 peptide was not the most enriched mimotope after the rounds of phage amplification; however, the most frequently selected mimotope will not necessarily be the most reactive – selection is independent, which means that increasing one does not eliminate the effect of the other [53]. Many events during viral infection may contribute to increasing the amplification of specific phages. The biological reasons for abundances and growth advantages of some mimotopes have been discussed in many reports and include events like the phages ability to bind to bacterial pili, and interference with packing or infection [54–56]. The M12 peptide sequence was further characterized by reverse binding using its specific IgG antibodies to capture and identify its antigen target by MS, which led us to the CAIII protein. The CAIII protein is a member of a multigene family that encodes carbonic anhydrase isozymes. It is an abundant muscle protein and plays an important role in facilitated CO_2

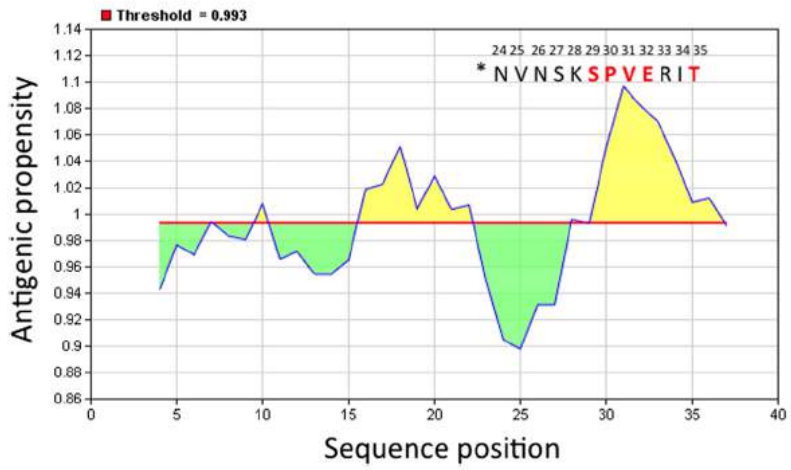


Fig. 5 Antigenicity predictions using the Kolaskar-Tongaonkar algorithm. Threshold (0.993); average (0.993); minimum antigenicity (0.898); maximum antigenicity (1.097). Regions with antigenic propensity scale above 0.993 are antigenic. Window size and center position were 7 and 4, respectively. *Localization of M12 peptide in the CAIII protein (aa 24–35). Matched aa residues are presented in red.

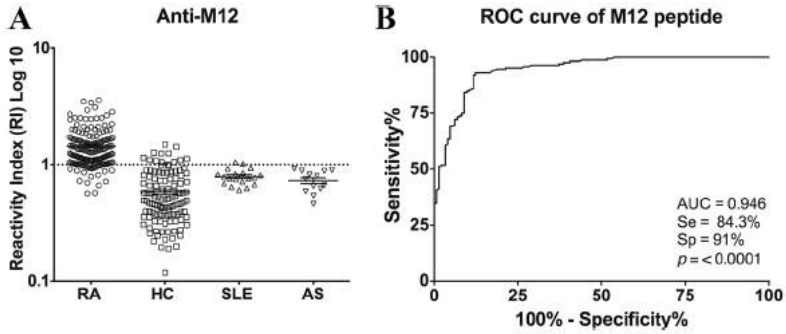


Fig. 6 Anti-M12 antibody detection by ELISA. Detection of anti-M12 antibodies in sera from rheumatoid arthritis (RA) patients (n = 172), healthy controls (HC) (n = 113), systemic lupus erythematosus (SLE) patients (n = 19) and ankylosing spondylitis (AS) patients (n = 13) and the respectively receiver operating characteristic (ROC) curve. **a** Sera from the four groups individually tested for their ability to bind to synthetic M12 peptide. Horizontal line = cut-off value; error bars show mean and standard deviation. **b** ROC curve constructed based on the control groups. The area under the curve (AUC), sensitivity (Se), specificity (Sp) and corresponding *p*-value are indicated inside the graph

diffusion and diverse processes involving H^+ and -HCO_3 transport [57]. The CAIII gene encodes a protein of 260 aa and a molecular weight of approximately 29 kDa, which is well conserved throughout humans and mice. This protein has already been identified as an autoantigen expressed in the synovial membrane of RA patients [58] and its circulating autoantibodies have been found in many diseases [59]. The Kolaskar and Tongaonkar antigenicity scale is a semi-empirical method that makes use of physicochemical properties of aa residues and their frequencies of occurrence in experimentally known segmental epitopes, and is used to predict antigenic determinants on proteins. Using this parameter, this method that can predict antigenic determinants with 75 % accuracy [41] has been used in many studies [60–62]. Amino acid residues of the M12 peptide matched conservative and semi-conservative residues in a predicted antigenic and exposed site of a putative epitope of the human CAIII protein, which probably gave this peptide the ability to be recognized by specific IgG during the mimotope selection. This hypothesis is supported by the fact that semi-conserved aa residues present similar physicochemical properties with the original residue allowing antibody binding [63]. Of interest, a synthetic peptide between 24–54 aa residues of the human CAIII, that encompasses the M12 epitope region, has been commercially used to generate antiCAIII antibodies in rabbits (Product ID: ABIN391954). This fact probably reinforces the importance of the CAIII protein in RA and the potential use of this peptide as an antigen for antibody detection to aid in diagnosis.

A wide range of serum biomarkers has been assessed to improve diagnosis and prognosis of RA. However, only the RF and antiCCP antibodies have gained wide acceptance [10]. The use of recombinant CAIII protein

Table 3 Antibodies positivity in the rheumatoid arthritis population

Antibody detection			Positivity	
Rheumatoid factor	AntiCCP	Anti-M12	n	%
+	−	−	8	4.6
−	+	−	0	0
−	−	+	45	26.2
+	+	−	7	4
+	−	+	32	18.6
−	+	+	13	7.6
+	+	+	55	32
−	−	−	12	7
Total			172	100

+ positive, − negative, *CCP* cyclic citrullinated peptide

Table 4 Clinical variables associated with detection of anti-M12 antibodies in patients with theumatoid arthritis

Variables	Anti-M12		
	Pearson *r*	*p*	*R* squared
Ethnicity	†	†	†
Gender	†	†	†
Age	†	†	†
Disease duration	0.1753	<0.05	0.03072
Rheumatoid factor	†	†	†
ESR	†	†	†
CRP	†	†	†
AntiCCP	†	†	†
Tender joints	†	†	†
Swollen joints	†	†	†
Biologic therapy	−0.2934	<0.005	0.08616
NSAIDs	†	†	†

Correlations were performed from Gaussian populations (Pearson) with a confidence interval of 95 %. †Values not statistically significant. *CCP* cyclic citrullinated peptide, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *NSAID* nonsteroidal anti-inflammatory drug

as an antigen has confirmed the presence of specific antiCAIII antibodies in RA sera [58, 59]; however, its sensitivity considering the entire CAIII protein was only 17 %. In fact, when compared with HCs, the specificity of circulating autoantibodies was high (100 %), but reached only medium to low specificity when compared with other autoimmune diseases, such as SLE (67 %) [58]. The crossreactivity and lack of sensitivity of the whole CAIII protein suggests the presence of multiple epitopes sharing common regions with other proteins, as well as the presence of immunodominant epitopes that surpass the response to the M12 critical epitope, which may have generated either false positive results or an insufficient reactivity. On the other hand, the use of the M12 peptide conferred more sensitivity and specificity in the detection of RA, since it was able to discriminate RA patients from HCs, and patients with AS and SLE, with high accuracy.

Several studies have measured the three different generations of antiCCP antibodies in RA patients in comparison with control groups. These antibodies present specificities in the range of 65 % to 100 % (mean of 93.4 %) and sensitivities in the range of 42 % to 92.2 % (mean of 64.3 %) [9, 64–71]. RF testing presents specificities in the range 85 % to 90.29 % and sensitivities in the range 46.26 % to 90 % [72–74]. The diagnostic parameters for the M12 peptide for RA diagnosis presented specificity of 91 % and sensitivity of 84.3 %, which is very close to the mean of values reported for antiCCP and RF antibodies, the two most currently used serum biomarkers for RA. On the other hand, anti-M12 antibodies were detected in 26.2 % of samples that are negative for antiCCP and RF. We speculate that the high percentage of positive samples for anti-M12 antibodies might be due to the different tests used to cut-off calculation, or even the patients disease activity at the time of sample collection or individual response to therapy. In this context, high levels of antiCCP and RF antibodies in RA patients have already been associated with an insufficient response to therapy [75, 76], while anti-M12 antibodies were weakly associated with therapeutic response in our RA cohort, which may help to explain the percentage of positive samples only for M12. It is widely accepted that the identification of RA at early stages and consequently the implementation of effective treatment strategies can significantly improve patient prognosis [77]. Anti-M12 antibodies showed a weak association with disease duration, suggesting its potential use as a biomarker in different stages during the process of RA development.

Conclusions

We have selected and identified a peptide that is capable of detecting specific circulating IgG in the serum of RA patients with high specificity and sensitivity. Although antibodies against synthetic M12 peptide were detected in patients with early and established RA, its potential use in the diagnosis at different stages of the disease remains to be studied during follow-up in a well characterized cohort of patients with early RA, and also its specificity as a serum biomarker should be further studied in a much larger cohort of patients with other inflammatory diseases. This peptide mimics a predicted antigenic region of the human carbonic anhydrase III by linear sequence analysis and could be used as an antigen for detection of specific RA autoantibodies.

Abbreviations

Aa: Amino acid; ACPA: Anti-Citrullinated peptide antibody; AS: Ankylosing spondylitis; AUC: Area under the curve; BSA: Bovine serum albumin; CAIII: Carbonic anhydrase III; CCP: Cyclic citrullinated peptides; CFA: Complete Freund's adjuvant; CIA: Collagen-induced arthritis; CII: Type II collagen; CRP: C-reactive protein; ELISA: Enzyme-linked immunosorbent assay; ESR: Erythrocyte sedimentation rate; HC: Healthy control; IgG: Immunoglobulin G; MS: Mass spectrometry; OD: Optical density; PBS: Phosphate-buffered saline; PD: Phage display; RA: Rheumatoid arthritis; RF: Rheumatoid factor; RI: Reactivity index; ROC: Receiver operating characteristic; SLE: Systemic lupus erythematosus; TBS: Tris-buffered saline; TFA: Trifluoroacetic acid.

Competing interests

GRA, PTF, LRG and CUV are co-inventors of a patent protecting the use of the 14 peptides selected in this study and its sequence for diagnostic use. The remaining authors declare that they have no competing interests.

Authors' contributions

GRA, JEF, ERV, LRG, JG and CUV conceived and designed the experiments. GRA, PTF and LMdL performed peptide selection. GRA, ERV and PTF performed immunoassays. GRA and PTF performed in silico analysis. GV, KHMC and VMC performed and analyzed mass spectrometry. GRA, PTF and HC contributed to statistical analyses. JEF, HC, MHN, LRG, JG and CUV contributed with reagents, materials, serum samples, and analysis tools. GRA, ERV, PTF, JEF, GV, LRG, MHN and CUV contributed to writing the paper. All authors read, edited and approved the final manuscript.

