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# *AVALIAÇÃO DA PRODUÇÃO DE ANTICORPOS ESPECÍFICOS, CITOCINAS E QUIMIOCINAS NA PARACOCCIDIOIDOMICOSE: CORRELAÇÃO COM A FORMA CLÍNICA E O TEMPO DE TRATAMENTO*

***Campinas***

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Este exemplar corresponde à versão final da Dissertação de Mestrado apresentada ao Curso de Pós-Graduação Ciências Médicas da Faculdade de Ciências Médicas da UNICAMP, para obtenção do título de Mestre em Ciências Médicas, Área Ciências Biomédicas.

Campinas, 01 de setembro de 2000.

Profa. Dra. Maria Heloisa S. L. Blotta
Orientadora

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*Dissertação de Mestrado apresentada ao Curso de Pós-Graduação em Ciências Médicas, da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Mestre em Ciências Médicas, na área de Ciências Biomédicas.*

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***Campinas***

***2000***

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**Data: 01.09.00**

## *DEDICATÓRIA*

*Para minha mãe, Elza.*

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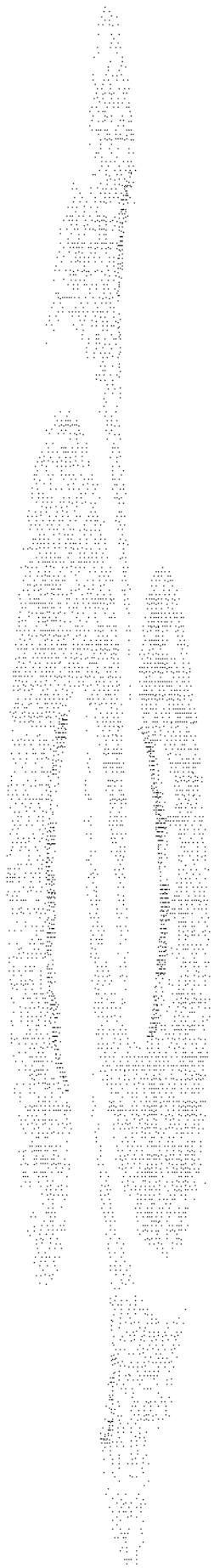
# *SUMÁRIO*

# *RESUMO*

A paracoccidioidomicose (PCM) é uma doença sistêmica, crônica e progressiva, causada pelo fungo *Paracoccidioides brasiliensis,* que apresenta-se sob duas formas clínicas principais: a forma Adulta ou Crônica (FA) e a forma Juvenil ou Aguda (FJ). O presente trabalho teve por objetivo estudar a correlação entre as formas clínicas e o padrão da resposta imunológica apresentada pelo hospedeiro na infecção humana pelo *P. brasiliensis.* Para tal, avaliamos a produção de mediadores imunológicos no soro de pacientes com a FJ ou a FA da PCM, nas diferentes fases da doença (antes, durante e após o tratamento). A resposta inflamatória foi avaliada pela dosagem das citocinas IL-6, TNF-α e TGF-β1 e quimiocinas IL-8 e MIP-1α pelo método de ELISA. A produção de anticorpos específicos (IgG, subclasses de IgG, IgE e IgA), também foi determinada utilizando-se diferentes técnicas de ELISA. Foi demonstrado que pacientes com a FJ da PCM apresentam níveis séricos aumentados de IgG, IgG4, IgE, IgA específicos para gp43 de *P. brasiliensis,* assim como elevado número de eosinófilos no sangue periférico. Os níveis de IgE específica se correlacionaram positivamente com os de IgG4 e IgA, assim como com o grau de eosinofilia periférica. Em pacientes com a FJ foram encontrados níveis mais elevados de TGF-β, um dos fatores que controla o "switch" para IgA, do que pacientes com FA, tendo sido observada uma correlação positiva entre a concentração sérica dessa citocina e da IgA específica. Por outro lado, os pacientes com a FA apresentaram altos níveis de IgG1 específica e concentrações inferiores dos outros isotipos de IgG e de IgA e IgE. Quanto a produção de IL-6, TNF-α e MIP-1α, apesar de não terem sido detectadas diferenças entre as formas clínicas, essas citocinas estavam aumentadas em pacientes com a FA disseminada, quando comparada às formas localizadas. Níveis diminuídos de IL-8 foram observados em pacientes com a FJ da PCM sugerindo que nestes indivíduos pode estar ocorrendo uma deficiência da atividade quimiotática para neutrófilos, bem como a uma alteração do mecanismo regulatório da produção de IgE. A dosagem das imunoglobulinas permitiu observar que todos os isotipos diminuíram ao longo do tratamento, com exceção da IgG4, cujos níveis permaneceram elevados para a maioria dos pacientes com a FJ, durante todo o período estudado (2 anos). Tomados em conjunto esses resultados refletem uma polarização da resposta imune na PCM, indicando um provável padrão Th2 nos pacientes com a FJ da doença.



# *1. INTRODUÇÃO*

## PARACOCCIDIOIDOMICOSE: A DOENÇA

Sendo endêmica da América Latina e apresentando-se como a micose sistêmica de maior prevalência no Brasil, a paracoccidioidomicose (PCM) é causada pelo fungo dimórfico *Paracoccidioides brasiliensis*. Em cultura à temperatura ambiente (25°C), o *P. brasiliensis* desenvolve-se sob a forma de micélio, caracterizada por hifas septadas sem conídios, com aspecto cotonoso. Em culturas a 37°C ou no organismo do hospedeiro assume a forma de levedura, arredondada ou oval com membrana bi-refringente. Esta forma varia em tamanho de 2 a 60 µm (comumente de 10 a 30 µm), apresentando-se como célula única ou com brotamentos laterais, o que lhe dá o aspecto de roda de leme e permite sua fácil identificação (RESTREPO, 1985; FRANCO *et al,* 1989).

Apesar do habitat natural do *P. brasiliensis* ainda ser controverso, a análise da ocorrência de casos permite a definição de áreas de endemicidade com características específicas: temperaturas médias de 17 a 24°C, clima temperado a quente (moderadamente úmido), índice pluviométrico de 800 a 2000 mm por ano, altitude variando de 47 a 1300 m, solo ácido e vegetação densa (floresta tropical e subtropical) (RESTREPO, 1985).

A PCM ocorre com maior freqüência em indivíduos do sexo masculino, cuja ocupação principal relaciona-se com o manuseio direto de solo, como agricultores e trabalhadores da construção civil (MOTA, 1996; BLOTTA *et al,* 1999). A aparente resistência das mulheres frente à infecção pelo *P. brasiliensis* deve-se ao fato de que os hormônios femininos inibem a transformação da forma miceliana para a forma de levedura, etapa essencial para que a infecção se estabeleça (RESTREPO *et al,* 1984; STOVER *et al,* 1986; SALAZAR, RESTREPO & STEVENS, 1988).

A infecção pelo *P. brasiliensis* se dá por via respiratória, através da inalação de propágulos, que se depositam nos alvéolos pulmonares. Nos pulmões o fungo passa da forma de micélio para a forma de levedura, através de uma série de mudanças na síntese de constituintes celulares (SAN-BLAS & SAN-BLAS, 1982; SALAZAR & RESTREPO, 1984; SAN-BLAS *et al,* 1987). Caso a infecção não seja controlada, o fungo pode permanecer latente por um período variável de meses a anos. Após a reativação, a infecção pode ficar restrita aos pulmões, ou então disseminar-se por via hematogênica e/ou linfática para vários órgãos do hospedeiro, sendo mais comum o acometimento da pele, mucosa oral e nasal,

linfonodos, fígado, baço, pâncreas e medula óssea. Os órgãos afetados variam principalmente de acordo com a forma clínica apresentada pelo paciente (FRANCO *et al*, 1989).

Vários fatores influenciam o aparecimento da doença, mas a freqüente associação da PCM à tuberculose e o fato de que os pacientes com PCM geralmente apresentam hábitos de etilismo e tabagismo, sugerem que uma alteração da resposta imunológica possa estar envolvida na suscetibilidade à doença (LONDERO & RAMOS, 1990; MARTINEZ & MOYA, 1992). Apesar de alguns trabalhos discutirem a predisposição genética como fator de risco para o desenvolvimento da doença, ainda não existe um consenso sobre esse tema (RESTREPO, RESTREPO & RESTREPO, 1983; LACERDA, ARCE-GOMEZ & QUEIROZ-TELLES, 1988; GOLDANI *et al*, 1991).

A maioria dos indivíduos que entra em contato com o fungo consegue conter a infecção, e não desenvolvem a doença. Este fato é evidenciado pela alta incidência de testes cutâneos de hipersensibilidade tardia positivos em moradores de regiões endêmicas para a PCM. Esses indivíduos não apresentam qualquer sinal da doença, sendo essa condição denominada PCM infecção (CAMPOS & FAVA-NETO, 1978; MOK & FAVA-NETO, 1978; ANDRADE *et al*, 1984; ZEMBRZUSKI *et al*, 1996). Outro indício de que a PCM pode se manifestar de forma assintomática ou subclínica é a presença do fungo em lesões parcialmente calcificadas, encontradas nos pulmões de indivíduos submetidos à necrópsia para investigações de outras patologias (ANGULO-ORTEGA, 1972; ALBORNOZ, 1982).

A PCM-doença apresenta-se sob duas formas clínicas principais: a Forma Adulta (FA) e a Forma Juvenil (FJ). A FA acomete principalmente indivíduos do sexo masculino, com idade superior a 30 anos. Nesses indivíduos a doença geralmente apresenta um longo período de latência e após a reativação, observa-se o acometimento preferencial do pulmão (90% dos casos), da pele e das mucosas oral e nasal. Já a FJ ocorre principalmente em indivíduos com até 30 anos, sendo rara em pacientes com idade superior. Esta forma tem evolução rápida (semanas a meses), é mais grave e afeta indistintamente ambos os sexos, caracterizando-se pelo acometimento do sistema fagocítico-mononuclear (linfonodos, baço, fígado e medula óssea) e raro envolvimento pulmonar (MONTENEGRO, 1986; FRANCO *et al*, 1987; FRANCO *et al* 1989).

O diagnóstico da PCM é feito pela observação do fungo em espécimes clínicos de pacientes (biópsias, raspado de lesões de pele ou mucosa, escarro e lavado broncoalveolar). O isolamento do fungo através da cultura desses materiais também é possível (NOGUEIRA-BOSCARDIN, BRANDÃO & BALLA, 1985; MATTOS *et al*, 1991). O diagnóstico sorológico apesar de não ser definitivo é bastante útil, principalmente para casos nos quais o achado do fungo é dificultado, devido a sua localização. Dentre os métodos sorológicos mais utilizados destacam-se a imunoprecipitação (imunodifusão radial e contra-imunoeletroforese), a hemaglutinação passiva e as técnicas imunoenzimáticas (ELISA e Imuno-Blot). Diversos tipos de preparações antigênicas têm sido empregadas nas técnicas imunológicas, como a gp43 de *P. brasiliensis*. Esta glicoproteína de 43 KDaltons, é produzida e excretada pelo fungo em cultura e apresenta-se como antígeno imunodominante, reconhecido por anticorpos presentes no soro da grande maioria dos pacientes com PCM (CAMARGO *et al*, 1984; CANO *et al*, 1986; CANO & RESTREPO, 1987; CAMARGO *et al*, 1988; CAMPOS *et al*, 1990; CAMARGO *et al*, 1991; BLOTTA & CAMARGO, 1993).

O tratamento da paracoccidioidomicose era inexistente até 1940, quando RIBEIRO introduziu o uso de sulfonamidas com bons resultados. A anfotericina-B, foi bastante utilizada, mas está sendo substituída por outras drogas que apresentam menor toxicidade e facilidade na aplicação, como os derivados imidazólicos: cetoconazol e itraconazol. Este último é aparentemente mais eficaz no controle do fungo, apresenta baixa toxicidade e alta atividade anti-fúngica, diminuindo o tempo de tratamento (NARANJO *et al* 1990). Apesar disso, seu alto custo não permite ampla utilização pelos pacientes, geralmente pessoas de baixa renda. Atualmente emprega-se com freqüência a combinação de duas drogas: a sulfadiazina e o trimetoprim devido à sua eficiência, baixo custo e disponibilidade na rede pública. O tratamento é eficaz para a maioria dos casos mas, como é de longa duração (mais de um ano), não são raros episódios de recidiva da doença, devido ao uso irregular ou mesmo abandono da medicação (RESTREPO *et al*, 1985; DILLON *et al*, 1986; BARRAVIERA *et al*, 1989; NARANJO *et al*, 1990).

## A RESPOSTA IMUNOLÓGICA NA PARACOCCIDIOIDOMICOSE

A resposta imunológica a agentes infecciosos envolve a participação efetiva e orquestrada da imunidade celular e humoral. Na PCM, como em outras micoses sistêmicas, o curso e desfecho da infecção dependem da resposta imune do hospedeiro e sua interação com o *P. brasiliensis.*

A resposta ao *P. brasiliensis* nos tecidos é caracterizada pela formação de granulomas, com a participação ativa de células T, que atuam tanto no recrutamento, como na ativação dos macrófagos. Estas estruturas relacionadas a contenção do agente patológico no sítio de infecção, são compostas por macrófagos e linfócitos, sendo que os primeiros localizam-se na porção central e os últimos ao redor destes. Células gigantes, formadas a partir da fusão de macrófagos, são caracteristicamente encontradas no interior dos granulomas (FRANCO *et al,* 1989; WYNN & CHEEVER, 1995).

Os macrófagos desempenham um papel de suma importância no controle da infecção por fungos como o *Blastomyces dermatitidis, Candida albicans* e *Paracoccidioides brasiliensis*, sendo que apenas macrófagos ativados conseguem fagocitar e digerir as células fúngicas (BRUMMER & STEVENS, 1987; BRUMMER *et al,* 1988; BRUMMER *et al,* 1990). Macrófagos de pacientes com PCM quando estimulados in vitro com IFN-γ fagocitam e digerem eficientemente as células do fungo, o mesmo não acontecendo na ausência de estimulação (RODRIGUES *et al,* 1999). Da mesma forma na infecção experimental em camundongos, a administração de IFN-γ recombinante potencializa a fagocitose de células do *P. brasiliensis* impedindo o progresso da infecção (BRUMMER, MORRISON & STEVENS, 1985; BRUMMER & STEVENS, 1987; BRUMMER *et al,* 1988; BRUMMER *et al,* 1989; CANO *et al,* 1992a; CANO *et al,* 1992b). O IFN-γ ativa os macrófagos estimulando seu metabolismo oxidativo (NATHAN *et al,* 1983) e sua capacidade de produzir TNF-α (LAKE *et al,* 1994). De forma semelhante, os fagócitos polimorfonucleares são diretamente influenciados por citocinas como o IFN-γ (GOIHMAN-YAHR *et al,* 1980; GOIHMAN-YAHR *et al,* 1989, KURITA *et al,* 1999), GM-CSF e IL-1β ((KURITA *et al,* 2000), que atuam potencializando a resposta de ingestão e destruição das células fúngicas.