Acknowledgements

The authors thank all members of the Biobanco-IMM from the Lisbon Academic Medical Center, for the work spent to separate blood samples and fill multiple worksheets containing relevant patient data and Renato Mendonça Borges for the assistance in designing the workflow figure.

Funding

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Foundation, Ministry of Education of Brazil (CSF SDW-2027/13-5) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Author details

[1]Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, MG, Brazil. [2]iMed.UL – Research Institute for Medicines and Pharmaceutical Sciences, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal. [3]Rheumatology Research Unit, Institute of Molecular Medicine, Lisbon, Portugal. [4]Rheumatology Department, Santa Maria Hospital, Lisbon Academic Medical Center, Lisbon, Portugal. [5]Laboratory of Genetics and Molecular Cardiology, Heart Institute, University of São Paulo Medical School, São Paulo, SP, Brazil. [6]Fleury group, São Paulo, SP, Brazil. [7]Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas, SP, Brazil. [8]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA. [9]IMM – Institute of Molecular Medicine, Lisbon, Portugal.

Received: 4 December 2014 Accepted: 12 June 2015
Published online: 23 June 2015

References

1. Rindfleisch JA, Muller D. Diagnosis and management of rheumatoid arthritis. Am Fam Physician. 2005;72:1037–47.
2. National Collaborating Centre for Chronic Conditions (UK). Rheumatoid Arthritis: National Clinical Guideline for Management and Treatment in Adults. London: Royal College of Physicians; 2009.
3. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP antibodies: the past, the present and the future. Nat Rev Rheumatol. 2011;7:391–8.
4. van Venrooij WJ, Hazes JM, Visser H. Anticitrullinated protein/peptide antibody and its role in the diagnosis and prognosis of early rheumatoid arthritis. Neth J Med. 2002;60:383–8.
5. Rantapaa-Dahlqvist S. Diagnostic and prognostic significance of autoantibodies in early rheumatoid arthritis. Scand J Rheumatol. 2005;34:83–96.
6. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum. 2000;43:155–63.
7. Coenen D, Verschueren P, Westhovens R, Bossuyt X. Technical and diagnostic performance of 6 assays for the measurement of citrullinated protein/peptide antibodies in the diagnosis of rheumatoid arthritis. Clin Chem. 2007;53:498–504.
8. Vander Cruyssen B, Cantaert T, Nogueira L, Clavel C, De Rycke L, Dendoven A, et al. Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays. Arthritis Res Ther. 2006;8:R122.
9. Goeb V, Jouen F, Gilbert D, Le Loet X, Tron F, Vittecoq O. Diagnostic and prognostic usefulness of antibodies to citrullinated peptides. Joint Bone Spine. 2009;76:343–9.
10. Taylor P, Gartemann J, Hsieh J, Creeden J. A systematic review of serum biomarkers anti-cyclic citrullinated peptide and rheumatoid factor as tests for rheumatoid arthritis. Autoimmune Dis. 2011;2011:815038.
11. van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Toes RE, Huizinga TW. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. Arthritis Res Ther. 2005;7:R949–58.
12. Syversen SW, Gaarder PI, Goll GL, Odegard S, Haavardsholm EA, Mowinckel P, et al. High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. Ann Rheum Dis. 2008;67:212–7.
13. Mjaavatten MD, van der Heijde DM, Uhlig T, Haugen AJ, Nygaard H, Bjorneboe O, et al. Should anti-citrullinated protein antibody and rheumatoid factor status be reassessed during the first year of followup in recent-onset arthritis? A longitudinal study. J Rheumatol. 2011;38:2336–41.
14. Park KH, Mun CH, Kang MI, Lee SW, Lee SK, Park YB. Treatment of collagen-induced arthritis using immune modulatory properties of human mesenchymal stem cells. Cell Transpl. 2015. doi:10.3727/096368915X687949.
15. Chow LN, Choi KY, Piyadasa H, Bossert M, Uzonna J, Klonisch T, et al. Human cathelicidin LL-37-derived peptide IG-19 confers protection in a murine model of collagen-induced arthritis. Mol Immunol. 2014;57:86–92.
16. Eneljung T, Tengvall S, Jirholt P, Henningsson L, Holmdahl R, Gustafsson K, et al. Antigen-specific gene therapy after immunisation reduces the severity of collagen-induced arthritis. Clin Dev Immunol. 2013;2013:345092.
17. Ding X, Hu J, Li J, Zhang Y, Shui B, Ding Z, et al. Metabolomics analysis of collagen-induced arthritis in rats and interventional effects of oral tolerance. Anal Biochem. 2014;458:49–57.
18. Rosloniec EF, Cremer M, Kang AH, Myers LK, Brand DD. Collagen-induced arthritis. Curr Protoc Immunol. 2010;89:15.5:15.5.1–15.5.25.
19. Wang M, Li X, Chen J, Zhou Y, Cao H, Wu X, et al. Screening and evaluating the mimic peptides as a useful serum biomarker of ankylosing spondylitis using a phage display technique. Rheumatol Int. 2011;31:1009–16.
20. Sergeeva A, Kolonin MG, Molldrem JJ, Pasqualini R, Arap W. Display technologies: application for the discovery of drug and gene delivery agents. Adv Drug Deliv Rev. 2006;58:1622–54.
21. Samoylova TI, Morrison NE, Globa LP, Cox NR. Peptide phage display: opportunities for development of personalized anti-cancer strategies. Anticancer Agents Med Chem. 2006;6:9–17.
22. Alves PT, Fujimura PT, Morais LD, Goulart LR. Revisiting the CD14: epitope mapping by phage display. Immunobiology. 2014;219:822–9.
23. Reis CF, Carneiro AP, Vieira CU, Fujimura PT, Morari EC, Silva SJ, et al. An antibody-like peptide that recognizes malignancy among thyroid nodules. Cancer Lett. 2013;335:306–13.
24. Santos PS, Sena AA, Nascimento R, Araujo TG, Mendes MM, Martins JR, et al. Epitope-based vaccines with the Anaplasma marginale MSP1a functional motif induce a balanced humoral and cellular immune response in mice. PLoS One. 2013;8:e60311.
25. Scott JK, Smith GP. Searching for peptide ligands with an epitope library. Science. 1990;249:386–90.
26. Huang J, Ru B, Dai P. Bioinformatics resources and tools for phage display. Molecules. 2011;16:694–709.
27. Somers K, Geusens P, Elewaut D, De Keyser F, Rummens JL, Coenen M, et al. Novel autoantibody markers for early and seronegative rheumatoid arthritis. J Autoimmun. 2011;36:33–46.
28. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham 3rd CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010;69:1580–8.
29. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nat Protoc. 2007;2:1269–75.
30. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
31. O'Valle F, Peregrina M, Crespo-Lora V, Galindo-Moreno P, Roman M, Padial-Molina M, et al. Osteoarticular expression of Musashi-1 in an Experimental model of arthritis. Biomed Res Int. 2015; Article ID 681456, in press.
32. BD Barbas CF, Scott JK, Silverman GJ. Phage display: a laboratory manual. NY: Cold Spring Harbor Laboratory Press; 2001.
33. Stothard P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques. 2000;28:1102–4.
34. Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, et al. ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res. 2012;40:W597–603.
35. ExPASy Proteomics and Sequence Analysis tool. http://web.expasy.org/translate/.
36. ClustalW2 online server. http://www.ebi.ac.uk/Tools/msa/clustalw2/.
37. Mayrose I, Penn O, Erez E, Rubinstein ND, Shlomi T, Freund NT, et al. Pepitope: epitope mapping from affinity-selected peptides. Bioinformatics. 2007;23:3244–6.
38. The Pepitope Server. http://pepitope.tau.ac.il/.
39. Immune Epitope Database and Analysis Resource. http://www.iedb.org/.
40. Pymol. http://www.pymol.org.
41. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990;276:172–4.
42. Kolaskar and Tongaonkar antigenicity scale. http://tools.immuneepitope.org/bcell/.
43. Camargo M, Intasqui Lopes P, Del Giudice PT, Carvalho VM, Cardozo KH, Andreoni C, et al. Unbiased label-free quantitative proteomic profiling and enriched proteomic pathways in seminal plasma of adult men before and after varicocelectomy. Hum Reprod. 2013;28:33–46.
44. Casey JL, Coley AM, Street G, Parisi K, Devine PL, Foley M. Peptide mimotopes selected from a random peptide library for diagnosis of Epstein-Barr virus infection. J Clin Microbiol. 2006;44:764–71.
45. Williams RO. Collagen-induced arthritis in mice: a major role for tumor necrosis factor-alpha. Methods Mol Biol. 2007;361:265–84.
46. Corthay A, Hansson AS, Holmdahl R. T lymphocytes are not required for the spontaneous development of entheseal ossification leading to marginal ankylosis in the DBA/1 mouse. Arthritis Rheum. 2000;43:844–51.
47. Eros G, Ibrahim S, Siebert N, Boros M, Vollmar B. Oral phosphatidylcholine pretreatment alleviates the signs of experimental rheumatoid arthritis. Arthritis Res Ther. 2009;11:R43.
48. Chiba A, Mizuno M, Tomi C, Tajima R, Alloza I, di Penta A, et al. A 4-trifluoromethyl analogue of celecoxib inhibits arthritis by suppressing innate immune cell activation. Arthritis Res Ther. 2012;14:R9.
49. Hong SH, Kwone JT, Lee JH, Lee S, Lee AY, Cho WY, et al. Ascidian tunicate extracts attenuate rheumatoid arthritis in a collagen-induced murine model. Nat Prod Commun. 2014;9:847–51.
50. Mural RJ, Adams MD, Myers EW, Smith HO, Miklos GL, Wides R, et al. A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome. Science. 2002;296:1661–71.
51. Weng X, Liao Q, Li K, Li Y, Mi M, Zhong D. Screening serum biomarker of knee osteoarthritis using a phage display technique. Clin Biochem. 2012;45:303–8.
52. Costa LE, Lima MI, Chavez-Fumagalli MA, Menezes-Souza D, Martins VT, Duarte MC, et al. Subtractive phage display selection from canine visceral

leishmaniasis identifies novel epitopes that mimic Leishmania infantum antigens with potential serodiagnosis applications. Clin Vaccine Immunol. 2014;21:96–106.
53. Derda R, Tang SK, Li SC, Ng S, Matochko W, Jafari MR. Diversity of phage-displayed libraries of peptides during panning and amplification. Molecules. 2011;16:1776–803.
54. Kuzmicheva GA, Jayanna PK, Sorokulova IB, Petrenko VA. Diversity and censoring of landscape phage libraries. Protein Eng Des Sel. 2009;22:9–18.
55. Rodi DJ, Soares AS, Makowski L. Quantitative assessment of peptide sequence diversity in M13 combinatorial peptide phage display libraries. J Mol Biol. 2002;322:1039–52.
56. Rodi DJ, Makowski L. Structure and Function of Macromolecular Assembly. Matsushita: Japan; 1997.
57. Edwards YH, Tweedie S, Lowe N, Lyons G. Carbonic anhydrase 3 (CA3), a mesodermal marker. Symp Soc Exp Biol. 1992;46:273–83.
58. Robert-Pachot M, Desbos A, Moreira A, Becchi M, Tebib J, Bonnin M, et al. Carbonic anhydrase III: a new target for autoantibodies in autoimmune diseases. Autoimmunity. 2007;40:380–9.
59. Liu C, Wei Y, Wang J, Pi L, Huang J, Wang P. Carbonic anhydrases III and IV autoantibodies in rheumatoid arthritis, systemic lupus erythematosus, diabetes, hypertensive renal disease, and heart failure. Clin Dev Immunol. 2012;2012:354594.
60. Srinivasan P, Kumar SP, Karthikeyan M, Jeyakanthan J, Jasrai YT, Pandya HA, et al. Epitope-based immunoinformatics and molecular docking studies of nucleocapsid protein and ovarian tumor domain of crimean-congo hemorrhagic fever virus. Front Genet. 2011;2:72.
61. Ranjbar MM, Ghorban K, Alavian SM, Keyvani H, Dadmanesh M, Roayaei Ardakany A, et al. GB virus C/hepatitis G virus envelope glycoprotein E2: computational molecular features and immunoinformatics study. Hepat Mon. 2013;13:e15342.
62. Sealey KL, Kirk RS, Walker AJ, Rollinson D, Lawton SP. Adaptive radiation within the vaccine target tetraspanin-23 across nine Schistosoma species from Africa. Int J Parasitol. 2013;43:95–103.
63. Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. Annu Rev Genomics Hum Genet. 2006;7:61–80.
64. Forslind K, Ahlmen M, Eberhardt K, Hafstrom I, Svensson B, Group BS. Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). Ann Rheum Dis. 2004;63:1090–5.
65. Herold M, Boeser V, Russe E, Klotz W. Anti-CCP: history and its usefulness. Clin Dev Immunol. 2005;12:131–5.
66. Wagner E, Skoumal M, Bayer PM, Klaushofer K. Antibody against mutated citrullinated vimentin: a new sensitive marker in the diagnosis of rheumatoid arthritis. Rheumatol Int. 2009;29:1315–21.
67. Mutlu N, Bicakcigil M, Tasan DA, Kaya A, Yavuz S, Ozden AI. Comparative performance analysis of 4 different anti-citrullinated protein assays in the diagnosis of rheumatoid arthritis. J Rheumatol. 2009;36:491–500.
68. Fernandez-Suarez A, Reneses S, Wichmann I, Criado R, Nunez A. Efficacy of three ELISA measurements of anti-cyclic citrullinated peptide antibodies in the early diagnosis of rheumatoid arthritis. Clin Chem Lab Med. 2005;43:1234–9.
69. Kudo-Tanaka E, Ohshima S, Ishii M, Mima T, Matsushita M, Azuma N, et al. Autoantibodies to cyclic citrullinated peptide 2 (CCP2) are superior to other potential diagnostic biomarkers for predicting rheumatoid arthritis in early undifferentiated arthritis. Clin Rheumatol. 2007;26:1627–33.
70. van Gaalen FA, Visser H, Huizinga TW. A comparison of the diagnostic accuracy and prognostic value of the first and second anti-cyclic citrullinated peptides (CCP1 and CCP2) autoantibody tests for rheumatoid arthritis. Ann Rheum Dis. 2005;64:1510–2.
71. Kim S, Kim JH, Lee JH, Kim HS. Evaluation of three automated enzyme immunoassays for detection of anti-cyclic citrullinated peptide antibodies in qualitative and quantitative aspects. Rheumatology. 2010;49:450–7.
72. Binesh F, Salehabadi HS, Behniafard N, Ranginkaman K, Behniafard N. A comparative assessment of the diagnostic value of anti-cyclic citrullinated peptide antibodies and rheumatoid factor in rheumatoid arthritis. J Clin Exp Pathol. 2014;4:158. doi:10.4172/2161- 0681.1000158.
73. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med. 2007;146:797–808.
74. Nell VP, Machold KP, Stamm TA, Eberl G, Heinzl H, Uffmann M, et al. Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis. Ann Rheum Dis. 2005;64:1731–6.
75. Visser K, Goekoop-Ruiterman YP, de Vries-Bouwstra JK, Ronday HK, Seys PE, Kerstens PJ, et al. A matrix risk model for the prediction of rapid radiographic progression in patients with rheumatoid arthritis receiving different dynamic treatment strategies: post hoc analyses from the BeSt study. Ann Rheum Dis. 2010;69:1333–7.
76. Mikuls TR, O'Dell JR, Stoner JA, Parrish LA, Arend WP, Norris JM, et al. Association of rheumatoid arthritis treatment response and disease duration with declines in serum levels of IgM rheumatoid factor and anti-cyclic citrullinated peptide antibody. Arthritis Rheum. 2004;50:3776–82.
77. Vermeer M, Kuper HH, Hoekstra M, Haagsma CJ, Posthumus MD, Brus HL, et al. Implementation of a treat-to-target strategy in very early rheumatoid arthritis: results of the Dutch Rheumatoid Arthritis Monitoring remission induction cohort study. Arthritis Rheum. 2011;63:2865–72.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

BioMed Central

Inflammation & Cell Signaling 2015; 2: e813. doi: 10.14800/ics.813; © 2015 by Galber R. Araujo
http://www.smartscitech.com/index.php/ics

REVIEW

Autoantibodies to type II collagen in rheumatoid arthritis and juvenile idiopathic arthritis: Meaning and clinical interest

Galber R. Araujo, Luiz R. Goulart, Carlos Ueira-Vieira

Laboratory of Nanobiotecnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil

Correspondence: Galber R. Araujo
E-mail: galber.araujo@gmail.com
Received: April 24, 2015
Published online: May 10, 2015

Type II collagen (CII) is the major protein in articular cartilage. Autoantibodies to native and denatured CII (anti-CII) have been reported in rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). The real meaning of the anti-CII antibodies appearance is still an open question. Anti-CII antibodies may occur more commonly very early in the disease course, suggesting that these autoantibodies could be associated with the pathophysiology of RA and JIA. This finding is supported by the fact that in collagen antibody-induced arthritis (CAIA) mouse model, immunization with anti-CII antibodies directed towards several epitopes on CII in joint cartilage can induce polyarthritis that shares several pathological features with RA. This review focuses on the inflammatory events that may be associated with anti-CII production and also the clinical application of these antibodies in RA and JIA.

Keywords: Type II collagen; autoantibodies; rheumatoid arthritis; juvenile idiopathic arthritis

To cite this article: Galber R. Araujo. Autoantibodies to type II collagen in rheumatoid arthritis and juvenile idiopathic arthritis: Meaning and clinical interest. Inflamm Cell Signal 2015; 2: e813. doi: 10.14800/ics.813.

Type II collagen (CII) is the predominant collagen type in joint cartilage, constituting more than 50% of its dry weight [1]. Native CII consists of a triple-helix composed by three identical α chains. The collagen fibrils resist stretching forces caused by hydrophilic proteoglycan molecules in the extracellular matrix of articular cartilage, contributing to cartilage integrity [2]. The degradation of CII is associated with cartilage degeneration and loss of function in RA patients [3]. When CII is denatured the α chains are separated, and the antigenic sites (epitopes) present in the molecule are lost after disruption of the three dimensional structure [4]. Autoantibodies to native and denatured CII have been reported in rheumatoid arthritis (RA) [5-8] and juvenile idiopathic arthritis (JIA) [9-11], two autoimmune systemic inflammatory disorders. The levels of anti-CII antibodies detected may vary in the same patient at different times and also between patients, suggesting that these antibodies might be associated with specific events during arthritis development or even genetic susceptibility.

In mice susceptible to experimental collagen-induced arthritis (CIA), an autoimmune polyarthritis that shares several pathological features with RA can be induced by immunization with CII. This experimental model is the most commonly studied autoimmune model of RA [12, 13]. In the collagen antibody-induced arthritis (CAIA) model, immunization with anti-CII antibodies, directed towards several epitopes on CII in joint cartilage, can also induce polyarthritis [14]. Thus it is clear that, somehow, CII is involved in the RA pathogenesis.

CII degradation and cartilage damage

Inflammation & Cell Signaling 2015; 2: e813. doi: 10.14800/ics.813; © 2015 by Galber R. Araujo
http://www.smartscitech.com/index.php/ics

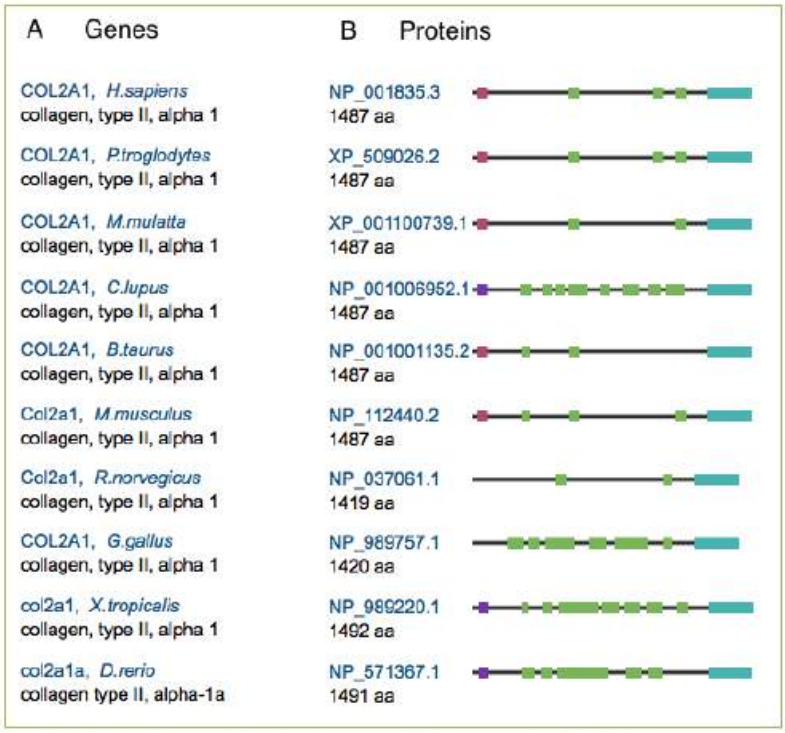


Figure 1. Multiple sequence alignment of CII molecule from different species. (A) Genes from different species identified as putative homologous of CII. (B) CII molecules used in sequence comparisons and their conserved domain architectures. Proteins accession number is provided before proteins sequence. Conserved domains: red - von Willebrand factor type C domain; blue - Fibrillar collagen C-terminal domain; purple - von Willebrand factor type C domain; green - Collagen triple helix repeat (20 copies). Multiple sequence alignment was carried out by the NCBI HomoloGene database (http://www.ncbi.nlm.nih.gov/homologene).