Em 1991 SILVA & FIGUEIREDO descreveram que pacientes com PCM apresentam níveis de TNF-α elevado no soro. Por outro lado, na infecção experimental, observou-se que camundongos inoculados com isolados avirulentos do *P. brasiliensis* produzem níveis de TNF-α muito maiores do que os encontrados por camundongos infectados por isolados virulentos, sugerindo que esta citocina possa ter um papel importante na modulação da resposta ao *P. brasiliensis* (FIGUEIREDO, ALVES & SILVA, 1993). Em trabalho complementar, SILVA *et al* (1995), observaram que além do TNF-α, a IL-1 e a IL-6 estão aumentadas no soro de pacientes com PCM disseminada. Recentemente FRANCO *et al* (1998), relacionaram a presença de TNF-α e TGF-β, à fibrose encontrada na PCM experimental. Essas duas citocinas também desempenham papel importante na leishmaniose, sendo o TNF-α associado à resistência à infecção, e o TGF-β à suscetibilidade à doença, devido a sua capacidade de inibir o metabolismo oxidativo dos macrófagos (BARRAL-NETO *et al*, 1991; BARRAL-NETO *et al*, 1992; CORRADIN *et al*, 1993). O TGF-β é produzido por diversos tipos de células, incluindo polimorfonucleares, macrófagos, linfócitos, sendo encontrado em grande quantidade em plaquetas e secretado em uma forma latente, cuja ativação *in vitro* pode ser feita com soluções de baixo pH. Apresenta uma enorme gama de funções, muitas delas antagônicas (MACCARTNEY-FRANCIS & WAHL, 1994). O TGF-β pode modular a expressão de moléculas de adesão e apresentar atividade quimiotática para células inflamatórias, atuando na migração celular. É associado a diversas desordens imunológicas como tumores, doenças autoimunes e à suscetibilidade a agentes infecciosos (LETTERIO & ROBERTS, 1998). Na tuberculose níveis elevados de TGF-β foram detectados em pacientes com formas mais severas e adiantadas da doença, estando relacionado ao desenvolvimento da fibrose característica dessa doença (TOOSSI *et al*, 1995; DLUGOVITZKY *et al*, 1999).

Pacientes com PCM apresentam resposta imune celular deprimida, demonstrada pela falta de reatividade em testes de hipersensibilidade tardia utilizando antígeno extraído do fungo, bem como através de testes in vitro, nos quais linfócitos apresentam proliferação diminuída frente à estimulação com mitógenos (PHA), antígenos de *Candida albicans* e de *P. brasiliensis* (MUSATTI *et al*, 1976). Em 1977, MOK & GREER em estudo semelhante demonstraram que a imunossupressão apresentada pelos pacientes estava relacionada à

gravidade e ao tempo de tratamento, havendo um restabelecimento parcial ou total das respostas após a cura clínica. Esses dados foram posteriormente confirmados por BENARD *et al* (1995), que também correlacionaram a diminuição da resposta celular com a forma clínica apresentada pelo paciente, encontrando maior supressão na FJ da PCM. MOTA *et al* (1988) e BAVA *et al* (1991) observaram a diminuição do número células T CD4+ e da relação CD4+/CD8+ no sangue periférico de pacientes com a forma mais grave da doença. Entretanto, a diminuição ocorreu de modo heterogêneo, não sendo observada em todos os indivíduos estudados.

Concomitante à supressão da resposta imune celular, pacientes com PCM apresentam ativação policlonal de células B, resultando na hipergamaglobulinemia característica da doença (CASTANEDA *et al*, 1988; CHEQUER-BOU-HABIB *et al*, 1989; BENARD *et al*, 1995; BENARD *et al*, 1996; BENARD *et al*, 1997). A resposta humoral na PCM é marcada pela elevada produção de imunoglobulinas. BIAGIONI *et al* (1984) demonstraram a ativação policlonal de células B através da dosagem de IgG anti-*P. brasiliensis* e da IgA e IgM total no soro de pacientes com PCM, encontrando correlação entre o aumento da produção de IgG específica e a gravidade da doença. YARZÁBAL *et al* (1980) observaram um aumento da síntese de IgE por pacientes com as formas mais severas da doença, dado confirmado por MENDES *et al* (1988), que além da IgE, encontraram um aumento de IgG4. De modo semelhante, a produção aumentada de IgE, também foi correlacionada com a gravidade da doença na coccidioidomicose (COX, BAKER & STEVENS, 1982).

A mudança de classe de imunoglobulinas (switch) produzidas pelas células B durante a resposta imune é controlada por citocinas. De modo geral a secreção de IgE e de IgG4 (IgG1 murina) é regulada pela IL-4, uma citocina produzida principalmente por células T, característica da resposta Th2 (SNAPPER, FINKELMAN & PAUL, 1988; DE KRUYFF *et al*, 1989; LUNDGREN *et al*, 1989; DE VRIES *et al*, 1991; GEHA, 1992; KING & NUTMAN, 1993). A IL-13, citocina estruturalmente relacionada à IL-4, também está envolvida no "switch" para IgE e IgG4 (PUNNONEN *et al*, 1993). Apesar da produção dessas imunoglobulinas ser controlada por mecanismos comuns, existem citocinas que atuam de modo seletivo, como a IL-12 (KINIWA *et al*, 1992; BOER *et al*, 1997), a IL-8

(KIMATA *et al*, 1992; KIMATA & LINDLEY, 1994) e a IL-10 (JEANNIN *et al*, 1998) que inibem seletivamente a secreção de IgE, não interferindo na secreção de IgG4. Por outro lado, a IL-5 (PENE *et al*, 1988) e a IL-6 (VERCELLI *et al*, 1989), parecem interagir sinergicamente com a IL-4, enquanto que o IFN-γ inibe a síntese de IgE e IgG4 e em camundongos estimula a síntese de IgG2a.

Recentemente BAIDA *et al* (1999), descreveram que pacientes com a FJ, produzem altos títulos de IgG específica para o *P. brasiliensis*, com predomínio da IgG4, enquanto que a IgG2 foi encontrada elevada em pacientes com a FA, que também apresentam níveis elevados de IgA específica. Paradoxalmente estudos recentes publicados por VAZ, SINGER-VERMES & CALICH (1998) e por KASHINO *et al* (2000) demonstraram que a secreção de IgA está aumentada em camundongos suscetíveis, que também produzem IgG2b, enquanto que camundongos resistentes produzem IgG2a. O controle da síntese de IgA está associado principalmente a duas citocinas: o TGF-β e a IL-5 (MURRAY *et al*, 1987; SONODA *et al*, 1989; DE FRANCE *et al*, 1992; VLASSELAER, PUNNONEN & DE VRIES, 1992; BAO *et al*, 1998), sendo que a IL-6 também atua de modo sinérgico (BEAGLEY *et al*, 1989; RAMSAY *et al*, 1994).

Eosinofilia periférica foi descrita em pacientes com PCM, principalmente naqueles com a FJ da doença (SHIKANAI-YASUDA *et al*, 1992). Os eosinófilos desempenham papel importante nas infecções causadas por helmintos, e são caracteristicamente associados à resposta alérgica. WAGNER *et al* (1998) observaram a presença de eosinófilos contendo grânulos de proteína básica principal (MBP) ao redor das células de *P. brasiliensis*, detectados em cortes histológicos de biópsias de mucosa oral e de pele de pacientes com PCM. A detecção da MBP secretada e depositada extracelularmente sobre os fungos indica uma possível participação de eosinófilos e seus produtos na fisiopatologia da PCM. Na esquistossomose os eosinófilos são responsáveis por grande parte das citocinas Th2 envolvidas na formação do granuloma (RUMBLEY *et al*, 1999). A ativação dos eosinófilos é controlada principalmente pela IL-5, que juntamente com a eotaxina atua na maturação e atração dessas células para o sítio de infecção (COLLINS *et al*, 1995; PALFRAMAN *et al*, 1998).

O desenvolvimento de modelos experimentais de resistência ou susceptibilidade à infecção pelo *P. brasiliensis* permitiu um avanço no conhecimento dos mecanismos imunológicos envolvidos na infecção (CALICH *et al*, 1985). A resistência apresentada pelos camundongos A/Sn é caracterizada pela presença de resposta celular preservada, pela produção de IFN-γ e de anticorpos da classe IgG2a. Esses animais apresentam resposta eficiente de defesa contra o fungo, impedindo sua disseminação (CANO *et al*, 1995). Mecanismos de resistência semelhantes são encontrados na infecção experimental por *Histoplasma capsulatum* (ZHOU *et al*, 1995) e por *Coccidioides imitis* (MAGEE & COX, 1996), nos quais a inoculação de IL-12 recombinante, citocina que estimula a produção de IFN-γ, resulta em maior resistência à infecção. Por outro lado, camundongos B.10 são suscetíveis à doença, apresentando deficiência da resposta celular e aumento da produção de anticorpos da classe IgG1 (equivalente a IgG4 humana), resultando na incapacidade de controlar a infecção e conseqüente disseminação do fungo para diversos órgãos. Além disso os camundongos B.10, apresentam maior produção de IL-4, compatível com resposta do tipo Th2 (CANO *et al*, 1995).

O perfil Th1, caracterizado pela produção preferencial de IFN-γ e IL-2, e Th2, cuja resposta é mediada por IL-4, IL-5 e IL-10, foi proposto por MOSMANN *et al* em 1986 e pode ser aplicado ao modelo experimental da infecção pelo *P. brasiliensis*, assim como acontece na leishmaniose (SCOTT *et al*, 1988; LIEW, HALE & HOWARD, 1982). O tipo de resposta imune desenvolvido pelo hospedeiro envolve uma série de fatores como as citocinas presentes no ambiente de diferenciação das células T, onde IFN-γ e IL-4 atuam de forma antagônica (GAJEWSKI, JOYCE & FITCH, 1989; MOSMANN & COFFMAN, 1989; PELEMAN *et al*, 1989; MARTINEZ *et al*, 1990; ROMAGNANI, 1991; MANETTI *et al*, 1993; PAUL & SEDER, 1994; O'GARRA & MURPHY, 1996), sinais coestimulatórios (ROCKEN *et al*, 1992; MCKNIGHT *et al*, 1994; KING *et al*, 1995; KUCHROO *et al*, 1995; LENSCHOW *et al*, 1995; THOMPSON, 1995), o tipo de antígeno apresentado (DEL PETRI *et al*, 1991) e também o tipo de célula apresentadora de antígeno (CHANG *et al*, 1990; GAJEWSKI *et al*, 1991). ALMEIDA *et al* (1998) demonstraram que em camundongos resistentes à infecção pelo *P. brasiliensis* as células apresentadoras de

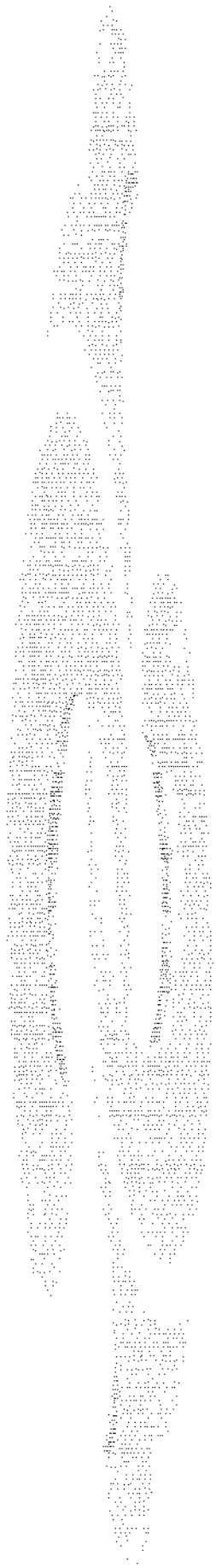
antígenos são preferencialmente macrófagos, enquanto que em camundongos suscetíveis a apresentação se dá por intermédio de células B.

O recrutamento das células do sistema imune para o sítio de infecção é controlado por citocinas com propriedades quimiotáticas denominadas quimiocinas. Estes mediadores são classificados em duas famílias principais dependendo da disposição dos dois primeiros resíduos de cisteína na molécula: as CXC-(α)-quimiocionas e as CC-(β)-quimiocinas. De modo geral as CXC-quimiocinas atraem neutrófilos e as CC-quimiocinas atuam sobre monócitos (TAUB *et al*, 1996; ROLLINS, 1997). A CXC-quimiocina mais bem estudada é a IL-8, que além de suas funções quimiotáticas sobre neutrófilos, linfócitos e eosinófilos, atua também sobre linfócitos B inibindo tanto a proliferação (KIMATA & LINDLEY, 1994), como a produção de IgE (KIMATA *et al*, 1992). LUKACS *et al* (1997), demonstraram que a CC-quimiocina MIP-1α atua sobre linfócitos Th2, estimulando sua proliferação, e ao mesmo tempo inibindo a produção de IL-4.

Recentemente foram detectados receptores específicos para quimiocinas em células Th1 ou Th2, demonstrando a importância destas proteínas no controle da resposta imune (BONECCHI *et al*, 1998; SALLUSTO, LANZAVECCHIA & MACKAY, 1998).

Analisados em conjunto, os dados disponíveis até o momento sugerem que, assim como na infecção experimental pelo *P. brasiliensis*, existe uma polarização da resposta imunológica também na doença humana, onde as formas mais severas apresentariam uma resposta Th2, enquanto que as formas mais brandas, assim como a melhora clínica dos indivíduos doentes estariam associadas a uma resposta do tipo Th1.

Com o intuito de gerar mais subsídios que contribuam para esta discussão, avaliamos a produção de mediadores séricos como anticorpos específicos ( IgG e subclasses, IgE e IgA), citocinas (TNF-α, IL-6, TGF-β) e quimiocinas (MIP-1α e IL-8), em pacientes com a FJ ou FA da PCM, em diferentes fases do tratamento. Os resultados obtidos acrescentam novas evidências à hipótese de que a forma juvenil da PCM representa o pólo Th2 da doença.



# *2. ARTIGO I*

**Enhanced Production of Specific IgG4, IgE, IgA and TGF-β in Sera from Patients with the Juvenile Form of Paracoccidioidomycosis**

**Running title: Serum mediators in paracoccidioidomycosis**

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**ABSTRACT**

Paracoccidioidomycosis (PCM) is a systemic, chronic disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*, which may be classified in two polar forms: acute or juvenile (JF) and chronic or adult form (AF). In order to further clarify this dichotomy, specific IgG, IgG subclasses, IgA, and IgE anti-gp43 were determined, by ELISA, in patients with both forms of PCM. The inflammatory response was evaluated by the detection of IL-6, TNF-α and TGF-β1 and the chemokines (IL-8 and MIP-1α), using a sandwich ELISA technique. We demonstrated that JF patients present significantly higher titers of IgE anti-gp43, the immunodominant and specific molecule of *P. brasiliensis*. Moreover, specific IgE levels strongly correlated with IgG4, IgA and eosinophilia. Increased levels of TGF-β (a switching factor for IgA) was also detected in sera from JF patients. In opposition, patients with AF were characterized by significantly higher levels of IgG1, lower levels of the other isotypes and lower number of eosinophils. The evaluation of the serum concentrations of IL-6, TNF-α, and MIP-1α did not distinguish the JF from the AF of the PCM, either before or after treatment. However, higher levels of these cytokines were detected in patients with disseminated AF compared with localized AF. The levels of IL-8 in patients with the JF of the disease were significantly lower than among AF patients. Taken together, the predominance of specific IgG4, IgE, and IgA antibodies, the associated eosinophilia and decreased levels of IL-8 are strong evidence for a Th2 type of response in the juvenile form of PCM.

## INTRODUCTION

Paracoccidioidomycosis (PCM), caused by the fungus *Paracoccidioides brasiliensis*, is the most prevalent deep-seated mycosis restricted to Latin America, with high incidence in Brazil. The greatest frequency of PCM is observed in previously healthy individuals between 30 and 50 years, being uncommon in childhood and relatively infrequent around puberty [7,14]. A wide spectrum of clinical manifestations has been described ranging from discrete pulmonary lesions to severe disseminated forms. According to the current classification, PCM may be considered a disease with two polar forms: Acute or Juvenile form (JF) and Chronic or Adult form (AF). The chronic progressive form almost always affects adult males, who show a high frequency of pulmonary, skin and visceral involvement. In clear contrast with the adult type, the juvenile type equally affects young patients of both sexes. The acute form is characterized by systemic lymph node involvement, hepatosplenomegaly, and bone marrow dysfunction, resembling a lymphoproliferative disease. From the immunopathologic point of view, the JF is more frequently associated with the anergic pole and the AF with the hyperergic pole. The anergic pole is characterized by necrotizing lesions with abundant parasites, impairment of cell-mediated immunity, and high titers of circulating antibodies. In the hyperergic pole, the lesions are less disseminated, with tuberculoid granulomata and small numbers of parasites; cell-mediated immunity is better preserved and the levels of antibodies are low [14,16].

Cellular immunity plays an essential role in PCM, since the severity of the disease runs parallel to with high antibody levels and impairment of T-cell function [4,5,25,26]. Presently, it is well established that the resistance against the fungi is based on T cell, macrophage, and B cell activities that are known to be mediated by interferon-γ and other Th1 type of cytokines. Conversely, susceptibility has been linked to the preferential elicitation of cytokines of the Th2 type: IL-4, IL-5, IL-10 [24]. Experimental evidence [10,12] strongly suggests that in paracoccidioidomycosis, resistance and susceptibility may be associated with a Th1 or Th2 pattern of cytokines, respectively. Considering that the isotypes of the immunoglobulins are determined by the patterns of cytokines present in microenvironment where B cells are being activated [11,23,30], the detection of particular isotypes can be considered as indicators of the corresponding pattern of inductive cytokines.

In this study we have investigated the levels of specific anti-*P.brasiliensis* isotypes and other serum factors that could further clarify the main immunological mechanisms operating in the two clinical forms of human PCM.

## MATERIAL AND METHODS

**Sera:** Patients cared for at the University Clinical Hospital of UNICAMP, Campinas – SP, Brazil, were grouped according to the PCM clinical forms and time of treatment. The diagnosis of PCM was confirmed by the histopathological exams of biopsy, either skin, ganglionary or pulmonary, and also the finding of the fungus in scrapings of skin lesions, linfonode secretion or in the sputum (direct examination). We analyzed 17 sera from patients with the JF (10 males and 7 females, age: 5-33 years) and 32 sera from patients with AF (30 males and 2 females, age: 30-72 years) of PCM. For some experiments the AF patients were grouped according to the clinical symptoms in localized (unifocal) form (symptoms referred to a single organ) or disseminated (multifocal) form (lesions in more than one organ or system) [16]. Sera were obtained before the beginning, during and after treatment (serum 1, 2, and 3, respectively). The average time between the collection of the serum 1 and the serum 2 was 11 months (10-13 months) and, between the serum 2 and the serum 3, 13 months (12-14 months). Twenty-three sera from healthy individuals, same age range, were used as control.

***P. brasiliensis*-gp43 antigen:** Purified gp43 was obtained by affinity chromatography of the crude exoantigen of *P. brasiliensis* B-339 on Sepharose 4B-IgG (rabbit anti-gp43 IgG). The gp43 was eluted from this column with 0.1 M glycine-HCl, pH 2.8, immediately neutralized with 2M Tris, pH 9.0, concentrated in an Amicon 10K apparatus and analyzed by SDS-PAGE [28]. Protein content was measured by the method of Bradford.

**Immunodiffusion test:** Immunodiffusion assay was performed using a crude *P. brasiliensis* antigen as previously described [6].

**Enzyme linked immunosorbent assays for the determination of:**

**a) Specific IgG:** 96-well plates (Greiner, Kremsmünster, Austria) were coated with gp43 of *P. brasiliensis*, at 2μg/ml. The assay, was performed as described previously [6]. The IgG titer was established by the highest dilution of the serum with absorbance superior to a cutoff established with sera from the control group. The samples were run in duplicates. The

cut-off was determined using the mean plus 2 SD of the absorbance obtained with serum from 23 healthy individuals.

**b) IgG subclasses:** 96-well plates were coated with gp43 of *P. brasiliensis*, in 0.1 M carbonate buffer (pH 9.6), at 2µg/ml, overnight at 4°C. The plates were washed with PBS-0.05% Tween 20 (PBS-T). The remaining binding sites were blocked with PBS-T-5% non-fat dry milk (PBS-T-M) for 2h at 37°C. After washing 3 times with PBS-T, serum samples diluted at 1:50 were added in duplicates, and the plates were incubated for 2h at 37° C. The wells were washed as described above and the antibodies anti-subclasses (anti-human IgG1, IgG2, IgG3 or IgG4, Sigma, St. Louis, USA) were added. After a 1h-incubation at 37°C, the plates were washed and the sheep anti-mouse IgG-peroxidase (dilution 1:1000; Sigma) was added. After a 1h-incubation at 37°C and three washes with PBS-T, the substrate solution (*o*-phenylediamine in 0.1M citrate-phosphate buffer, pH 5.0; plus 0.03% $H_2O_2$) was added and the reaction was interrupted by the addition of 2N $H_2SO_4$. The optical densities were read in a ELISA reader (SLT Spectra, SLT Instruments, Austria) at 492 nm. Results were expressed as the absorbance index (AI), a numeric value calculated by dividing the net absorbance of each test serum by the net absorbance of a positive reference serum pool on each plate, multiplied by 100. The AI is an arbitrary value that is linearly related to the antibody concentration and allows the comparison of sera tested on different plates and in different experiments [17].

**c) Specific IgE and IgA:** 96-well plates were coated with anti-IgE or anti-IgA (Sigma) in 0.1 M carbonate buffer (pH 9.6), at 2µg/ml, overnight at 4°C. The plates were washed with PBS-T and the remaining binding sites were blocked with PBS- 10% fetal calf serum (PBS-FCS) for 1h at RT. After washing 3 times with PBS-T, serum samples, diluted at 1:21 for IgE and 1:25 for IgA determination, were added in duplicates, and the plates were incubated for 2h at 37° C. A positive reference serum pool was included in each plate. After washing 3 times with PBS-T, *P. brasiliensis* gp43 was added at 2µg/ml, for 1 hour at RT. The wells were washed as described above, treated for 1 h at RT with anti-gp43 mAb-peroxidase (dilution 1:350) and washed again. Substrate OPD was added and after color development, optical density readings were taken as described above. The results were expressed as absorbance index as for the subclasses.

**d) IL-6, TNF-α, IL-8 and MIP-1α:** The antibodies used for coating 96-well plates were MAB206 (anti-human IL-6), MAB210 (anti-human TNF-α), MAB208 (anti-human IL-8) and MAB670 (anti-human MIP-1α). Second-step biotynilated detection mAbs were, respectively: BAF206, BAF210, BAF208 and BAF270 (all from R&D Systems, Minneapolis, USA). The concentration of the cytokines in the samples was determined by the standard curve, obtained with recombinant cytokines. The minimum detection limits for IL-6, TNF-α, IL-8 and MIP-1α were 3.9, 7.8, 32 and 32 pg/ml, respectively.

**e) TGF-β:** To quantify the amount of TGF-β1 in the serum samples, a commercial ELISA kit (Amershan Pharmacia Biotech) was used. Samples were acid activated to release all TGF-β activity by treatment with 1 N HCl (30 min) and neutralization with 1 N NaOH/HEPES. The assay was sensitive to 4 pg/ml.

**Statistical methods:** In order to compare the variables between each phase of the treatment, for each clinical form, the Wilcoxon non-parametric test was used. Patients with the JF and AF of PCM as well as the control group were compared using the Kruskal-Wallis non-parametric test. The relationship between the parameters was examined using Spearman's rank correlation coefficients. Significance was defined as $p \leq 0.05$.

## RESULTS

Sera from patients with JF of the PCM, analyzed previously to treatment presented higher titers of antibodies detected by immunodifusion (variation from 1/16 to 1/1024), than sera from patients with the AF (1/1 to 1/256). The difference between the groups was significant (JF vs. AF, $p<0.0001$) (Figure 1, left). Using the ELISA technique for the detection of IgG anti-gp43 of *P. brasiliensis*, the difference observed between the two groups was also significant (JF vs. AF, $p=0.01$) (Figure 1, right). During treatment a decrease in the level of specific antibodies detected by ID and ELISA (total IgG) was observed. The initial levels of antibodies detected by ID in sera from patients with the JF were higher and decreased more rapidly. Using the ELISA, a high sensitivity assay, high titers of antibodies were detected, even after 3 years of treatment (data not shown).

In order to evaluate the IgG isotypes produced in response to the *P. brasiliensis* infection, sera were analyzed for the detection of IgG1, IgG2, IgG3 and IgG4. As shown in Fig. 2, patients with the JF of PCM produced more IgG4 than patients with the AF (AI: 130.7 vs. 15.5, $p<0.0001$). On the other hand, patients with the AF produced high levels of IgG1 anti-*P.brasiliensis* (196.2 vs. 124.6, $p<0.0001$) (Figure 2). In relation to the other subclasses (IgG2 and IgG3), no significant differences were observed between the two clinical forms.

The evaluation of IgE and IgA isotypes levels showed higher levels in sera of patients with the JF of PCM (IgE-AI: 90.0 vs. 16.3, $p<0.0001$ and IgA-AI: 92.9 vs. 35.1, $p<0.0001$). (Figure 3, left and right, respectively).

The number of peripheral blood eosinophils was also higher in patients with the JF compared with the AF of PCM (2.0 to 67%, median: 10,0 vs. 0 to 20.4%, median: 2.4, $p<0.0001$). There was a positive correlation between the levels of specific IgE and the number of peripheral blood eosinophils ($r=0.42$, $p=0.0075$, serum 1).

The follow up of the patients demonstrated that IgG1 levels dropped during treatment for both clinical forms of the disease. The same pattern was observed for IgE and IgA, referring to JF patients. The decrease of the number of peripheral blood eosinophils

during treatment was also very evident for patients with the JF of PCM. For the AF patients, who presented low levels of IgE and IgA since the beginning of infection, no differences were detected during treatment. Differently from IgG1, IgE and IgA, the levels of specific IgG4 remained high during the whole treatment (Figure 4).

When IgG4 levels were compared with IgG1, before treatment, a negative correlation was observed clearly showing two separate groups of patients: the JF with high levels of IgG4 and low levels of IgG1, and the AF, with low levels of IgG4 and high levels of IgG1 (Figure 5A). Seven patients of the AF group also presented high levels of specific IgG4 and IgE (Figures 5A and B) and corresponded to subjects with a disseminated and severe form of PCM. These results suggested that IgG4 and IgE isotypes can be used as a disease severity marker.

The IgE antibody response to the *P. brasiliensis* gp43 antigen positively correlated with the IgG4 ($r= 0.75$, $p<0.0001$) and IgA ($r=0.64$, $p<0.0001$) (Figure 5 B and C).

Considering the prominent production of IgA in sera of JF patients and based on studies showing that TGF-β is an IgA switch factor for human B cells, we next examined TGF-β levels in sera from patients with both clinical forms of PCM. Detectable levels of TGF-β were found in patients with both clinical forms of the disease as well as in the control group (Figure 6, left). However, patients with the JF produced significantly higher levels of TGF-β, in comparison with the AF ($p= 0.016$) and the control group ($p= 0.04$). A positive correlation was found between IgA and TGF-β ($r= 0.55$, $p <0.0001$).

The evaluation of the serum concentration of IL-6, TNF-α e MIP-1α did not show significant differences between the AF and the JF of PCM. The values obtained varied in a large scale, not allowing any correlation with the clinical forms (data not shown). Considering patients with the AF however, it was possible to detect differences between the disseminated and localized (focal) form of the disease, with higher levels of the inflammatory cytokines in the former (TNF-α: 136.4 vs. 4.0 pg/ml, $p=0.01$, IL-6: 65.1 vs. 22.3 pg/ml, $p=0.03$ and MIP-1α: 613.7 vs. 122.4 pg/ml, $p=0.03$, data not shown). In relation

to IL-8, a CXC chemokine described to inhibit IL-4 induced IgE and IgG4 production, lower levels were detected in sera of JF patients as compared with AF patients ($p=0.01$) and the control group ($p=0.001$) (Figure 6, right).