Articular cartilage damage is one of the key features of RA, ultimately leading to a loss of joint function [2]. In healthy joints, a thin layer of proteinaceous material covers the cartilage surface [15] inhibiting anti-CII antibodies binding [16]. In inflamed joints, CII epitopes are exposed to antibodies due to disruption of this proteinaceous layer. High levels of anti-CII antibodies might degrade CII molecules and induce an acute inflammation mediated by surface-bound immune complexes (ICs) containing antibodies against CII [7], which leads to the complement system activation and stimulate the production of proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and IL-8 [17], contributing for perpetuation of joint inflammation and consequently, cartilage damage. Antibodies to major CII epitopes are present at the inflammation site in RA patients, and have been detected in serum and synovial fluid samples from these patients, supporting the notion of a local increased immune response to CII in the joints [18]. Thus, although the involvement of anti-CII antibodies in the RA pathogenesis is still an open question, they could be useful as markers for the biomonitoring of joint destruction in some patients.

CII structure

The *COL2A1* gene encodes an important component of the CII molecule, called pro-alpha1(II) chain [19]. The sequence of CII chains is conserved between different species, sharing many epitope sites and conserved domains (Figure 1). In CIA model, polyarthritis can be induced by immunization with CII from different species [20-24], indicating that arthritogenic epitopes are highly conserved. Furthermore, antibodies from RA patients can react to various heterologous CII molecules, such as mice, chicken, bovine, porcine and monkey [5, 18, 25]. Analysis of the CII triple-helix showed that numerous epitopes could be target by autoantibodies and responses against different CII epitopes may vary at different stages of the disease [26], which could help to explain the difference in the levels of anti-CII antibodies detected in different studies.

Clinical interest of anti-CII antibodies detection in RA and JIA patients

Many studies have evaluated the diagnostic performance of anti-CII antibodies detection in cohorts composed by RA

Inflammation & Cell Signaling 2015; 2: e813. doi: 10.14800/ics.813; © 2015 by Galber R. Araujo
http://www.smartscitech.com/index.php/ics

Table 1. Prevalence of anti-CII antibodies in different studies

Test	Positivity	References
RA patients		
Anti-CII native	22.2%	[33]
Anti-CII native	20%	[34]
Anti-CII denatured	27%	[34]
Anti-CII native	27%	[35]
Anti-CII denatured	82%	[35]
Anti-CII native	8.8%	[7]
Anti-CII native	31.5%	[5]
Anti-CII denatured	21.5%	[5]
Anti-Citrullinated CII	24%	[11]
Anti-CII native	14.6%	[36]
Anti-Citrullinated CII	78.5%	[36]
Anti-CII native HLA-DR4/1+	66.7%	[37]
Anti-CII native HLA-DR4/1-	30.3%	[37]
JIA patients		
Anti-CII native	47%	[10]
Anti-CII denatured	42%	[10]
Anti-CII native	3.1%	[30]
Anti-CII native	44%	[9]
Anti-Citrullinated CII	24%	[11]

and JIA patients. The detection of these autoantibodies varies depending of the methodology employed and the CII type used as the antigen. Increased levels of anti-CII have been found in RA [27-29] and JIA [9, 30] patients during the early phase of the disease. DBA/1J CIA mice immunized with CII bovine also presented high titles of anti-CII antibodies immediately after the presentation of polyarthritis signs [31]. These findings support a major role of autoantibodies against CII in the pathophysiology of RA. It has been reported that in RA patients, high levels of autoantibodies specific for native human CII detected in the time of RA diagnosis were associated with early but not later signs of inflammation. This event could be explained by proinflammatory cytokine induction driven by surface-bound ICs containing anti-CII in early inflammatory processes [7]. High levels of anti-CII antibodies were also associated with an elevated degree of joint destruction at the time of diagnosis in RA patients [32]. On the other hand, anti-CII antibodies detected early in the disease course of JIA predicted joint damage when assessed eight years after disease diagnosis [30]. In a study about prevalence and avidity of anti-CII, autoantibodies displaying high avidity to CII were associated with disease activity in JIA patients [9]. The authors hypothesize that this event might be associated with treatment-resistant patients where the ICs production is not being blocked properly, resulting in active disease. They suggest that, a target therapy based on this mechanism could be highly promising for the treatment of RA and JIA patients with poor remission rate.

The prevalence of anti-CII has been reported between 8.8% and 88% in RA [5, 7, 33-36] patients and between 3.1% and 47% in JIA [9-11, 30] patients. In an investigation about the relationship between HLA-DR4/1 subtypes and T cell responses to CII antibodies in RA patients was observed that the HLA-DR4/1 positive group presented much higher positivity to CII antibodies (66.7%) than HLA-DR4/1 negative group (34.8%) [37], suggesting that genetic susceptibility may be associated with high levels of anti-CII antibodies detection. Table 1 provides a summary of autoantibodies detected against native and denatured CII in different studies involving RA and JIA patients. Due to the high differences in the levels of anti-CII reported in several studies and due to the lack of disease specificity, anti-CII antibodies are not considered as useful diagnostic biomarkers. In this context, studies focusing on the standardization of assays for anti-CII antibodies detection could be of great clinical interest.

In conclusion, it's still not clear what the presence of anti-CII antibodies in RA and JIA patients means, and their use as biomarker to aid in diagnosis is still very controversial. From CIA mice studies we can deduce that type II collagen presents arthritogenic epitopes, which are capable of inducing an acute inflammation response very similar to that observed in RA patients. Anti-CII appearing around the early phase of the disease indicates that these autoantibodies may play a pivotal role in the immunopathogenesis of RA and JIA.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their support.

Inflammation & Cell Signaling 2015; 2: e813. doi: 10.14800/ics.813; © 2015 by Galber R. Araujo

http://www.smartscitech.com/index.php/ics

References

1. Kelley WN. Textbook of rheumatology 5th edition. Philadelphia: W.B. Saunders; 1997: 626-685.
2. Kuettner KE, Aydelotte MB, Thonar EJ. Articular cartilage matrix and structure: a minireview. J Rheumatol Suppl 1991; 27:46-48.
3. Dodge GR, Pidoux I, Poole AR. The degradation of type II collagen in rheumatoid arthritis: an immunoelectron microscopic study. Matrix 1991; 11:330-338.
4. Morgan K. What do anti-collagen antibodies mean? Ann Rheum Dis 1990; 49:62-65.
5. Clague RB, Morgan K, Reynolds I, Williams HJ. The prevalence of serum IgG antibodies to type II collagen in American patients with rheumatoid arthritis. Brit J Rheumatol 1994; 33:336-338.
6. Kim WU, Yoo WH, Park W, Kang YM, Kim SI, Park JH, *et al.* IgG antibodies to type II collagen reflect inflammatory activity in patients with rheumatoid arthritis. J Rheumatol 2000; 27:575-581.
7. Mullazehi M, Mathsson L, Lampa J, Ronnelid J. High anti-collagen type-II antibody levels and induction of proinflammatory cytokines by anti-collagen antibody-containing immune complexes in vitro characterise a distinct rheumatoid arthritis phenotype associated with acute inflammation at the time of disease onset. Ann Rheum Dis 2007; 66:537-541.
8. Morgan K, Clague RB, Collins I, Ayad S, Phinn SD, Holt PJ. Incidence of antibodies to native and denatured cartilage collagens (types II, IX, and XI) and to type I collagen in rheumatoid arthritis. Ann Rheum Dis 1987; 46:902-907.
9. Araujo GR, Fonseca JE, Fujimura PT, Cunha-Junior JP, Silva CH, Mourao AF, Canhao H, *et al.* Anti-type II collagen antibodies detection and avidity in patients with oligoarticular and polyarticular forms of juvenile idiopathic arthritis. Immunol Lett 2015; 165:20-25.
10. Rosenberg AM, Hunt DW, Petty RE. Antibodies to native and denatured type II collagen in children with rheumatic diseases. J Rheumatol 1984; 11:425-431.
11. Gilliam BE, Chauhan AK, Moore TL. Evaluation of anti-citrullinated type II collagen and anti-citrullinated vimentin antibodies in patients with juvenile idiopathic arthritis. Pediatr Rheumatol Online J 2013; 11:31.
12. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nat Protoc 2007; 2:1269-1275.
13. Srinivasan M, Eri R, Zunt SL, Summerlin DJ, Brand DD, Blum JS. Suppression of immune responses in collagen-induced arthritis by a rationally designed CD80-binding peptide agent. Arthritis Rheum 2007; 56:498-508.
14. Nandakumar KS, Svensson L, Holmdahl R. Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. Am J Pathol 2003; 163:1827-1837.
15. Noyori K, Jasin HE. Inhibition of human fibroblast adhesion by cartilage surface proteoglycans. Arthritis Rheum 1994; 37:1656-1663.
16. Noyori K, Koshino T, Takagi T, Okamoto R, Jasin HE. Binding characteristics of antitype II collagen antibody to the surface of diseased human cartilage as a probe for tissue damage. J Rheumatol 1994; 21:293-296.
17. Mullazehi M, Mathsson L, Lampa J, Ronnelid J. Surface-bound anti-type II collagen-containing immune complexes induce production of tumor necrosis factor alpha, interleukin-1beta, and interleukin-8 from peripheral blood monocytes via Fc gamma receptor IIA: a potential pathophysiologic mechanism for humoral anti-type II collagen immunity in arthritis. Arthritis Rheum 2006; 54:1759-1771.
18. Lindh I, Snir O, Lonnblom E, Uysal H, Andersson I, Nandakumar KS, *et al.* Type II collagen antibody response is enriched in the synovial fluid of rheumatoid joints and directed to the same major epitopes as in collagen induced arthritis in primates and mice. Arthritis Res Ther 2014; 16:R143.
19. Cheah KS, Stoker NG, Griffin JR, Grosveld FG, Solomon E. Identification and characterization of the human type II collagen gene (COL2A1). Proc Natl Acad Sci USA 1985; 82:2555-2559.
20. Svensson L, Jirholt J, Holmdahl R, Jansson L. B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). Clin Exp Immunol 1998; 111:521-526.
21. Suszko A, Obminska-Mrukowicz B. Influence of polysaccharide fractions isolated from Caltha palustris L. on the cellular immune response in collagen-induced arthritis (CIA) in mice. A comparison with methotrexate. J Ethnopharmacol 2013; 145:109-117.
22. Miyata M, Sasajima T, Sato H, Saito A, Saito A, Iriswa A, *et al.* Suppression of collagen induced arthritis in mice utilizing plasmid DNA encoding interleukin 10. J Rheumatol 2000; 27:1601-1605.
23. Andersson M, Cremer MA, Terato K, Burkhardt H, Holmdahl R. Analysis of type II collagen reactive T cells in the mouse. II. Different localization of immunodominant T cell epitopes on heterologous and autologous type II collagen. Scand J Immunol 1991; 33:505-510.
24. Andersson M, Holmdahl R. Analysis of type II collagen-reactive T cells in the mouse. I. Different regulation of autoreactive vs. non-autoreactive anti-type II collagen T cells in the DBA/1 mouse. Eur J Immunol 1990; 20:1061-1066.
25. Terato K, DeArmey DA, Ye XJ, Griffiths MM, Cremer MA. The mechanism of autoantibody formation to cartilage in rheumatoid arthritis: possible cross-reaction of antibodies to dietary collagens with autologous type II collagen. Clin Immunol Immunopathol 1996; 79:142-154.
26. Rowley MJ, Nandakumar KS, Holmdahl R. The role of collagen antibodies in mediating arthritis. Mod Rheumatol 2008; 18:429-441.
27. Cook AD, Rowley MJ, Stockman A, Muirden KD, Mackay IR. Specificity of antibodies to type II collagen in early rheumatoid arthritis. J Rheumatol 1994; 21:1186-1191.
28. Pereira RS, Black CM, Duance VC, Jones VE, Jacoby RK, Welsh KI. Disappearing collagen antibodies in rheumatoid arthritis. Lancet 1985; 2:501-502.
29. Fujii K, Tsuji M, Kitamura A, Murota K. The diagnostic significance of anti-type II collagen antibody assay in rheumatoid arthritis. Int Orthop 1992; 16:272-276.
30. Berntson L, Nordal E, Fasth A, Aalto K, Herlin T, Nielsen S, *et al.* Anti-type II collagen antibodies, anti-CCP, IgA RF and IgM RF are associated with joint damage, assessed eight years after onset of juvenile idiopathic arthritis (JIA). Pediat Rheumatol Online J 2014; 12:22.