## DISCUSSION

In order to study the characteristics of the immune response in the PCM infection, we examined some parameters representative of Th1 and Th2 profiles, in the two clinical forms of the disease. In accordance with previous reports [9,2], in the present study we observed that all the patients presented anti-gp43 IgG antibodies before treatment, detected either by ID or by ELISA. However, the antibody levels were significantly higher in the juvenile form than in the adult form of PCM. In spite of the greater production, the effectiveness of the antibodies in the juvenile form in combating the fungus is probably impaired, considering the isotypes produced. In this study, we observed that IgG4 to *P. brasiliensis* reached higher levels in patients with JF in comparison to those with AF. Inversely, patients with AF presented higher levels of antibodies of the IgG1 subclass. However, in a recent paper, a significant increase of IgG2, instead of IgG1, was reported in AF patients [2]. Therefore, further studies will be needed to establish which IgG subclass better characterizes the adult chronic form of PCM.

The excellent correlation between IL-4 and help for IgE suggests that IgE production can be used as a good marker for the existence of a strong Th2-like response [11,30]. In the present study we were able to show that JF patients present significantly higher titers of IgE anti-gp43, the immunodominant and specific molecule of *P.brasiliensis* [6]. The gp43 is a glycoprotein of 43,000 Da isolated from the supernatant fluid of yeast cultures by affinity chromatography. This molecule specifically binds to the extracellular matrix protein laminin, leading to enhancement of fungal pathogenesis [22,34]. A follow up on the patients showed that while IgE antibodies consistently dropped in treated patients, the levels of IgG4 tended to remain stable. Our data are in agreement with previous observations concerning the association between high IgE levels and impairment of protective cell mediated immunity [1,18,37]. Less attention has been given to IgG4, and only very recently its occurrence was described in PCM by Baida et al [2]. These authors found anti-gp43 IgG4 antibodies in the sera in 100% of the patients with the JF, and in only 12% of those with the AF of PCM. In full agreement with these data, we also observed that all the patients with JF presented anti-*P.brasiliensis* antibodies of the IgG4 isotype, in levels significantly higher than those presented by patients with the AF. However, a group of 7

subjects with the AF, easily identified in Figure 2, produced levels of specific IgG4 equivalent to those seen in the JF group. These same patients presented high levels of IgE, as well as lower levels of IgG1, as can be seen in the right half of Figure 5. From the clinical point of view, these were patients with disseminated disease with lymph node involvement. This group is illustrative of the heterogeneity of the AF of PCM, ranging from isolated lesions in the respiratory tract to widely disseminated forms. Therefore, we conclude that IgG4 and IgE may be considered useful markers for the severity of the disease and impairment of the protective immunity.

Antibodies of the IgG4 isotype against some antigens are functionally monovalent and produce small non-precipitating immunecomplexes that do not fix complement [36] and, therefore, have a low potential for destroying pathogens. All the signals recognized to be involved in IgE switching "in vitro" also control IgG4, suggesting that IgE and IgG4 synthesis are regulated by common mechanisms [30,33]. However, there are many observations indicating that "in vivo" these isotypes may be independently regulated [8,19]. In chronic helminthic infections, IgG4 antibodies are the predominant IgG subclass and probably play an important role as "blocking antibodies" of IgE mediated response. In addition, the development of a potent and specific IgG4 response, during allergic desensitization, has been associated with a positive outcome [33,27]. Taking into account these observations, we hypothesize that specific IgG4 may present a regulatory role upon the IgE mediated response in PCM.

The most interesting observation of our study was the strong positive correlation between specific IgE levels and IgG4, IgA, and eosinophilia. We observed significantly higher numbers and percentages of peripheral eosinophils in JF compared to AF patients. The degree of eosinophilia was positively correlated with IgE levels and both parameters decreased with treatment. These results are in accordance with those of Benard et al [4], who showed an inverse correlation between eosinophil levels and T cell function, evaluated by the "in vitro" proliferative response to antigens from *P.brasiliensis*. In coccidioidomycosis, peripheral blood eosinophilia and microabscesses with large numbers of eosinophils, were related to the disseminated form of the disease and to a poor prognosis [15]. A recent paper suggested that eosinophils, through toxic granule proteins, could participate in the

pathophysiology of PCM [35]. These observations could be taken as evidence for IL-5 production, since the number of eosinophils in the circulation is directly influenced by this cytokine [29].

The synergistic effect of IL-4 and IL-5 in enhancing IgA secretion was previously described [23]. In addition, in experimental PCM a preferential secretion of specific IgA was associated with progressive disease in susceptible mice [20]. Accordingly, we and other authors [9] have detected higher IgA-specific antibodies levels in the juvenile form rather than in the adult form of PCM. Moreover, we have also detected increased levels of TGF-β in the sera of JF patients. TGF-β is the cytokine responsible for IgA switch [32], in addition to a variety of other immunologic effects, many of them paradoxical [13]. In leishmaniasis, endogenous TGF-β production correlates with susceptibility to infection and with the development of a nonhealing Th2-type response [3].

Silva et al [31] suggested a role for inflammatory cytokines in the genesis and control of PCM. These authors observed increased levels of TNF, IL-1, and IL-6 in the serum of adult PCM patients with disseminated disease, associated with low lymphoproliferative response and high antibody titers. In the present study, the evaluation of the serum concentrations of IL-6, TNF-α, and MIP-1α did not distinguish the JF from the AF of the PCM, either before or after treatment. However, higher levels of these cytokines were detected in patients with disseminated AF (lesions in two or more organs) compared with localized AF (lesion in only one organ). Interestingly, the levels of IL-8 in patients with the JF of the disease were significantly lower than among AF patients. This result may be considered as an additional indicator of the participation of a Th2 type response in JF. IL-8 modulates the IgE synthesis induced by IL-4 [21] and, therefore, a reduction in the production of IL-8 could contribute to a shift toward a Th2 response in the juvenile form of PCM. Its inhibitory effect on IgE production may represent a regulatory pathway.

In conclusion, the predominance of specific IgG4, IgE, and IgA antibodies and the associated eosinophilia are strong evidence for a Th2 type of response in the juvenile form of PCM. In contrast, patients with the adult form of the disease were characterized by high levels of IgG1, lower levels of the other immunoglobulin isotypes, and lower numbers

of eosinophils. A final confirmation that Th1 and Th2 patterns are related to AF and to JF, respectively, would be the assessment of the levels of the cytokines that characterize these patterns, a study that is presently being conducted by our group.

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through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. Infect. Immun. **62:** 1465-1469.

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**LEGENDS TO THE FIGURES:**

**Figure 1:** Detection of antibodies anti-*P. brasiliensis* by immunodiffusion (ID) and ELISA-IgG in sera from patients with adult form (AF) and juvenile form (JF) of PCM, before treatment and the control group (C). The values represent relative titers, in which the dilution of the serum was substituted by absolute numbers ID: 1=Not detected; 2= 1:1; 3= 1:2; 4= 1:4, 5= 1:8; etc. ELISA: 1= Not detected; 2= 1:400; 3= 1:800; 4= 1:1.600, etc. The horizontal bars represent the median. Kruskal-Wallis, * $p< 0.05$

**Figure 2:** Levels of IgG subclasses in patients with adult form (AF) and juvenile form (JF) of PCM, before treatment and in the control group (C). The results were expressed as absorbance index. The horizontal bars represent the median. Kruskal-Wallis, * $p<0,05$

**Figure 3:** Levels of specific IgE (left) and IgA (right) in sera of patients adult form (AF) and juvenile form (JF) of PCM, before treatment and in the control group (C). The results were expressed as absorbance index. The horizontal bars represent the median. Kruskal-Wallis, * $p <0,05$

**Figure 4:** Levels of specific IgG1, IgG4, IgE and IgA in consecutive sera from patients with the adult form (■) and the juvenile form (□) of PCM. Sera were obtained before the beginning (S1), during (S2) and after treatment (S3). The average time between the collection of sera was 12 months. Comparison between AF and JF: Kruskal-Wallis , * $p<0.05$. Significant differences between sera of the same patient: (Wilcoxon, $p< 0.05$): FA-IgG1: S1 vs. S2, S2 vs. S3, S1 vs. S3; FA-IgE: S1 vs. S2, S1 vs. S3; FA-IgA: S1 vs. S2, S1vs. S3; FJ-IgG1: S1 vs. S3; IgE: S1 vs. S3; IgA: S1 vs. S3. The results were expressed as absorbance index (median ± SD).

**Figure 5:** Correlation between IgG4 and IgG1 (A), IgG4 and IgE (B), IgA and IgE (C) levels in sera of patients with the adult form (□) and juvenile form (●) of PCM, before treatment. The results were expressed as absorbance index (AI). Spearman's rank correlation coefficient were: $r=-0.42$, $p<0.05$; $r= 0.75$, $p<0.05$; $r=0.64$, $p<0.05$, respectively.

**Figure 6:** Levels of TGF-β (left) and IL-8 (right) in sera from patients with the adult form (AF) and juvenile form (JF) of PCM and the control group (C). Kruskal-Wallis, *$p<0,05$. The horizontal bars represent the median.

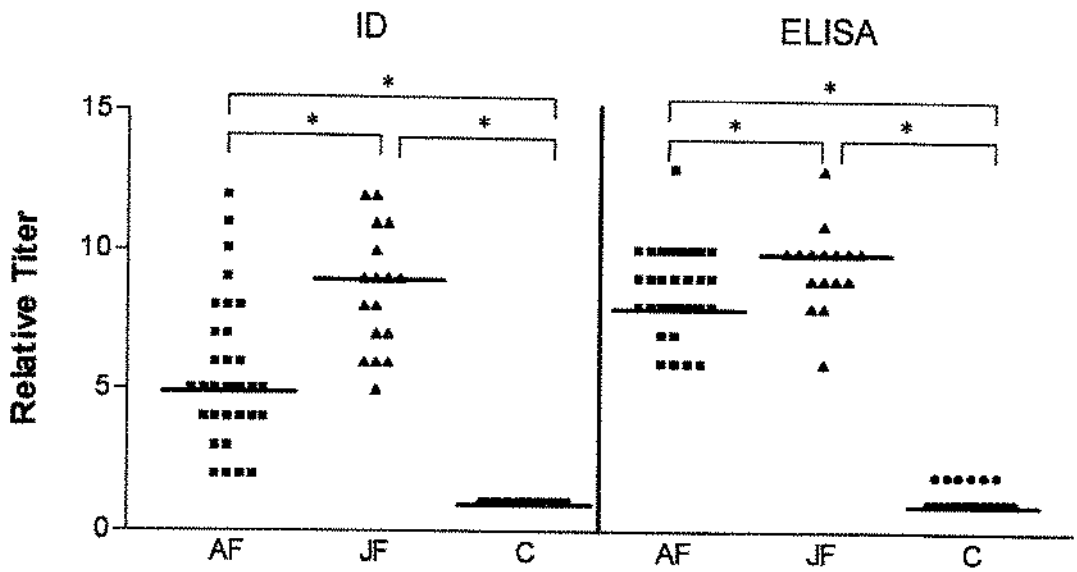



Figure 1

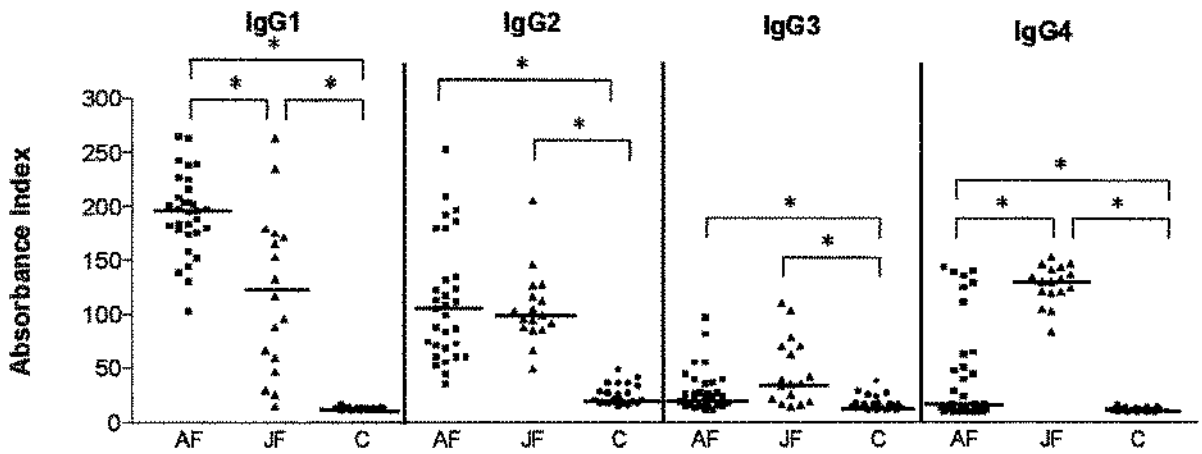

IgG1
IgG2
IgG3
IgG4
Absorbance Index
300
250
200
150
100
50
0
AF
JF
C
*

Figure 2

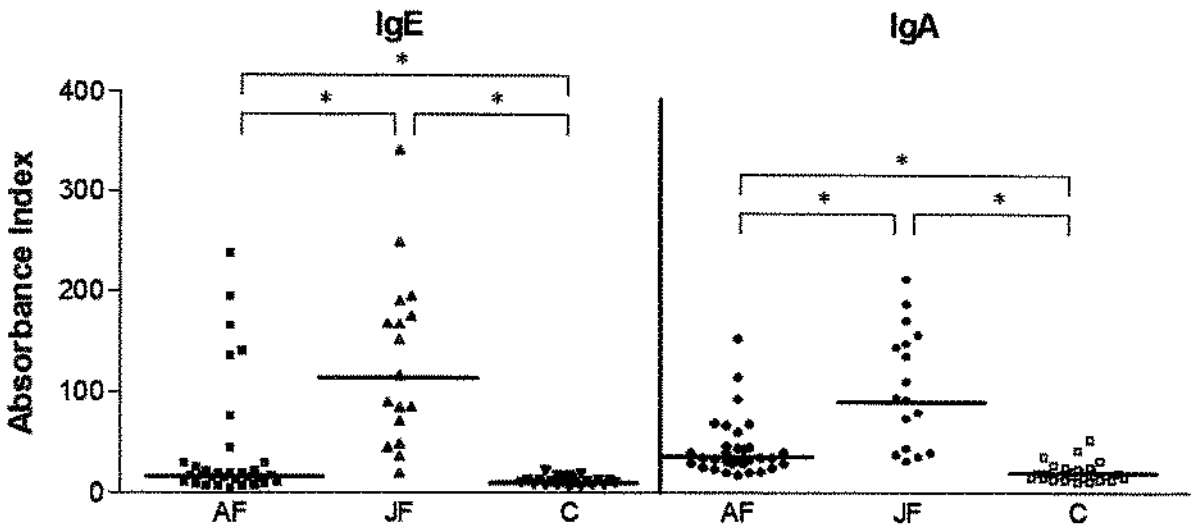



Figure 3

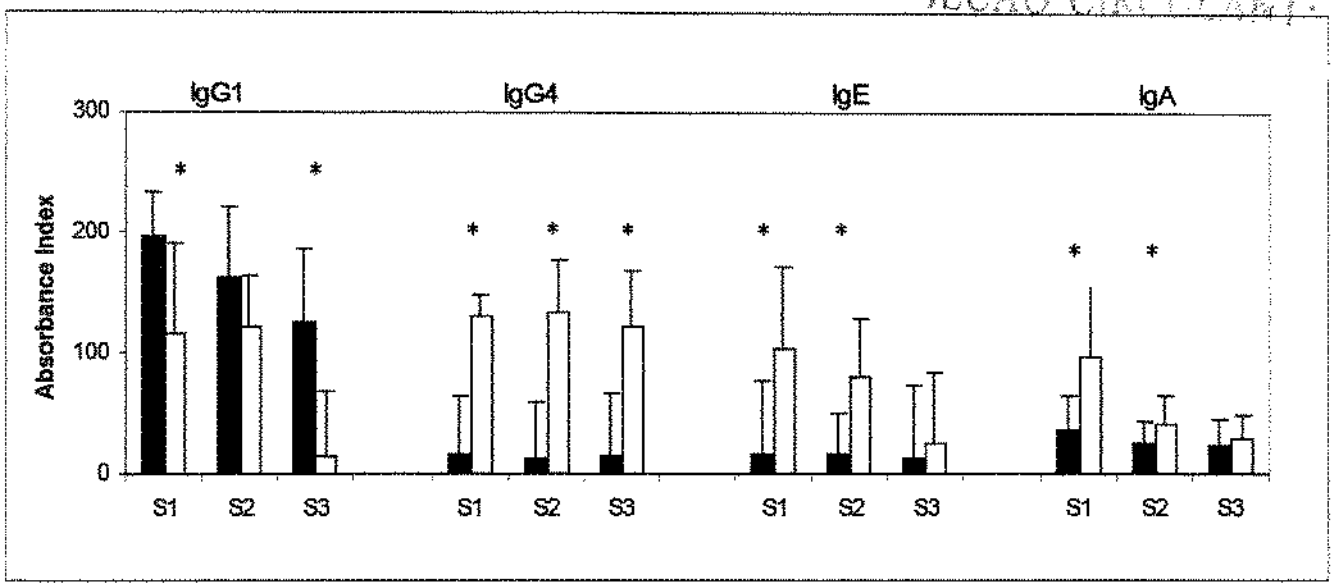
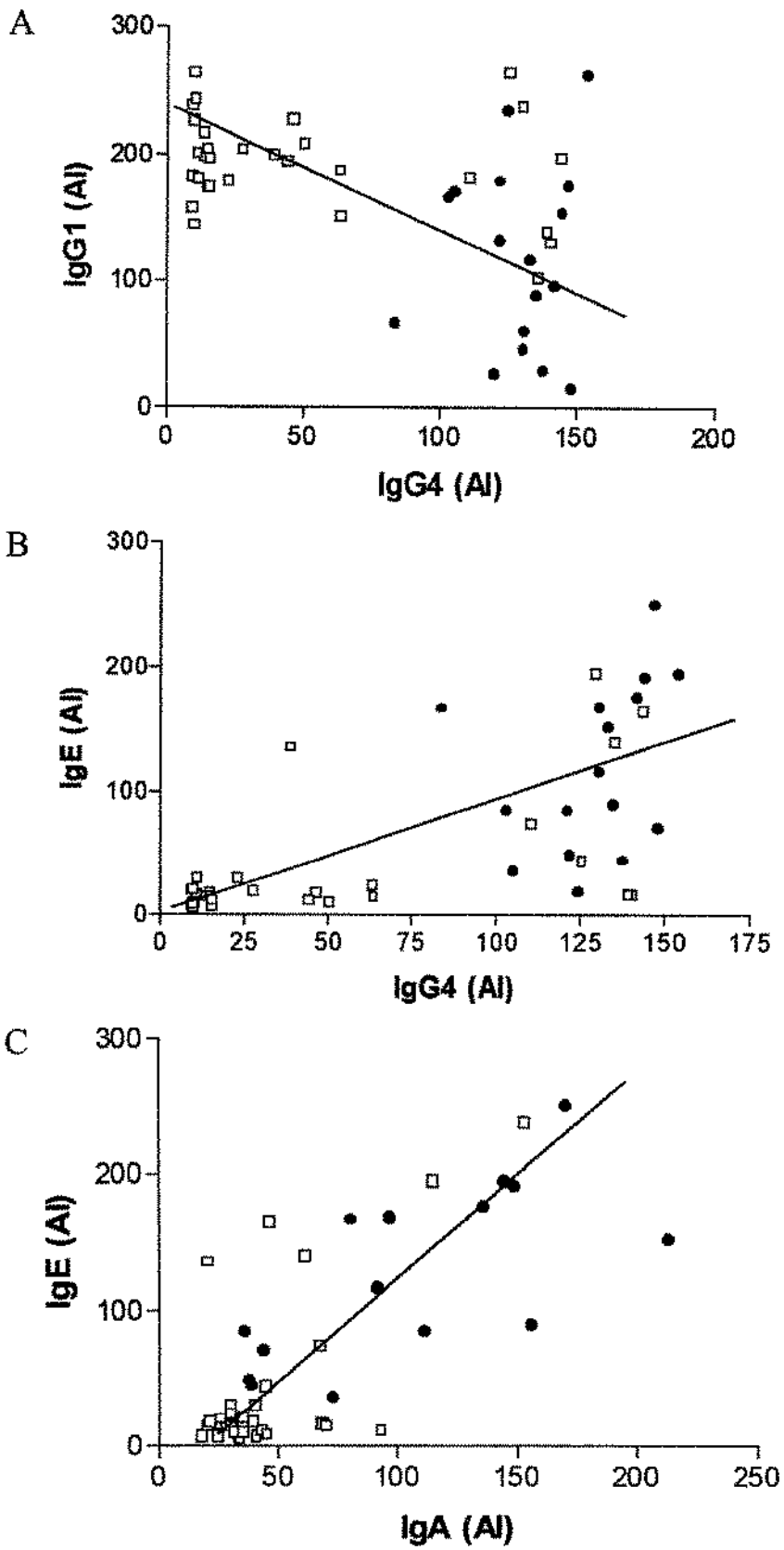



Figure 4


A
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IgG1 (AI)
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IgG4 (AI)
B
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IgE (AI)
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25
50
75
100
125
150
175
IgG4 (AI)
C
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IgE (AI)
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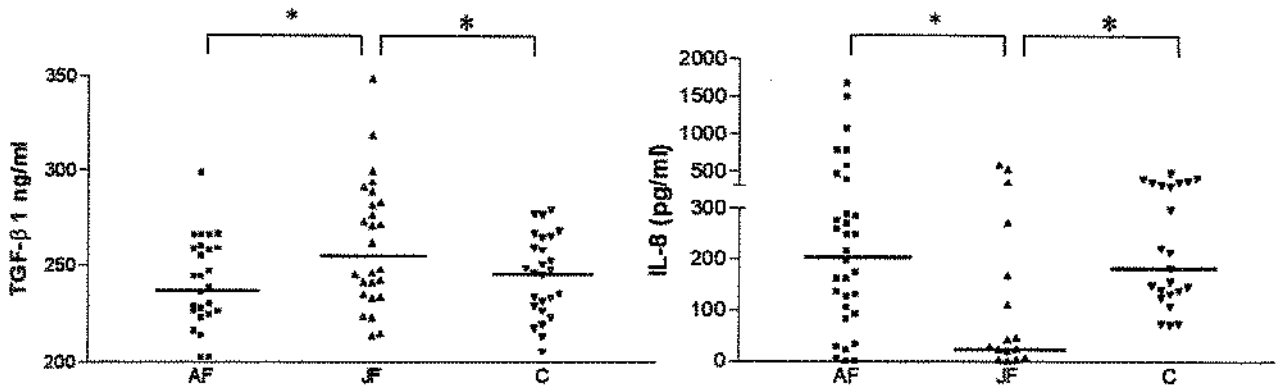
250
IgA (AI)

Figure 5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350
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TGF-β 1 ng/ml
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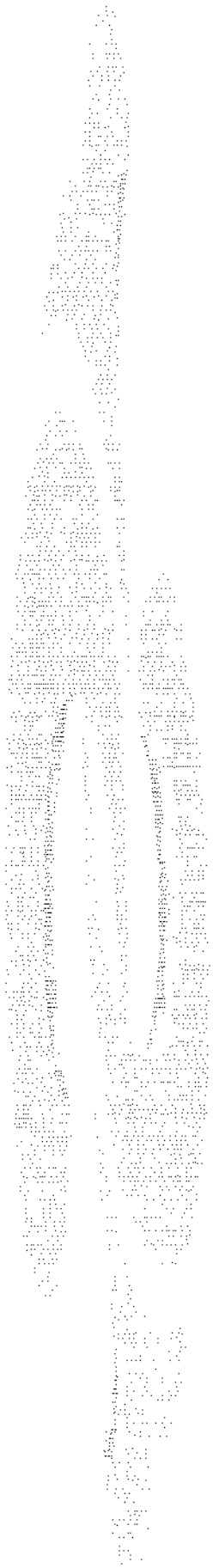
0
IL-8 (pg/ml)
AF
JF
C

Figure 6



# *3. ARTIGO II*

**A capture ELISA assay to detect specific IgE in sera of patients with paracoccidioidomycosis**

**Running head: Detection of *P. brasiliensis* IgE by capture ELISA**

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**ABSTRACT**

Paracoccidioidomycosis (PCM) is the most frequent systemic mycosis in South America. The disease is characterized by a polyclonal activation of B cells, resulting in hyperimmunoglobulinemia. The production of IgE in deep mycosis has been related to the severity of the disease. However, the detection of specific IgE in the serum of patients is difficult, due to the competition with the IgG. In the present work, we compared a capture and an indirect-ELISA technique to detect *P. brasiliensis*-IgE. We found that the capture-ELISA presented higher performance and lower background values than the indirect assay, resulting in a significant quantitative discrimination between sera from patients with the two major clinical forms of PCM. Patients with the juvenile form presented significantly higher levels of *P. brasiliensis*-IgE. The capture-ELISA was utilized to follow up patients under treatment, showing that the levels of specific IgE decreased as the patient's clinical conditions improved.

## INTRODUCTION

Helminth infections like atopic disorders show characteristic elevations of serum IgE.[1, 2] This condition has been associated with predominance of T-helper 2 (Th2) cells and IL-4 production over T-helper 1 (Th1) cells and IFN-γ secretion.[1, 3] Elevated serum IgE has also been found in patients with fungal infection like blastomycosis, coccidioidomycosis and paracoccidioidomycosis.[4, 5, 6] Experimental evidence strongly suggests that, in humans, paracoccidioidomycosis resistance and susceptibility may be associated with a Th1 or Th2 pattern of cytokines, respectively.[7, 8] Considering that the isotypes of the immunoglobulins are determined by the patterns of cytokines present in microenvironment where B cells are being activated, the detection of particular isotypes can be considered as indicators of the corresponding pattern of inductive cytokines.[9, 10, 11] The excellent correlation between IL-4 and help for IgE suggests that IgE production can be used as a good marker for the existence of a strong Th2-like response.[10, 11]

Paracoccidioidomycosis is characterized by a polyclonal activation of B cells resulting in high levels of serum IgG.[12, 13] The detection of antigen-specific IgE in diseases associated with polyclonal B cell activation and hypergammaglobulinemia is difficult due to the IgG antibody competition for the same epitopes.

In the indirect ELISA, the test antigen adsorbed in the solid phase binds specific antibodies of all isotypes in amounts proportional to their concentration in the test sample. Bound specific IgE is subsequently detected with a secondary antibody. The main limitations of the indirect ELISA are false negative or false-low IgE results due to competition with specific IgG for antigenic sites. This limitation may be overcome by the capture principle, as the selective binding of patient IgE eliminates IgG competition.

Here we described a simple and specific capture ELISA to detect IgE anti-*P. brasiliensis* gp43, and compared it with an indirect ELISA, by using serum specimens from patients with the juvenile and adult forms of paracoccidioidomycosis.

## MATERIAL AND METHODS

***P. brasiliensis*-gp43 antigen:** Purified gp43 was obtained by affinity chromatography of the crude exoantigen of *P. brasiliensis* B-339 on Sepharose 4B-IgG (mAb anti-gp43 IgG). The gp43 was eluted from this column with 0.1 M glycine-HCl, pH 2.8, immediately neutralized with 2M Tris, pH 9.0, concentrated in an Amicon 10K apparatus and analyzed by SDS-PAGE.[14] Protein content was measured by the method of Bradford.

**Serum specimens:** Patients with proven PCM cared for at the University Clinical Hospital of UNICAMP, Campinas – SP, Brazil, were classified into two groups according to the clinical forms: the acute or juvenile form (JF) and the chronic or adult form (AF).[15] Sera from 16 patients with the JF and from 16 patients with AF of PCM, obtained before the beginning of the treatment were analyzed. The diagnosis was confirmed by the histopathological exams of biopsy, tegumentary, ganglionary or pulmonary and also by the finding of the fungus in scrapings of tegumentary lesions, linfonode secretion or in the sputum (direct examination). Serological tests were positive in all patients with titers ranging from 1/2 to 1/4,096 for immunodiffusion and 1/800 to 1/409,000 for ELISA (IgG). Fifteen sera from healthy individuals were used as control. For some experiments we used consecutive sera from 6 patients of each clinical form obtained before, during and after treatment.

**Indirect ELISA:** 96-well polystyrene flat-bottomed plates (Greiner, Kremsmünster, Austria) were coated with *P. brasiliensis* gp43 at 2µg/mL in 0.1% carbonate buffer and incubated for 2 hours at room temperature (RT) and overnight at 4°C. The plates were washed once with PBS containing 0.1% Tween 20 (PBS-T) and the remaining sites were blocked with PBS-T containing 10% fetal calf serum for 1 h at RT. After 2 washings with PBS-T, the serum samples diluted 1/20 in PBS-T containing 0.25% gelatin (PBS-T-G) were added in duplicates and the plates were incubated for 2 h at 37°C. The wells were washed as described above and mouse anti-human anti IgE (Sigma, St Louis, USA) was added at 2µg/mL in PBS-T-G. The plates were incubated 1 h at 37° C and then washed again before receiving the conjugate (anti-mouse IgG-peroxidase, Sigma) at 1:1000. After 1h-incubation at 37°C, the plates were washed 3 times and then, 100 µl of substrate solution (5 mg of O-

phenylenediamine in 10 ml of 0.1 M citrate-phosphate buffer, pH 5.0, plus 10 µl of 30% $H_2O_2$) was added to each well. After color development the reaction was interrupted by the addition of 50 µl of 4N $H_2SO_4$. The optical densities were read in an ELISA reader (SLT Spectra, SLT Instruments, Austria) at 492 nm.