Inflammation & Cell Signaling 2015; 2: e813. doi: 10.14800/ics.813; © 2015 by Galber R. Araujo

http://www.smartscitech.com/index.php/ics

31. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, *et al*. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. J Clin Invest 2006; 116:961-973.

32. Mullazehi M, Wick MC, Klareskog L, van Vollenhoven R, Ronnelid J. Anti-type II collagen antibodies are associated with early radiographic destruction in rheumatoid arthritis. Arthritis Res Ther 2012; 14:R100.

33. Gioud M, Meghlaoui A, Costa O, Monier JC. Antibodies to native type I and II collagens detected by an enzyme linked immunosorbent assay (ELISA) in rheumatoid arthritis and systemic lupus erythematosus. Coll Rel Res 1982; 2:557-564.

34. R owley MJ, Mackay IR, Brand CA, Bateman JF, Chan D. Epitope specificity of antibodies to type II collagen in rheumatoid arthritis and systemic lupus erythematosus. Rheumatol Int 1992; 12:65-69.

35. Cook AD, Rowley MJ, Mackay IR, Gough A, Emery P. Antibodies to type II collagen in early rheumatoid arthritis. Correlation with disease progression. Arthritis Rheum 1996; 39:1720-1727.

36. Yoshida M, Tsuji M, Kurosaka D, Kurosaka D, Yasuda J, Ito Y, *et al*. Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. Mod Rheumatol 2006; 16:276- -281.

37. Li X, Li R, Ning XR, Li ZG. [Relationship between HLA-DR4/1 subtypes and T cell responses to type II collagen or anti-C II 263-272 antibodies]. Beijing da xue xue bao 2005; 37:582-584.

Immunology Letters 165 (2015) 20–25

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Anti-type II collagen antibodies detection and avidity in patients with oligoarticular and polyarticular forms of juvenile idiopathic arthritis

Galber R. Araujo[a,b,*], João E. Fonseca[c,d], Patricia T. Fujimura[a], Jair P. Cunha-Junior[e], Carlos H.M. Silva[f], Ana F. Mourão[c,g], Helena Canhão[c,d], Luiz R. Goulart[a,h], João Gonçalves[b,i], Carlos Ueira-Vieira[a]

[a] Laboratory of Nanobiotecnology, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Uberlândia, Brazil
[b] iMed – Instituto de Medicina, Faculdade de Farmácia da Universidade de Lisboa, Lisbon, Portugal
[c] Rheumatology Research Unit, Instituto de Medicina Molecular, Lisbon, Portugal
[d] Rheumatology Department, Lisbon Academic Medical Center, Lisbon, Portugal
[e] Laboratory of Immunotecnology and Imunochemistry, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, Brazil
[f] Pediatric Rheumatology Department, Hospital de Clínicas, Universidade Federal de Uberlândia, Uberlândia, Brazil
[g] Rheumatology Department, Hospital Egas Moniz, Lisbon, Portugal
[h] Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA
[i] IMM – Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 16 January 2015
Received in revised form 14 February 2015
Accepted 11 March 2015
Available online 20 March 2015

Keywords:
Juvenile idiopathic arthritis
Type II collagen
Serodiagnosis

ABSTRACT

Juvenile idiopathic arthritis (JIA) refers to a heterogeneous group of illnesses that have in common the occurrence of chronic joint inflammation in children younger than 16 years of age. The diagnosis is made only on clinical assessment. The identification of antibody markers could improve the early diagnosis, optimizing the clinical management of patients. Type II collagen is one potential autoantigen that has been implicated in the process of arthritis development. The aims of our study were to investigate the occurrence of anti-type II collagen antibodies and also to determine the avidity of the antibody–antigen binding. Ninety-six patients with oligoarticular or polyarticular JIA, 13 patients with ankylosing spondylitis (AS) and 61 healthy controls (HC) were tested for anti-type II collagen antibodies by ELISA and avidity ELISA. Sensitivity and specificity were determined by the receiver operating characteristic (ROC) curve analysis. Forty-two JIA patients (44%) were positive for antibodies against type II collagen. Its detection was significantly higher in JIA patients than in AS patients ($p = 0.006$) and HCs ($p < 0.0001$). Furthermore, anti-type II collagen antibody detection was significantly more frequent in patients with JIA of ≤6 months duration ($p = 0.0007$). Antibodies displaying high avidity to type II collagen were associated with disease activity ($p = 0.004$). This study demonstrates that antibodies against type II collagen are present in the serum of patients with oligoarticular and polyarticular JIA, being its presence more prevalent in patients with early disease. It also demonstrates that JIA patients with active disease present antibodies with high avidity against type II collagen.

© 2015 European Federation of Immunological Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Juvenile idiopathic arthritis (JIA), the most common chronic inflammatory rheumatic disorder of childhood, is characterized by the onset of chronic arthritis of unknown etiology in children younger than 16 years of age [1]. According to the International League of Associations for Rheumatology criteria, JIA comprises seven subtypes, being oligoarticular and polyarticular the most common [2]. Arthritis that affects four or fewer joints after the first six months of the disease is classified as persistent oligoarticular JIA. Although sometimes thought of as a benign condition, persistent oligoarticular JIA may, in fact, lead to a wide spectrum of outcomes, ranging from complete remission after discontinuation of medication to development of severe damage to affected joints. The extended oligoarticular JIA subtype has a worse prognosis and includes patients who have five or more joints affected after the first 6 months of disease [3]. Arthritis that affects five or more joints during the first 6 months of the disease is called polyarticular JIA, which is classified as rheumatoid factor-negative (RF^-) or rheumatoid factor-positive (RF^+). JIA also encompasses juvenile psoriatic

* Corresponding author. Tel.: +55 34 3218 2478; fax: +55 34 3218 2203.
E-mail address: galber.araujo@gmail.com (G.R. Araujo).

http://dx.doi.org/10.1016/j.imlet.2015.03.006
0165-2478/© 2015 European Federation of Immunological Societies. Published by Elsevier B.V. All rights reserved.

arthritis, enthesis-related arthritis and systemic arthritis. All JIA subtypes are of unknown causes. Diagnosis is based on the combined evaluation of medical history, clinical presentation and, to some extent, laboratory abnormalities [4,5].

Type II collagen (CII) is the predominant collagen type in joint cartilage and anti-CII antibodies have been reported to be present in serum, synovial fluid and eluted from cartilage explants of Rheumatoid Arthritis (RA) patients [6,7]. Studies have shown that antibodies from patients with RA react to various species of CII such as chicken, bovine, porcine and humans [6,8,9]. However, few studies have evaluated the presence of anti-CII antibodies in JIA [10–13]. Avidity ELISA has been used for measuring antibodies–antigen interaction in many diseases, including RA, in which different profiles could be detected with or without washing with denaturing agents [6,14–16]. There are no published studies evaluating the avidity of anti-CII antibodies in JIA.

In the present study we investigated whether antibodies present in serum of children with oligoarticular and polyarticular JIA reacts with type II collagen. In addition, we measured the level of avidity between type II collagen and IgG antibodies by using the avidity ELISA assay.

2. Materials and methods

2.1. Patients

For this study, serum samples were obtained from Brazilian and Portuguese donors. A total of 96 patients (72 female and 24 male, mean age 13.6 ± 6.8 years) who fulfilled the diagnostic criteria for oligoarticular and polyarticular JIA, according to the International League of Associations for Rheumatology [2], 13 patients with ankylosing spondylitis (AS) were used as an inflammatory rheumatic disease control (2 female and 11 male, mean age 49.3 ± 7.9 years) and 61 Healthy Controls (HC) (41 female and 20 male, mean age 11.7 ± 4 years), were enrolled in this study. Twenty-seven (27.8%) patients had samples collected in the early phase of the disease (≤6 months duration). Seventy-one (73.2%) patients had active disease at the time of sample collection. The criteria adopted to classify active disease were that defined by Consolaro et al. [17] and include 4 measures: positivity for CRP or ESR into a continuous measure of inflammation; presence of swollen joints; a physician's global assessment of disease activity, and a parent/patient global assessment of well-being.

Serum samples collected from AS patients were used as a rheumatic disease control group and healthy blood donors were used as the HC group. The study protocol involving Brazilian patients was approved by the ethics committee of the Federal University of Uberlândia whereas the study protocol involving Portuguese patients was approved by the ethics committees of the Centro Hospitalar Lisboa Norte, Hospital de Santa Maria, Lisbon Academic Medical Centre. The Portuguese samples were obtained from the Biobanco-IMM, Lisbon Academic Medical Centre. After approval of the consent procedure by the ethics committee, written informed consent was obtained from every parent of each child included in this study. The school-aged children provided their verbal and written informed consent to participate in this study, while children under 6 years of age only provided their verbal informed consent. After signed all informed consent were scanned and the originals were filed. The study was conducted in accordance with the Declaration of Helsinki.

2.2. Blood tests for inflammation detection

C-reactive protein (CRP) detection was determined by latex agglutination test (LAT) and a value of ≥0.8 mg/dl was considered elevated. Latex fixation test was utilized for the detection of IgM RF positivity and erythrocyte sedimentation rate (ESR) was determined by modified Westergren technique and considered elevated at ≥15 mm/h.

2.3. ELISA and avidity ELISA

For antibody analysis we used serum samples stored at −80, collected between 2011 and 2014. The best serum dilution, CII concentration, coating/blocking/washing buffers and anti-Human IgG were determined after several tests. The best ELISA condition for CII measurement was chosen based on the difference of reactivity between patients with JIA and HCs. ELISA and avidity ELISA were performed simultaneously. The avidity of CII-specific IgG antibodies was determined as previously described [18], with some modifications. Briefly, 96-well microtiter plates (NUNC MaxiSorp) were coated overnight at 4 °C with bovine CII 1.5 μg/ml (Becton Dickinson Biosciences, San Jose, CA) previously diluted in 0.06 M carbonate buffer (pH 9.6). To verify the presence of cross-reaction between antibodies and the ELISA plate, each serum sample was tested alone as blank control (without CII). The microplates were blocked with PBS supplemented with 3% of Bovine Serum Albumin (BSA) for 1 h at 37 °C. Serum samples diluted at 1:100 in PBS–BSA 1% plus 0.05% Tween 20 were added in duplicate on separate plates. After incubation for 1 h at 37 °C, the plates were washed with PBS Tween 0.05% and then subjected to differential washing as follows: one plate was incubated with 6 M urea solution diluted in PBS for 10 min, while the other plate was incubated with PBS Tween 0.05% for 10 min. Anti-CII antibodies were detected using horseradish peroxidase-conjugated anti-IgG (Sigma Chemical Co., St. Louis, Mo.) diluted 1:1000 and incubated for 1 h at 37 °C. The reaction was revealed with a substrate solution consisting of ortho-phenylenediamine (Sigma Chemical Co.) at 1 mg/ml in 0.01 M citrate–phosphate buffer (pH 5.0) and 0.03% H_2O_2. After incubation for 15 min at room temperature, the reaction was stopped with 2 N H_2SO_4 and read at 492 nm.

For ELISA analysis, the final optical density (OD) was normalized by the ratio of OD readings for each JIA and AS sample divided by the mean of OD value obtained by the HC group. Receiver operating characteristic (ROC) curve was constructed comparing the ELISA results from JIA with HC group. Based on the ROC curve, a cut-off point was determined as the value corresponding to the highest sensitivity without lowering the specificity. Differences in anti-CII antibodies reactivity between JIA patients and control groups are expressed in Reactivity index (RI), which was calculated based on the cut-off point (RI = normalized OD of each sample/cut-off). Samples with RI > 1.49 (cut-off) were considered positive.

The criteria adopted to classify the avidity index (AI) were defined by Marcolino et al. [18]. AIs were calculated as the ratio between the absorbance (Abs) obtained for the plate washed with urea (U^+) and the plate without urea (U^-), and was expressed as a percentage: $AI(\%) = Abs(U^+)/Abs(U^-) \times 100$. Avidity index was arbitrarily defined as: less than 30%, which was considered low avidity, between 30% and 60%, classified as average avidity and higher than 60%, corresponding to high avidity. Data were presented as a mean value ± standard deviation.

2.4. Statistical analysis

Unpaired *t* test with Welch's correction was used to evaluate the differences among JIA vs HC and JIA vs AS groups. Kruskall–Wallis test was used when three or more groups were compared. ELISA data were normalized based on the overall average absorbance obtained in the detection of anti-CII antibodies in the HC group. Cut-off that allowed best sensitivity and specificity was determined using the ROC curve. The area under the curve (AUC) was also

Table 1
Demographic and laboratory features of the studied population.

	Juvenile idiopathic arthritis = 96				*p*-Value (between JIA subtypes)	Ankylosing spondylitis	Healthy children
	Persistent oligoarticular	Extended oligoarticular	Polyarticular RF⁻	Polyarticular RF⁺			
n (*B*/*P*)	41 (21/20)	15 (8/7)	25 (5/20)	15 (6/9)		13 (0/13)	61 (38/23)
Gender (F/M)	31/10	10/5	21/4	11/4		2/11	41/20
Mean age ± SD	10.5 ± 6.8	12.8 ± 8	14.1 ± 12	22.7 ± 14	0.0038	49.3 ± 7.9	11.7 ± 4
Mean disease duration (years) ± SD	6.2 ± 5.6	9.1 ± 5.5	10.1 ± 8.1	16 ± 14.2	NS	12 ± 9.1	
CRP ± SD (mg/dl)	1.4 ± 1.8	1.1 ± 0.9	0.9 ± 1.2	2.2 ± 1.3	NS	2.7 ± 0.8	
Positive/negative	9/32	7/8	6/19	8/7			
ESR ± SD (mm/h)	20.1 ± 18	19.5 ± 10.7	20.8 ± 14.8	19.6 ± 12.7	NS	Missing	
Positive/negative	30/11	10/5	14/9	11/4			

n, number of subjects; *B*, Brazilian population; *P*, Portuguese population; F, female; M, male; SD, standard deviation; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; NS, not significant. $p < 0.05$ is statistically significant. Variables are expressed as means ± SD. Differences among groups were assessed by Kruskal–Wallis test.

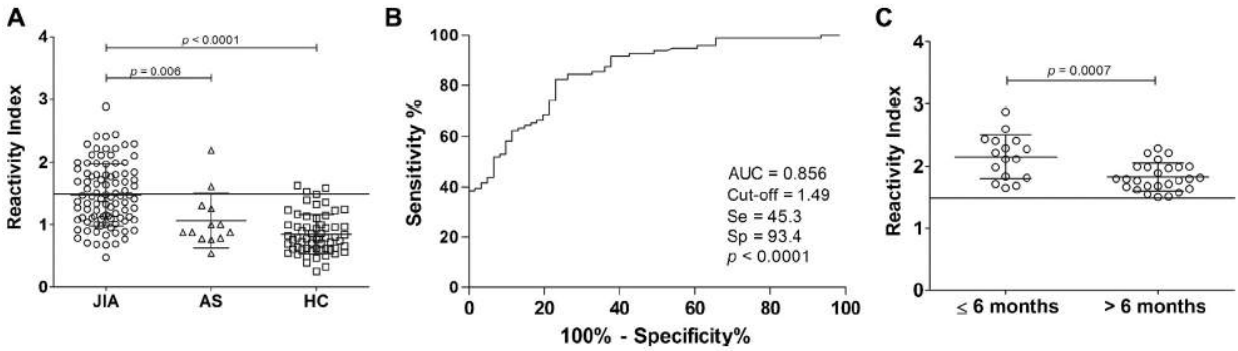


Fig. 1. Anti-collagen type II antibodies detection by ELISA. (A) Detection of anti-CII antibodies in serum samples from JIA patients (96), AS patients (13) and healthy children (61) by ELISA and the respective ROC curve (B). The area under the curve (AUC), cut-off, sensitivity (se), specificity (sp) and corresponding *p*-value are indicated inside the graph. (C) Anti-CII antibody levels in patients with early and established JIA. The horizontal bar indicates the cut-off (1.49). Reactivity index = normalized OD of each sample/cut-off.

determined. Correlations were assessed using Pearson's correlation (r). A value of $p < 0.05$ was considered statistically significant. Data were analyzed by using the GraphPad software package 5.0 (GraphPad Software Inc., San Diego, USA).

3. Results

3.1. Demographic characteristics

The studied population is presented in Table 1. At the time of samples collection, 73 JIA patients were receiving treatment. Thirty-nine (40.6%) were receiving treatment with non-steroidal anti-inflammatory drugs, 46 (47.9%) were on treatment with disease modifying anti-rheumatic drugs and 13 (13.5%) were receiving treatment with biologic agents. Nine (9.4%) patients were untreated because it was their first visit to the rheumatology clinic at the time of sample collection or because they were in disease remission. Fifteen (15.6%) patients were RF positive (mean concentration of 91 ± 260 IU/ml). A total of 30 (31.2%) patients had raised CRP (mean concentration of 1.4 ± 1.3 mg/dl) and 65 (67.7%) had raised ESR (mean concentration 20.2 ± 10.7 mm/h). JIA patients had mean disease duration of 10 ± 7 years.

3.2. Immunoreactivity of collagen type II against IgG from JIA patients and controls

Anti-CII antibodies levels were significantly higher in JIA than in AS patients ($p = 0.006$) and HC group ($p < 0.0001$) (Fig. 1A and B). We found that 42 (44%) JIA patients were positive to CII in ELISA (RI = 1.920 ± 0.3; AUC = 0.856; $p < 0.0001$). Although we observed differences in positivity across JIA subtypes, no significant differences were observed in the levels of anti-CII reactivity. The performance of the test was evaluated for sensitivity and specificity and found to be 45.3% and 93.4%, respectively. On the other hand, we found that 2 AS patients (15%) and 4 HCs (6.5%) were also anti-CII positives. These results are summarized in Table 2.

We did not observe significant differences in reactivity to CII in JIA patients across gender, ethnicity, number of joints affected or

Table 2
Positivity for anti-collagen type II antibodies in the studied population.

	Juvenile idiopathic arthritis				*p*-Value	Ankylosing spondylitis *n* = 13	Healthy children *n* = 61
	Persistent oligoarticular *n* = 41	Extended oligoaritular *n* = 15	Polyarticular RF⁻ *n* = 25	Polyarticular RF⁺ *n* = 15			
Reactivity index	1.35 ± 0.5	1.53 ± 0.3	1.6 ± 0.5	1.6 ± 0.4	NS	1.1 ± 0.4	0.85 ± 0.3
Positivity *n* (%)	16 (39%)	7 (46.6%)	10 (40%)	9 (60%)		2 (15%)	4 (6.5%)

NS, not significant. $p < 0.05$ is statistically significant. Variables are expressed as means ± SD. Differences among JIA subtypes were assessed by Kruskal–Wallis test.

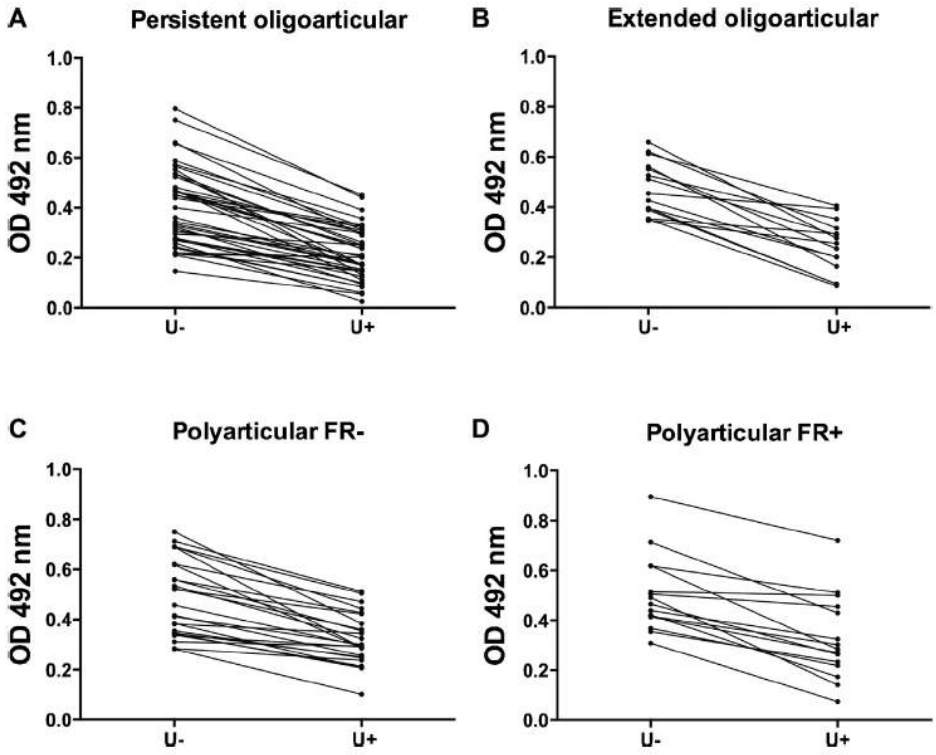


Fig. 2. Anti-CII antibody levels detected in JIA before and after treatment with 6 M urea. Optical density differences before (U^-) and after (U^+) treatment with urea are shown for each patient separately for each JIA subtype (A, B, C, D). The paired U^- and U^+ samples from individual patients are identified by a line between points.