**Indirect ELISA with RF Absorbent-treated sera (RF-Indirect):** The same protocol above was used except that the serum samples were previously treated with RF-Absorbent (Behring Diagnostic, Marburg, Germany). Serum samples were diluted 1:10 in PBS-T-G, combined 1:1 to the RF-Absorbent, mixed well and incubated for 10 min at RT. The mixture was then added directly in the ELISA plate (100 µl/well).

**Anti-gp43 mAb conjugate:** Monoclonal antibody anti-gp43 was conjugated with peroxidase (Sigma, St. Louis, USA), and used at 1:350.[16]

**Capture ELISA:** 96-well plates were coated with 100 µl of mouse anti-human IgE in 0.1 M carbonate buffer (pH 9.6), at 2µg/ml, overnight at 4°C. The plates were washed with PBS-T and the remaining binding sites were blocked with PBS- 10% fetal calf serum (PBS-FCS) for 1h at RT. After washing 3 times with PBS-T, serum samples, diluted at 1:20, were added in duplicates, and incubated for 2 h at 37° C, and plates washed. *P. brasiliensis* gp43 (2µg/ml) was added to the wells, for 1 h at RT. The wells were washed again and anti-gp43 mAb-peroxidase (1:350 dilution) was added for 1 h at RT, and washed again. OPD substrate was added and after color development, optical density readings were taken as described above.

**Statistical analysis:** In order to compare the variables among the ELISAs tests and the phases of treatment for each clinical form, the Wilcoxon non-parametric test was used. Patients with the JF and AF of PCM were compared using the Mann-Whitney non-parametric test. Significance was defined as $p \leq 0.05$.

## RESULTS

In order to detect specific IgE antibodies to *P.brasiliensis* gp43 in sera from patients with PCM we compared two types of ELISAs: indirect and capture. Initially a dose response test was performed by examining a serial twofold dilution of a pool consisting of 4 sera from patients with proven PCM, not considering the clinical form. One negative serum from a healthy donor was used as control. The indirect ELISA was performed with unabsorbed sera (ELISA) and serum samples treated with RF-absorbent (RF-ELISA). The RF-Absorbent is currently used to remove IgM rheumatoid factors from samples of serum or plasma before they are used in a test for specific IgM antibodies by the indirect method (ELISA or Immunofluorescence). It is an anti-human IgG-antibody preparation that is directed against IgG, forming immune complexes to which the IgM-RF is bound and thus removed. Since any pathogen-specific IgG present is also removed by the action of RF-Absorbent, competition between specific IgG and IgM (or other isotypes), which may be of importance in some tests, is also minimized.

As can be seen in Figure 1, using the indirect assay (ELISA and RF-ELISA) and the capture assay (cap-ELISA), the absorbances decreased with the increasing serum dilution. The capture technique yielded much higher OD signals than indirect ELISA, even when RF-Absorbent treated serum samples were used. For the following experiments we chose the 1/20 dilution, since at this point the ELISA techniques showed the highest differences in the absorbance values

Next, we compared sera from patients with the JF and AF of PCM. The indirect ELISA resulted in high background (OD: 0.326) and only low levels of IgE were detected for both clinical forms (Figure 2: A and B). The background of the indirect assay using RF-treated serum samples was also high (0.353) and although higher levels were detected as compared with the test performed with non treated samples, no major improvement was observed. The capture ELISA, in which plates were coated with anti-IgE selecting only this isotype, enable the detection of higher levels of specific IgE, mainly in the JF patients. Using this technique the background was very low (OD: 0.076). The capture ELISA

produced higher OD signals than indirect ELISA, resulting in a significant quantitative discrimination between samples from JF and AF patients.

Figure 3 shows the results of the specific IgE detection in consecutive serum samples from 6 patients with the JF and 6 patients with the AF of PCM. The patients were followed for two years while receiving antifungal treatment. The IgE levels decreased significantly for patients with the JF. No differences were observed for patients with the AF, considering they had presented low levels of IgE since the beginning of the treatment. The differences between JF and AF were significant considering serum 1 and 2.

## DISCUSSION

A major problem in most studies of the IgE response in infections is a lack of a simple, sensitive technique to detect specific IgE antibodies. Many times the presence of excessive amounts of specific antibodies of the IgG class lead to competitive inhibition of IgE binding to antigenic determinants in indirect type of assays, such as indirect ELISA. This would greatly limit the sensitivity of the technique. The use of an antibody capture technique solves this problem since antibodies of the IgE class are separated from other classes of antibodies before the incubation with the antigen. Therefore competition with other classes of antibody is avoided.

The RF-absorbent is frequently used to remove the IgM rheumatoid factors from samples of serum before they are used in a test for specific IgM antibodies detection. Additionally it removes the excess of IgG present in the samples. Souza -Atta et al (1999) related that the use of RF-absorbent permitted the detection of specific IgE in sera from patients with visceral leishmaniasis and schistosomiasis.[17] In the present study, the treatment of the serum samples with RF-absorbent was not enough to improve the IgE detection.

Since the introduction of the μ-capture ELISA in 1978, this technique has shown to be very useful for the detection of specific IgM.[18] More recently the capture principle has been applied for the detection of other immunoglobulin isotypes such as IgE [19, 20] and IgA.[21, 22] The binding of a representative part of the total IgE and subsequent detection of anti- *P. brasiliensis* IgE reflects the relative amount of specific versus total IgE, and not the absolute concentration of specific IgE as measured by the indirect ELISA. An increase in the relative amount of specific IgE seemed to be a more sensitive parameter than an increase in the absolute concentration of specific IgE in serum measured by an indirect ELISA.[23]

Another relevant point is the antigen used in the immunoassay. The *P. brasiliensis* gp43 is the immunodominant antigen, recognized by the majority of sera (IgG) from patients with PCM.[24] This molecule, isolated from the supernatant fluid of yeast cultures by affinity chromatography, specifically binds to the extracellular matrix protein laminin, leading to enhancement of fungal pathogenesis.[25, 26]

In general, the juvenile form of PCM is more severe and disseminated than the adult form. In the present study, we showed that JF patients present significantly higher titers of IgE anti-gp43. Therefore, these results might give support to the suggestion that a relationship exists between the onset and the magnitude of the anti-fungus IgE and the severity of disease symptoms, as has been observed by others.[27,28]

Finally, the IgE capture ELISA was suitable for monitoring the specific IgE in consecutive samples. A follow up on the patients showed that IgE antibodies consistently dropped in patients with the JF during treatment. On the other hand, for AF patients the IgE levels were low and constant during the whole treatment.

It is tempting to speculate on the significance of the *P.brasiliensis*-IgE response and its possible role in immunological defense or fungus pathogenesis. However, further studies are required to define more clearly the IgE response in patients with different clinical manifestations of PCM. The method described in this report is simple and easy to perform and permits carrying out these investigations.

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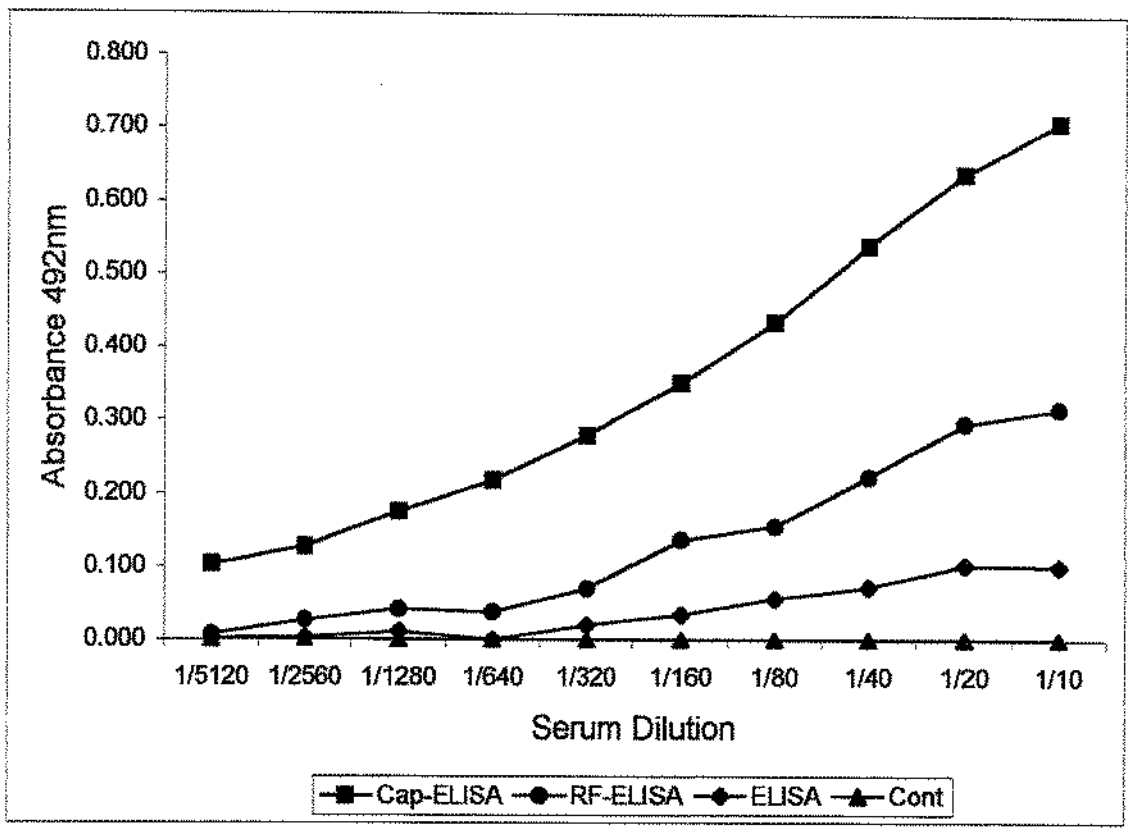
## LEGENDS TO THE FIGURES

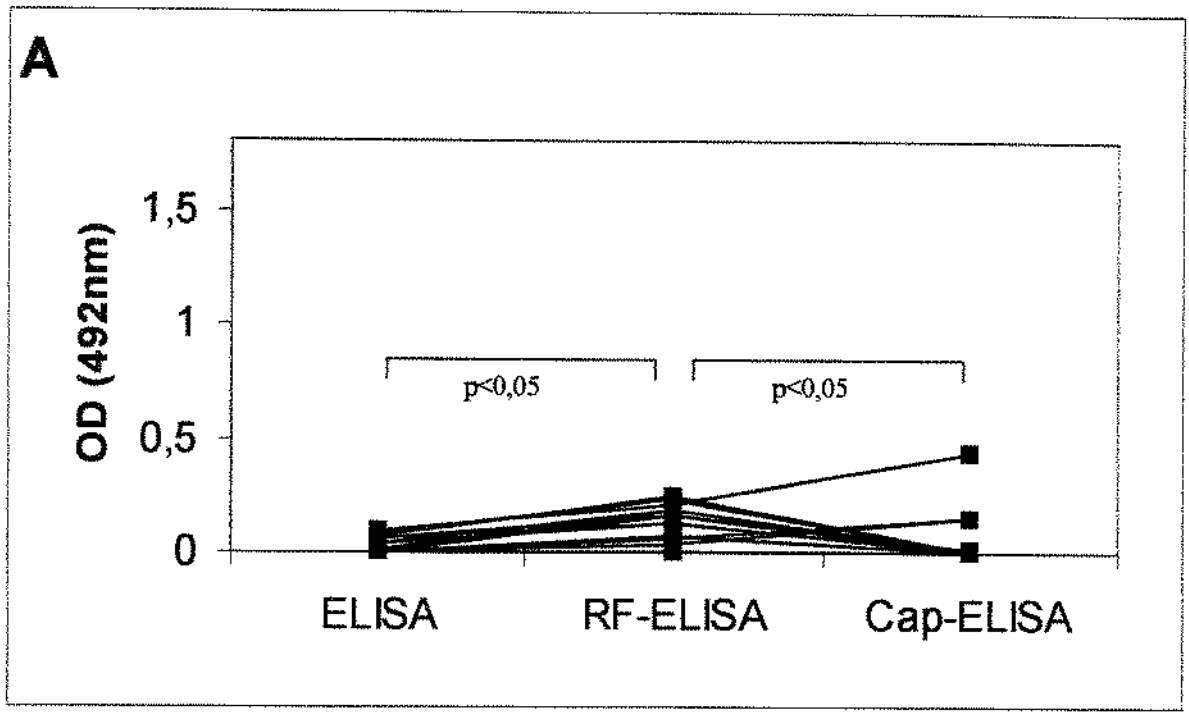
**Figure 1**: Detection of IgE antibodies to *P. brasiliensis* gp43 in a pool of 4 sera from patients with PCM, using the indirect technique (◆), indirect ELISA with RF treated serum samples (●) and Capture-ELISA (■). One negative serum from a healthy donor was used as control (▲). The sera were diluted from 1/10 to 1/5120.

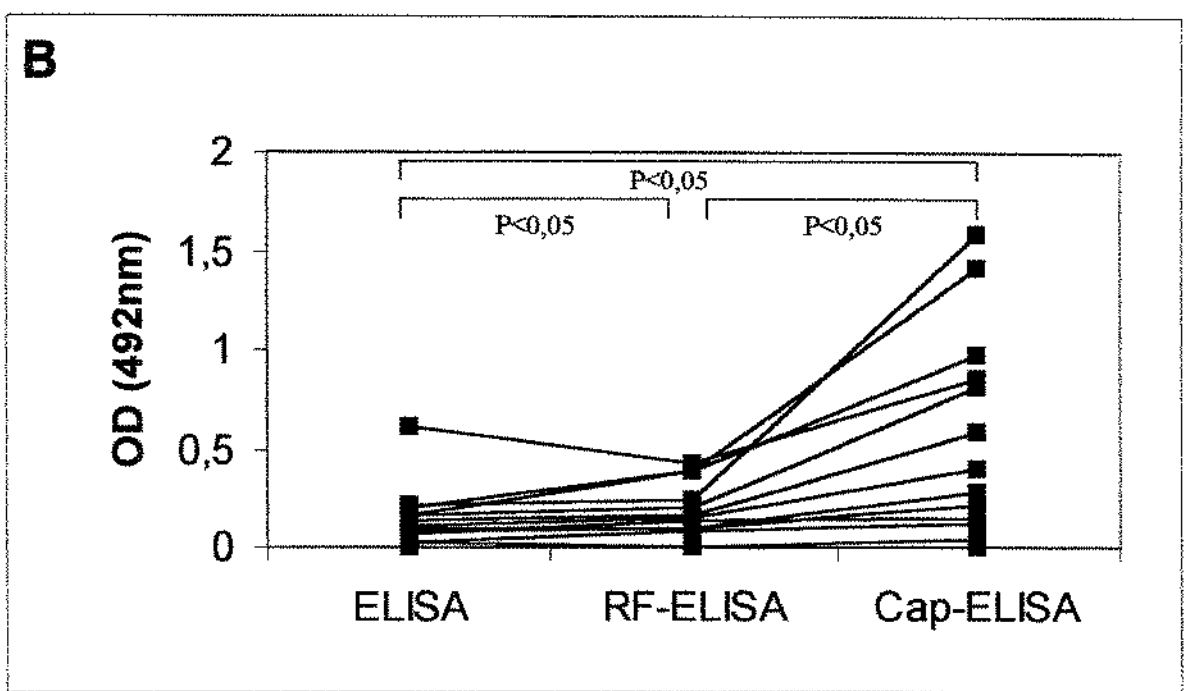
**Figure 2:** Detection of IgE antibodies to *P. brasiliensis* gp43 in sera from 16 patients with the Adult Form (A) and 16 patients with the Juvenile Form (B) of PCM, analyzed by an indirect ELISA, indirect ELISA with RF-treated serum samples and Capture-ELISA. A) ELISA vs. RF-ELISA: $p<0,05$; RF-ELISA vs. Cap-ELISA: $p<0,05$. B) ELISA vs. RF-ELISA: $p<0,05$; ELISA vs. Cap-ELISA: $p<0,05$; RF-ELISA vs. Cap-ELISA: $p<0,05$.

**Figure 3:** Detection of IgE antibodies to *P. brasiliensis* gp43 in consecutive sera from patients with the Juvenile Form (JF) of PCM and control group (C), analyzed by Capture-ELISA. Sera were obtained before the beginning, during and after treatment (serum 1, 2, and 3, respectively). The average time between the collection of the serum 1 and the serum 2 was 11 months and, between the serum 2 and the serum 3, 13 months. Comparison between sera: A) NS. B) S1 vs. S2: $p=0,0156$, S1 vs. S3: $p=0,0156$, S2 vs. S3: $p=0,02$ (Wilcoxon). Comparison between clinical form: FJ-S1 vs. FA-S1: $p=0,0006$, FJ-S2 vs. FA-S2: $p=0,0175$, FJ-S3 vs. FA-S3:$p=0,7104$ (NS) (Mann-Whitney).




**Mamoni et al, Figure 1**

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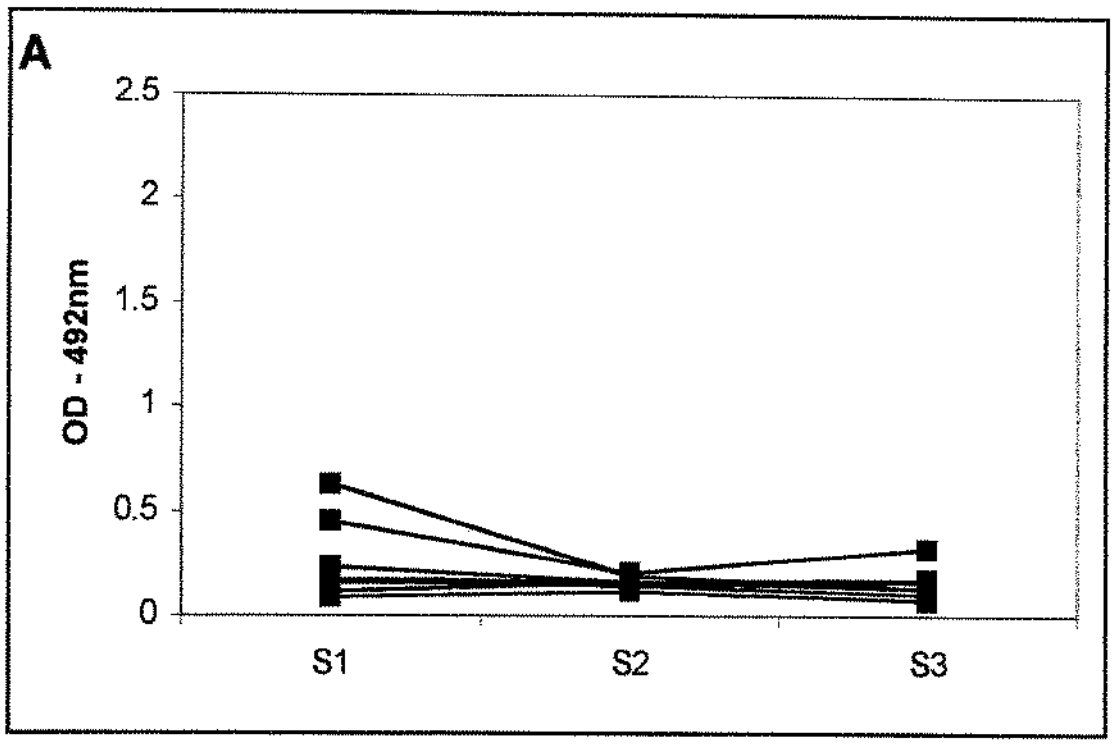


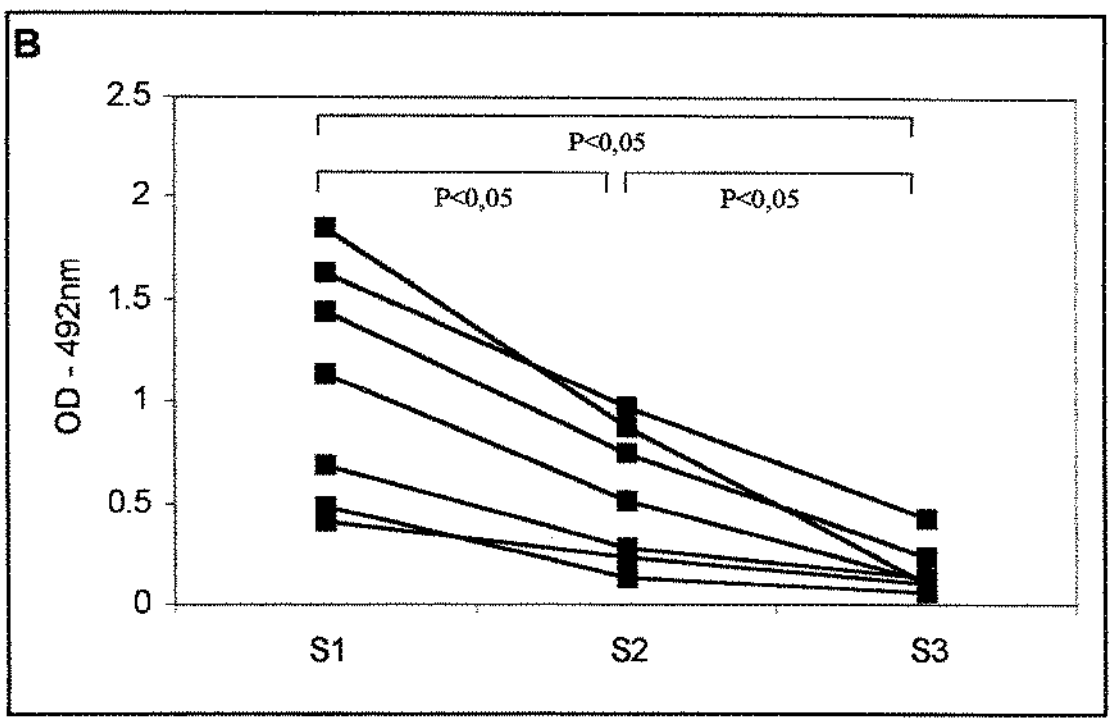


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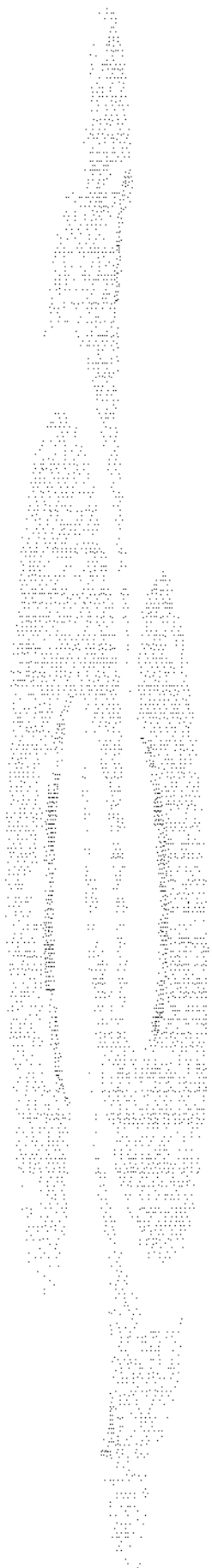


# *4. DISCUSSÃO*

Com o intuito de melhor caracterizar a resposta imune na infecção humana pelo *P. brasiliensis*, foram analisados alguns parâmetros séricos em pacientes com as duas formas clínicas principais da doença. Confirmando trabalhos anteriores (BUENO *et al*, 1997; BAIDA *et al*, 1999), observamos que pacientes com a FJ da PCM produzem níveis elevados de anticorpos IgG anti-gp43, detectados por IDR ou por ELISA, no início do tratamento. Apesar da maior produção de anticorpos, pacientes com a FJ apresentam uma infecção mais severa, demonstrando que esses anticorpos são aparentemente incapazes de controlar a infecção. Uma das explicações para este fato seria a composição dos isotipos produzidos em resposta ao fungo. Observamos que os pacientes com a FJ apresentam produção aumentada de IgG4, enquanto que os pacientes com a FA apresentam níveis maiores de IgG1. Anticorpos do isotipo IgG4 são funcionalmente monovalentes e produzem imunocomplexos pequenos, não-precipitantes, e não fixam complemento apresentando baixo potencial para a destruição de patógenos (VAN DER ZEE, VAN SWIETEN & AALBERSE, 1986; XU, OOMEN & KLEIN, 1994). BAIDA *et al* (1999) observaram o aumento de IgG2 ao invés de IgG1 em pacientes com a FA. Esses resultados conflitantes poderiam ser explicados pelo amplo espectro clínico apresentado pelos pacientes com a FA da PCM.

Neste trabalho foi demonstrado que pacientes com a FJ da PCM apresentam níveis significantemente aumentados de IgE específica anti-gp43 de *P. brasiliensis*. Como a síntese de IgE é diretamente influenciada pela IL-4, a predominância deste isotipo no soro de pacientes com a FJ da PCM é um forte indício da existência de uma resposta do tipo Th2 (CALLARD & TURNER, 1990; ROMAGNANI, 1997). Vários trabalhos demonstram a associação entre níveis elevados de IgE e deficiência na resposta imune mediada por células (ARANGO & YARZABAL, 1982; YARZABAL *et al*, 1990; HOSTETLER *et al*, 1993). Em nosso trabalho, o acompanhamento dos pacientes mostrou que os níveis de IgE específica caem durante o tratamento, enquanto que os níveis de IgG4 permanecem mais estáveis, principalmente nos pacientes com a FJ. Recentemente BAIDA *et al* (1999) relataram a predominância de anticorpos do tipo IgG4 anti-gp43 no soro de pacientes com a FJ da PCM. De modo semelhante, nosso grupo de pacientes com a FJ também apresentou níveis significantemente maiores de IgG4 do que pacientes com a FA. Entretanto, em 7 pacientes com a FA, foram detectados níveis de IgG4 comparáveis aos pacientes com a FJ,

assim como níveis de IgE elevados e baixos níveis de IgG1. Do ponto de vista clínico, tais pacientes apresentavam a forma disseminada da doença, todos com o envolvimento de linfonodos. Estes achados indicam que a IgG4 e a IgE podem ser utilizadas como marcadores da severidade da doença e deficiência da resposta imune protetora.

Estudos in vitro indicam que o controle da produção de IgG4 e IgE ocorre através de mecanismos comuns (ROMAGNANI, 1997; VERCELLI *et al,* 1998), mostrando a forte influência da IL-4 e IL-13. Entretanto, in vivo, a síntese destes isotipos, pode ter regulação independente, com o envolvimento de outras citocinas (BOER *et al*, 1997; JEANNIN *et al*, 1998). Em infecções crônicas por helmintos, anticorpos IgG4 são predominantes, e desempenham papel importante como moduladores da resposta de defesa mediada por IgE. Na resposta alérgica, a produção de IgG4 tem sido associada a um bom prognóstico, sendo o principal isotipo produzido na imunoterapia de dessensibilização a alérgenos (VERCELLI *et al*, 1998; OEHLING, SANZ & RESANO, 1998). Portanto, mais do que um papel de defesa, a IgG4 também deve ter um papel regulador sobre a resposta mediada pela IgE na PCM.

Em nosso trabalho demonstramos que existe correlação significativa entre os níveis de IgE específica, IgG4, IgA e número de eosinófilos no sangue periférico. A participação de eosinófilos como células de defesa em infecções por helmintos é bem estudada. Na coccidioidomicose, a presença de eosinofilia periférica e de tecidos apresentando microabcessos contendo eosinófilos foi associada a forma disseminada da doença e pior prognóstico (ECHOLS, PALMER & LONG, 1982). Nos pacientes estudados, verificamos número aumentado de eosinófilos no sangue periférico, principalmente na forma juvenil. O grau de eosinofilia correlacionou-se significativamente com os níveis de IgE específica, sendo que ambos decrescem com o tratamento. Estes resultados confirmam os achados de BENARD *et al* (1996), que demonstraram uma correlação inversa entre o número de eosinófilos e a resposta mediada por células T, avaliada pela resposta proliferativa in vitro a antígenos do *P. brasiliensis*. Em relato recente, WAGNER *et al*, 1998 sugerem que eosinófilos, através da secreção de proteínas tóxicas, podem participar da destruição de células de *P. brasiliensis* em sítios inflamatórios. Como a ativação e migração de eosinófilos são eventos diretamente influenciados pela produção de IL-5 por células T

CD4+, a presença de elevado número destas células na circulação de pacientes com a FJ da PCM é mais uma evidência indireta da participação preferencial da resposta Th2 nesta forma clínica da doença (ROBOZ & RAFII, 1999).

Confirmando os achados de BUENO *et al* (1997) encontramos níveis maiores de IgA específica em pacientes com a FJ, quando comparados aos pacientes com a FA. Na PCM experimental a secreção preferencial de IgA foi associada à progressão da doença em camundongos suscetíveis (KASHINO *et al*, 2000). Em nosso trabalho, encontramos correlação positiva entre os níveis séricos de IgA e TGF-β, uma das citocinas que participam do processo de "switch" para esta classe de imunoglobulina, atuando em conjunto com a IL-5 (SONODA *et al*, 1992), e com a IL-4 (MCINTYRE, KEHRY & SNAPPER, 1995). O TGF-β apresenta uma ampla gama de ações, muitas das quais antagônicas (CLARK & COKER, 1998) e na leishmaniose, a sua produção também foi associada à suscetibilidade à infecção e ao desenvolvimento de uma resposta Th2 (BARRAL *et al*, 1992).

SILVA *et al* (1995) observaram níveis aumentados de TNF, IL-1 e IL-6 no soro de pacientes com a FA que apresentavam disseminação da doença, associada a uma resposta proliferativa deficiente e altos títulos de anticorpos, indicando um possível papel das citocinas inflamatórias na gênese e controle da PCM Em nosso trabalho a avaliação da concentração sérica de IL-6, TNF-α e MIP-1α, não distinguiu a FJ da FA da PCM, não tendo sido detectadas alterações significativas durante o tratamento. Entretanto, ao analisar o grupo de pacientes com a FA, encontramos níveis mais elevados das três citocinas naqueles indivíduos com a forma disseminada da doença, quando comparados aos pacientes com a forma localizada.

Outro resultado interessante foi a detecção de níveis diminuídos de IL-8 no soro de pacientes com a FJ em relação aos pacientes com a FA da doença. A IL-8 é uma quimiocina envolvida na resposta inflamatória, pois atua diretamente na migração de neutrófilos, atraindo-os para o sítio de infecção. Desta forma, uma diminuição de sua produção poderia comprometer a mobilização de células de defesa, alterando a composição do exsudato inflamatório. Além da função quimiotática, a IL-8 modula a síntese de IgE induzida por IL-4 (KIMATA *et al*, 1992) e por conseguinte uma redução de seus níveis na

circulação poderia contribuir para a manutenção da resposta Th2, encontrada nos pacientes com a FJ da PCM.

Em conclusão, a produção predominante de IgG4, IgE e IgA, associada à eosinofilia periférica, níveis elevados de TGF-β e diminuídos de IL-8, são fortes indícios da presença de uma resposta Th2 na FJ da PCM. Estes fatores em conjunto estariam prejudicando a resposta de defesa do hospedeiro, levando a uma forma mais grave e disseminada da doença. Por outro lado, pacientes com FA teriam resposta imune celular mais preservada, com alta produção de IgG1 e número normal ou pouco aumentado de eosinófilos no sangue periférico. Para este grupo, entretanto, não foi observado um padrão de resposta imune característico, provavelmente devido ao modo heterogêneo com que a doença se manifesta nestes pacientes.

# *5. CONCLUSÕES*

Pacientes com a FJ da PCM apresentam níveis mais elevados de anticorpos IgG anti-gp43 de *P. brasiliensis*, do que pacientes com a FA da doença. A subclasse predominante desta forma clínica é a IgG4, enquanto que para a FA é a IgG1. Não foram detectadas diferenças entre as duas formas clínicas da PCM quanto à produção de IgG2 e IgG3. Anticorpos das classes IgE e IgA também se encontram em níveis mais elevados em pacientes com a FJ, assim como o número de eosinófilos no sangue periférico. Foi observado que todos estes parâmetros decrescem com o tratamento, com exceção da IgG4, cujos níveis permanecem elevados por mais tempo (pelo menos 2 anos). Foi detectada correlação significativa entre os níveis de IgE e IgG4, IgA e número de eosinófilos. Por outro lado, os níveis de IgG1 e IgG4 correlacionaram-se negativamente.

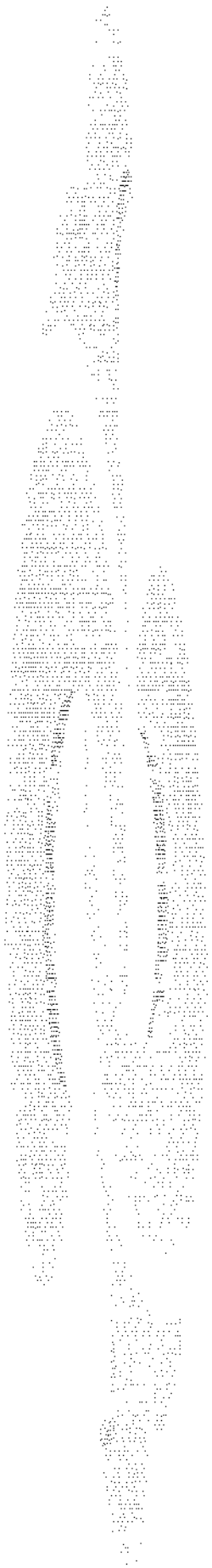
A avaliação da concentração sérica das citocinas inflamatórias IL-6 e TNF-α e da quimiocina MIP-1α, não mostrou diferença entre as duas formas clínicas. Entretanto, quando considerados somente os pacientes adultos, estas estavam aumentadas nos que apresentavam a forma mais severa (disseminada) da doença.

Os níveis de TGF-β estavam aumentados nos pacientes com a FJ e houve correlação positiva desta citocina com os níveis de IgA. A CXC-quimiocina IL-8 foi encontrada em menor quantidade no soro dos pacientes com a FJ da PCM.

A produção dominante de IgG4, IgE e IgA, associada a eosinofilia periférica, produção de TGF-β e baixos níveis de IL-8, são fortes indícios da presença de uma resposta Th2 na FJ da PCM. Estes fatores em conjunto estariam prejudicando a resposta de defesa do hospedeiro, levando a uma forma mais grave e disseminada da doença. Por outro lado, pacientes com FA teriam resposta imune celular mais preservada, com alta produção de IgG1 e número normal ou pouco aumentado de eosinófilos no sangue periférico. Para este grupo, entretanto, não foi observado um padrão de resposta imune característico, provavelmente devido ao modo heterogêneo com que a doença se manifesta nestes pacientes.

# *6. SUMMARY*

Paracoccidioidomycosis (PCM) is a systemic, chronic disease caused by the dimorphic fungus *Paracoccidioides brasiliensis,* which may be classified in two polar forms: acute or juvenile (JF) and chronic or adult form (AF). In order to further clarify this dichotomy, specific IgG, IgG subclasses, IgA, and IgE anti-gp43 were determined, by ELISA, in patients with both forms of PCM. The inflammatory response was evaluated by the detection of IL-6, TNF-α and TGF-β1 and the chemokines (IL-8 and MIP-1α), using a sandwich ELISA technique. We demonstrated that JF patients present significantly higher titers of IgE anti-gp43, the immunodominant and specific molecule of *P. brasiliensis*. Moreover, specific IgE levels strongly correlated with IgG4, IgA and eosinophilia. Increased levels of TGF-β (a switching factor for IgA) was also detected in sera from JF patients. In opposition, patients with AF were characterized by significantly higher levels of IgG1, lower levels of the other isotypes and lower number of eosinophils. The evaluation of the serum concentrations of IL-6, TNF-α, and MIP-1α did not distinguish the JF from the AF of the PCM, either before or after treatment. However, higher levels of these cytokines were detected in patients with disseminated AF compared with localized AF. The levels of IL-8 in patients with the JF of the disease were significantly lower than among AF patients. IL-8 modulates the IgE synthesis induced by IL-4 and, therefore, a reduction in the production of IL-8 could contribute to a shift toward a Th2 response. Taken together, the predominance of specific IgG4, IgE, and IgA antibodies, the associated eosinophilia and decreased levels of IL-8 are strong evidence for a Th2 type of response in the juvenile form of PCM.



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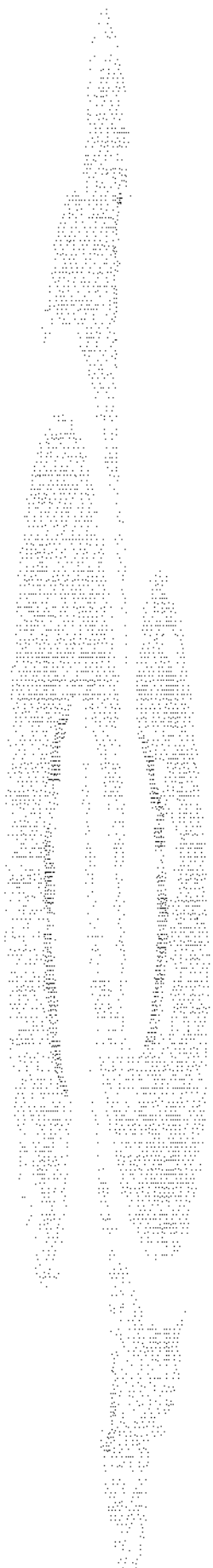

# *8. APÊNDICE*

## Tabela I – Pacientes com FA da PCM

| Paciente | Sexo[1] | Idade | Cor[2] | Órgãos Afetados[3] | Diagn.[4] | Tratamento Adotado[5] | Evolução |
|---|---|---|---|---|---|---|---|
| AB | M | 47 | B | P | OD | S+T | Melhora |
| BFP | M | 44 | B | P / M | BX | S+T / C | Cura |
| BP | M | 61 | B | P | OD | S+T | Cura |
| BPO | M | 44 | B | P / M | BX | S+T / C | Melhora |
| CFC | M | 54 | B | P / M / LN | BX | S+T | Inalterado |
| CL | F | 38 | B | M | BX | S+T | Cura |
| DJM | M | 69 | B | P / C / M | BX | S+T | Cura |
| DJD | M | 43 | B | M / LN | BX | S+T | Melhora |
| ER | M | 38 | B | P / M | BX | S+T | Melhora |
| FADS | M | 33 | M | M / LN | OD | S+T | Cura |
| HAO | M | 43 | B | P | OD | S+T | Melhora |
| JL | M | 34 | B | P / M / SN | OD | S+T | Inalterado |
| JAO | M | 34 | B | P / C / LN | BX | S+T | Melhora |
| JBC | M | 40 | B | P | OD | S+T | Cura |
| JCG | M | 35 | B | P | OD | S+T | Melhora |
| JCH | M | 46 | B | P / LN | BX | S+T | Inalterado |
| JCL | M | 47 | B | P / M / LN | BX | S+T | Melhora |
| JMS | M | 35 | B | P | OD | S+T | Cura |
| JR | M | 39 | M | M / LN | BX | S+T / C | Melhora |
| JSS | M | 54 | B | P / M / LN | BX | S+T / C / I | Melhora |
| JV | M | 41 | B | P | OD | S+T | Melhora |
| LCM | M | 44 | B | P / M | BX | S+T / C | Cura |
| LM | M | 55 | B | P / M | BX | S+T | Melhora |
| LR | M | 49 | B | P / M / B | BX | S+T | Melhora |
| LSS | M | 32 | N | P / M / LN | OD | S+T / I | Melhora |
| MP | M | 53 | B | P / M | BX | S+T | Melhora |
| NMP | F | 30 | N | M | BX | S+T | Melhora |
| OASN | M | 31 | B | P / M | BX | S+T | Melhora |
| PRS | M | 61 | B | P / M / H | BX | S+T | Inalterada |
| RA | M | 72 | B | M | BX | S+T / C | Melhora |
| SMA | M | 46 | N | P/M/C/LN | BX | S+T | Cura |
| VL | M | 41 | B | P | OD | S+T | Cura |

1) Sexo: M=Masculino; F=Feminino.
2) Cor: B=Branca; N=Negra; M= Mestiço.
3) Órgãos Afetados: P=Pulmão; M=Mucosa; LN=Linfonodo; C=Pele; SN=Sistema Nervoso; B=Baço; H=Fígado.
4) Diagnóstico feito por: BX=Biópsia; OD=Observação Direta.
5) Medicamento Utilizado: S+T= Sulfametoxazol+Trimetoprim a (Bactrin ®); C=Cetoconazol; I=Itraconazol.

**Tabela II – Pacientes com a FJ da PCM**

| Paciente | Sexo[1] | Idade | Cor[2] | Órgãos Afetados[3] | Diagn.[4] | Tratamento Adotado[5] | Evolução |
|---|---|---|---|---|---|---|---|
| AAR | M | 18 | B | LN / O | OD | A / S+T | Melhora |
| ADF | M | 23 | N | LN / H | OD | S+T | Cura |
| ALGP | M | 18 | B | LN / C | BX | I | Melhora |
| CDL | F | 14 | B | LN | BX | S+T | Cura |
| EAZB | M | 24 | B | LN / B / C | BX | S+T / C | Cura |
| ECM | F | 18 | N | LN / C | OD | S+T / A | Cura |
| ESZB | M | 7 | B | LN / O | BX | S+T | Cura |
| FLOM | M | 20 | B | LN / M | BX | S+T | Melhora |
| GR | M | 16 | M | LN / H / B | BX | A / S+T | Cura |
| JCFS | M | 33 | N | LN/SN/H/B/P | BX | A / S+T | Cura |
| JHPL | M | 5 | B | LN / H / B | BX | S+T | Cura |
| JRG | F | 15 | B | LN | OD | S+T | Melhora |
| MLL | M | 15 | B | LN / M | BX | S+T | Melhora |
| RB | F | 12 | B | LN | BX | S+T | Cura |
| RMC | F | 13 | B | LN / MO | OD | S+T | Cura |
| SRF | F | 18 | B | LN | BX/OD | A / S+T | Melhora |
| TCSR | F | 13 | B | LN, H, B | BX | S+T | Melhora |

1. Sexo: M=Masculino; F=Feminino.
2. Cor: B=Branca; N=Negra; M= Mestiço.
3. Órgãos Afetados: P=Pulmão; M=Mucosa; LN=Linfonodo; C=Pele; SN=Sistema Nervoso; B=Baço; H=Fígado, O=Medula Óssea
4. Diagnóstico feito por: BX=Biópsia; OD=Observação Direta.
5. Medicamento Utilizado: S+T= Sulfametoxazol+Trimetoprin (Bactrin ®), C=Cetoconazol, I=Itraconazol; A=Anfotericina B.