therapy. There was no statistically significant difference between the Portuguese and the Brazilian population. JIA patients with ≤6 months of disease duration that were positives for anti-CII antibodies ($n = 16$) presented levels of anti-CII antibodies significantly higher ($p = 0.0007$) than patients with established disease ($n = 26$) (Fig. 1C). Of interest, 32 (76%) of the anti-CII positive patients had active disease. Anti-CII antibodies were directly correlated with RF (Pearson $r = 0.7207$, $p < 0.005$). No correlations were found when levels of anti-CII antibodies were analyzed separately for the values of CRP, ESR and the number of inflamed joints. However, a 13-year-old girl with polyarticular RF^+, who presented inflammation in more than 5 joints, high CRP concentration (2.8 mg/dl), increased ESR (38 mm/h), and very high levels of rheumatoid factor (1118.80 Ul/ml), presented the highest levels of anti-CII antibodies in ELISA (RI = 2.9).

3.3. Avidity of anti-CII antibodies and controls

The levels of anti-CII antibodies detected in the serum from different subtypes of JIA without and after treatment with 6 M urea are shown in Fig. 2. In the serum samples from JIA patients, AIs ranged from 9.6% to 97% (mean of 52.2% ± 18) (Fig. 3A). Forty-six (47.9%) samples presented antibodies with high avidity to CII (mean of 73.9% ± 10.4), 39 (40.6%) samples presented antibodies with medium avidity (mean of 47.4% ± 8.1) and 11 (11.4%) samples presented antibodies with low avidity (mean of 24.1% ± 5.3). Serum samples from AS patients presented AIs ranging from 20.3% to 52% (mean of 36.2% ± 7.9), with most patients displaying antibodies with medium avidity to CII. Serum from HC presented AIs ranging from 7% to 47.1% (mean of 26% ± 8.5), with most of patients displaying antibodies with low avidity to CII. When JIA patients were subdivided according to different populations, gender, ethnicity, treatment or disease duration, no significant difference was observed in the avidity index in any disease subtype. In the group

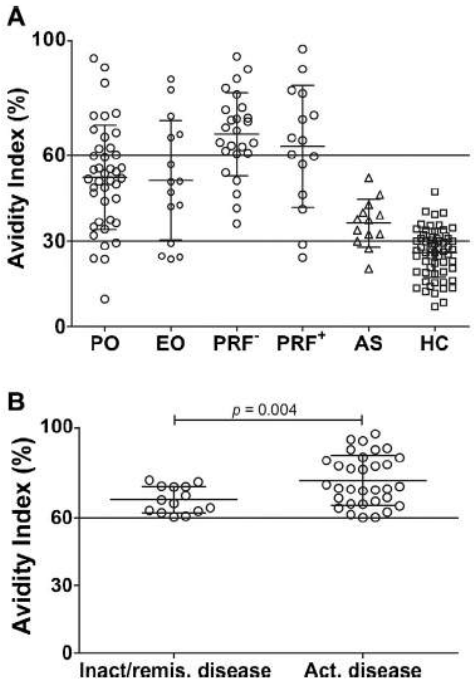


Fig. 3. Avidity profile of anti-collagen type II antibodies. (A) Avidity profiles of anti-CII antibodies observed in 96 patients with oligoarticular and polyarticular forms of JIA, 13 patients with AS and 61 HCs. (B) Antibodies displaying high avidity to type II collagen in patients with inactive and active JIA. The avidity index values were defined as: <30% = low avidity; between 30% and 60% = medium avidity; >60% = high avidity. Avidity indexes are indicated as lines.

displaying antibodies with high avidity against CII, we observed that patients with active disease ($n = 32$; mean of 76.5% ± 11) at the time of sample collection presented AIs significantly higher in comparison with patients in inactive/remission disease ($n = 14$; mean of 68% ± 5.6) ($p = 0.004$) (Fig. 3B). Interestingly, polyarticular JIA patients presented AIs significantly higher than other JIA subtypes ($p = 0.006$). We did not observe significant association between high avidity antibodies and levels of RF for polyarticular JIA patients. The avidity of antibodies toward CII was significantly higher in JIA patients when compared with AS ($p < 0.0001$) or HC group ($p < 0.0001$).

4. Discussion

Most of what we know about autoimmune response to CII comes from experimental disease models. Particularly, from the collagen-induced arthritis (CIA) experimental models, obtained by immunization with autologous or heterologous type II collagen with adjuvant [19]. Several studies have attempted to identify antibodies to CII in RA [6–9,20–22], however, only a small number of published studies aimed to determine the prevalence of anti-CII antibodies in JIA patients [10–13]. The current work reports the occurrence and avidity of anti-type II collagen antibodies in patients with JIA compared with healthy individuals.

Different compositions of type II collagen have been used as the antigen to antibodies detection in RA and JIA in its native or denatured forms. The vast majority of published studies about the occurrence of anti-CII in JIA and RA, aimed to determine the prevalence of antibodies to citrullinated CII [12,23,24]. Antibodies to native citrullinated CII have already been detected in 78.5% of RA [24] and 24% of JIA patients [12]. Antibodies to native human CII were detected in 20% [25] and 70% of RA [11], 47% [11] of JIA patients and recently, in 3.1% [13]. Antibodies to native bovine CII (the same we have used in our study) were detected in 31.5% of RA patients [6]. Based on the most adequate cut-off value determined by the ROC curve, we found that 44% of our JIA patients were positives to anti-CII antibodies. The positivity of anti-CII antibodies in the AS population (15%) was lower than the results reported in another study [26], which showed positivity in 33.3% of the patients. Although anti-CII antibodies were present in the AS group its detection was much higher in JIA patients. The differences in the prevalence of anti-CII in the several published studies may be due to the different species of CII used as the antigen to antibodies detection, since studies that aimed to analyze CII specificity revealed that autoantibodies are generally associated with specific arthritogenic epitopes present in the CII molecules from different species [27,28]. The different methodologies of data analysis (normalization and cut-off determination), the number of patients in which these anti-CII antibodies have been measured, or even patient's disease activity [6] could also have contributed to these heterogeneous results.

Our results also showed increased levels of anti-CII antibodies in patients with early JIA, with the vast majority of individuals presenting active disease at the time of sample collection. These data are consistent with previous findings reporting higher frequencies of anti-CII antibodies in early RA [29–31] and recently, in JIA patients [13]. Similar results were also observed in CIA models after arthritis induction by immunization with CII, where the levels of circulating anti-CII antibodies in MRL/l mice were as well highest at 6 months, thus immediately after the development of arthritis [32]. Anti-CII appearing early in the course of the disease suggests that these autoantibodies might play a role in JIA physiopathology. However, the clinical relevance of its detection in early JIA is still an open question. We hypothesize that in patients with early JIA, high levels of anti-CII antibodies might induce an acute inflammation mediated by surface-bound immune complexes (IC) containing anti-CII, as has already been described in RA [9]. Purified CII-specific monoclonal antibodies were shown to induce an acute form of arthritis in mice [33], which demonstrates that anti-CII autoantibodies are indeed directly pathogenic *in vivo*. These anti-CII antibodies appearing around clinical onset suggests that these autoantibodies play an early role in the process of disease development. If it proves to be true, the detection of these antibodies could lead to faster diagnosis and treatment. Our results also showed a high percentage of anti-CII positive patients presenting active disease (76%). Taking into account what has already been reported [34–36], we believe that circulating ICs containing anti-CII can activate the complement system and stimulate the production of cytokines of pathogenic importance in the inflammatory process, such as interleukin-1β (IL-1β), IL-6, IL-10 and tumor necrosis factor α (TNF-α). This event could perpetuate joint inflammation and consequently maintain the disease activity. This hypothesis is corroborated by Mullazehi et al. [34] who reported that in RA patients the production of the cytokines TNF-α, IL-1β and IL-8 decreased after the specific blockade of ICs containing anti-CII.

Our results also showed a high percentage of anti-CII antibody positive samples in the group with polyarticular RF^+ and also a direct correlation between anti-CII antibodies and RF. This is in line with the fact that this subtype corresponds basically to a juvenile RA onset, with a more persistent and severe course of the disease as compared to other JIA subtypes [37]. Furthermore, we reported a patient with polyarticular onset displaying high levels of RF, CRP and ESR who presented the highest level of anti-CII antibodies. Although when analyzed separately no correlation between levels of anti-CII and values of CRP and ESR was found, these findings are in agreement with what has already been reported in RA patients [7], where levels of anti-CII antibodies were higher in patients with high CRP and ESR, and partially, with another study [13] involving JIA patients, where anti-CII antibodies was associated with high levels of CRP.

In the present study, we calculated the AI of specific anti-CII antibodies, determined from the avidity ELISA. A brief 6 M urea treatment allowed the dissociation of the low-avidity interactions, retaining the antibodies that bind with higher avidity to CII. This is the first study investigating the nature of anti-CII antibodies avidity in patients with JIA. We have shown that levels of antibodies displaying high avidity to CII were significantly higher in patients with active disease and in polyarticular patients. The driving force in the antibody binding and progressive destruction of cartilage is still obscure. However, it has been demonstrated that anti-CII antibodies form immune complexes with CII joint cartilage that, when not effectively removed, can activate complement and contribute to chronic inflammation [36]. Treatment-resistant JIA results in joint destruction, especially in patients with polyarticular subtype, which have the poorest prognosis with a remission rate of only 15%. More than 50% of JIA patients do not achieve remission despite treatment and require further rheumatological care as adults [38]. A possible explanation for the high occurrence of antibodies binding with high avidity against CII in samples from patients with active JIA could be that IC's production is not being blocked properly, resulting in active disease. In fact, it has been reported that the blocking of the complement system resulted in decrease of inflammatory cytokine, showing that complement plays a role in arthritis [36]. If this proves to be true, a target therapy based on this mechanism could be highly promising for both RA and JIA.

In conclusion, the present study showed that anti-CII antibodies are present in patients with oligoarticular and polyarticular forms of JIA. Consistent with RA data, high levels of anti-CII antibodies were detected in patients with JIA of ≤6 months duration, suggesting that anti-CII antibodies in JIA patients are associated with the early inflammatory process. Patients with active disease had more frequently high avidity antibodies against CII. Our data

support the hypothesis that anti CII antibodies may play a role in the physiopathology of JIA.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgements

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) funded by the Ministry of Education of Brazil (2027/13-5), and by the Universidade Federal de Uberlândia.

References

[1] A. Ravelli, A. Martini, Juvenile idiopathic arthritis, Lancet 369 (2007) 767–778.
[2] R.E. Petty, T.R. Southwood, P. Manners, J. Baum, D.N. Glass, J. Goldenberg, et al., International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001, J. Rheumatol. 31 (2004) 390–392.
[3] C. Huemer, P.N. Malleson, D.A. Cabral, M. Huemer, J. Falger, T. Zidek, et al., Patterns of joint involvement at onset differentiate oligoarticular juvenile psoriatic arthritis from pauciarticular juvenile rheumatoid arthritis, J. Rheumatol. 29 (2002) 1531–1535.
[4] G.E. Dannecker, P. Quartier, Juvenile idiopathic arthritis: classification, clinical presentation and current treatments, Horm. Res. 72 (Suppl 1) (2009) 4–12.
[5] M. van Rossum, R. van Soesbergen, S. de Kort, R. ten Cate, A.H. Zwinderman, B. de Jong, et al., Anti-cyclic citrullinated peptide (anti-CCP) antibodies in children with juvenile idiopathic arthritis, J. Rheumatol. 30 (2003) 825–828.
[6] R.B. Clague, K. Morgan, I. Reynolds, H.J. Williams, The prevalence of serum IgG antibodies to type II collagen in American patients with rheumatoid arthritis, Br. J. Rheumatol. 33 (1994) 336–338.
[7] W.U. Kim, W.H. Yoo, W. Park, Y.M. Kang, S.I. Kim, J.H. Park, et al., IgG antibodies to type II collagen reflect inflammatory activity in patients with rheumatoid arthritis, J. Rheumatol. 27 (2000) 575–581.
[8] K. Terato, D.A. DeArmey, X.J. Ye, M.M. Griffiths, M.A. Cremer, The mechanism of autoantibody formation to cartilage in rheumatoid arthritis: possible cross-reaction of antibodies to dietary collagens with autologous type II collagen, Clin. Immunol. Immunopathol. 79 (1996) 142–154.
[9] M. Mullazehi, L. Mathsson, J. Lampa, J. Ronnelid, High anti-collagen type-II antibody levels and induction of proinflammatory cytokines by anti-collagen antibody-containing immune complexes in vitro characterise a distinct rheumatoid arthritis phenotype associated with acute inflammation at the time of disease onset, Ann. Rheum. Dis. 66 (2007) 537–541.
[10] C.B. Lindsley, L.L. Janecek, W.A. Horton, H.B. Lindsley, IgG anticollagen antibodies in sera of patients with childhood rheumatic diseases [abstract], Arthritis Rheum. 23 (1980) S56.
[11] A.M. Rosenberg, D.W. Hunt, R.E. Petty, Antibodies to native and denatured type II collagen in children with rheumatic diseases, J. Rheumatol. 11 (1984) 425–431.
[12] B.E. Gilliam, A.K. Chauhan, T.L. Moore, Evaluation of anti-citrullinated type II collagen and anti-citrullinated vimentin antibodies in patients with juvenile idiopathic arthritis, Pediatr. Rheumatol. Online J. 11 (2013) 31.
[13] L. Berntson, E. Nordal, A. Fasth, K. Aalto, T. Herlin, S. Nielsen, et al., Anti-type II collagen antibodies, anti-CCP, IgA RF and IgM RF are associated with joint damage, assessed eight years after onset of juvenile idiopathic arthritis (JIA), Pediatr. Rheumatol. Online J. 12 (2014) 22.
[14] J.S. Remington, P. Thulliez, J.G. Montoya, Recent developments for diagnosis of toxoplasmosis, J. Clin. Microbiol. 42 (2004) 941–945.
[15] M.M. Newkirk, M.J. Fournier, J. Shiroky, Rheumatoid factor avidity in patients with rheumatoid arthritis: identification of pathogenic RFs which correlate with disease parameters and with the gal(0) glycoform of IgG, J. Clin. Immunol. 15 (1995) 250–257.
[16] R. Penkova, I. Goshev, S. Gorinstein, P. Nedkov, Stability of collagen during denaturation, J. Protein Chem. 18 (1999) 397–401.
[17] A. Consolaro, N. Ruperto, A. Bazso, A. Pistorio, S. Magni-Manzoni, G. Filocamo, et al., Development and validation of a composite disease activity score for juvenile idiopathic arthritis, Arthritis Rheum. 61 (2009) 658–666.
[18] P.T. Marcolino, D.A. Silva, P.G. Leser, M.E. Camargo, J.R. Mineo, Molecular markers in acute and chronic phases of human toxoplasmosis: determination of immunoglobulin G avidity by Western blotting, Clin. Diagn. Lab. Immunol. 7 (2000) 384–389.
[19] R.O. Williams, Collagen-induced arthritis as a model for rheumatoid arthritis, Methods Mol. Med. 98 (2004) 207–216.
[20] K. Morgan, R.B. Clague, I. Collins, S. Ayad, S.D. Phinn, P.J. Holt, Incidence of antibodies to native and denatured cartilage collagens (types II, IX, and XI) and to type I collagen in rheumatoid arthritis, Ann. Rheum. Dis. 46 (1987) 902–907.
[21] A.D. Cook, M.J. Rowley, I.R. Mackay, A. Gough, P. Emery, Antibodies to type II collagen in early rheumatoid arthritis. Correlation with disease progression, Arthritis Rheum. 39 (1996) 1720–1727.
[22] M. Mullazehi, M.C. Wick, L. Klareskog, R. van Vollenhoven, J. Ronnelid, Anti-type II collagen antibodies are associated with early radiographic destruction in rheumatoid arthritis, Arthritis Res. Ther. 14 (2012) R100.
[23] P. Suwannalai, L.A. van de Stadt, H. Radner, G. Steiner, H.S. El-Gabalawy, C.M. Zijde, et al., Avidity maturation of anti-citrullinated protein antibodies in rheumatoid arthritis, Arthritis Rheum. 64 (2012) 1323–1328.
[24] M. Yoshida, M. Tsuji, D. Kurosaka, D. Kurosaka, J. Yasuda, Y. Ito, et al., Autoimmunity to citrullinated type II collagen in rheumatoid arthritis, Mod. Rheumatol. 16 (2006) 276–281.
[25] M.J. Rowley, I.R. Mackay, C.A. Brand, J.F. Bateman, D. Chan, Epitope specificity of antibodies to type II collagen in rheumatoid arthritis and systemic lupus erythematosus, Rheumatol. Int. 12 (1992) 65–69.
[26] E.K. Choi, P.A. Gatenby, N.W. McGill, J.F. Bateman, W.G. Cole, J.R. York, Autoantibodies to type II collagen: occurrence in rheumatoid arthritis, other arthritides, autoimmune connective tissue diseases, and chronic inflammatory syndromes, Ann. Rheum. Dis. 47 (1988) 313–322.
[27] K. Terato, Y. Shimozuru, K. Katayama, Y. Takemitsu, I. Yamashita, M. Miyatsu, et al., Specificity of antibodies to type II collagen in rheumatoid arthritis, Arthritis Rheum. 33 (1990) 1493–1500.
[28] C. Buckee, K. Morgan, S. Ayad, I. Collins, R.B. Clague, P.J. Holt, Diversity of antibodies to type II collagen in patients with rheumatoid arthritis: detection by binding to alpha-chains and to cyanogen bromide peptides, Br. J. Rheumatol. 29 (1990) 254–258.
[29] A.D. Cook, M.J. Rowley, A. Stockman, K.D. Muirden, I.R. Mackay, Specificity of antibodies to type II collagen in early rheumatoid arthritis, J. Rheumatol. 21 (1994) 1186–1191.
[30] R.S. Pereira, C.M. Black, V.C. Duance, V.E. Jones, R.K. Jacoby, K.I. Welsh, Disappearing collagen antibodies in rheumatoid arthritis, Lancet 2 (1985) 501–502.
[31] K. Fujii, M. Tsuji, A. Kitamura, K. Murota, The diagnostic significance of anti-type II collagen antibody assay in rheumatoid arthritis, Int. Orthopaed. 16 (1992) 272–276.
[32] A. Tarkowski, R. Holmdahl, K. Rubin, L. Klareskog, L.A. Nilsson, K. Gunnarsson, Patterns of autoreactivity to collagen type II in autoimmune MRL/l mice, Clin. Exp. Immunol. 63 (1986) 441–449.
[33] K.S. Nandakumar, L. Svensson, R. Holmdahl, Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes, Am. J. Pathol. 163 (2003) 1827–1837.
[34] M. Mullazehi, L. Mathsson, J. Lampa, J. Ronnelid, Surface-bound anti-type II collagen-containing immune complexes induce production of tumor necrosis factor alpha, interleukin-1beta, and interleukin-8 from peripheral blood monocytes via Fc gamma receptor IIA: a potential pathophysiologic mechanism for humoral anti-type II collagen immunity in arthritis, Arthritis Rheum. 54 (2006) 1759–1771.
[35] J. Ronnelid, A. Tejde, L. Mathsson, K. Nilsson-Ekdahl, B. Nilsson, Immune complexes from SLE sera induce IL10 production from normal peripheral blood mononuclear cells by an FcgammaRII dependent mechanism: implications for a possible vicious cycle maintaining B cell hyperactivity in SLE, Ann. Rheum. Dis. 62 (2003) 37–42.
[36] L. Mathsson, A. Tejde, K. Carlson, M. Hoglund, B. Nilsson, K. Nilsson-Ekdahl, et al., Cryoglobulin-induced cytokine production via FcgammaRIIa: inverse effects of complement blockade on the production of TNF-alpha and IL-10. Implications for the growth of malignant B-cell clones, Br. J. Haematol. 129 (2005) 830–838.
[37] R. Schneider, M.H. Passo, Juvenile rheumatoid arthritis, Rheum. Dis. Clin. N. Am. 28 (2002) 503–530.
[38] K. Minden, U. Kiessling, J. Listing, M. Niewerth, E. Doring, J. Meincke, et al., Prognosis of patients with juvenile chronic arthritis and juvenile spondyloarthropathy, J. Rheumatol. 27 (2000) 2256–2263.

Peptídeos selecionados no Capítulo II

Autores: ARAUJO, G. R.; VAZ. E. R.; GOULART, L. R.; VIEIRA, C. U.

Título: Peptídeos e sua utilização no imunodiagnóstico da artrite reumatoide e artrite idiopática juvenil

Número do registro: BR10201500180

Data de depósito: 27/01/2015

Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial

Peptídeos selecionados no Capítulo IV

Autores: ARAUJO, G. R.; FUJIMURA, P. T.; GOULART, L. R.; SILVA, C. H. M.; VIEIRA, C. U.

Título: Peptídeos miméticos a autoantígenos e sua utilização em imunodiagnóstico da artrite idiopática juvenil

Número do registro: BR1020130176397

Data de depósito: 10/07/2013

Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial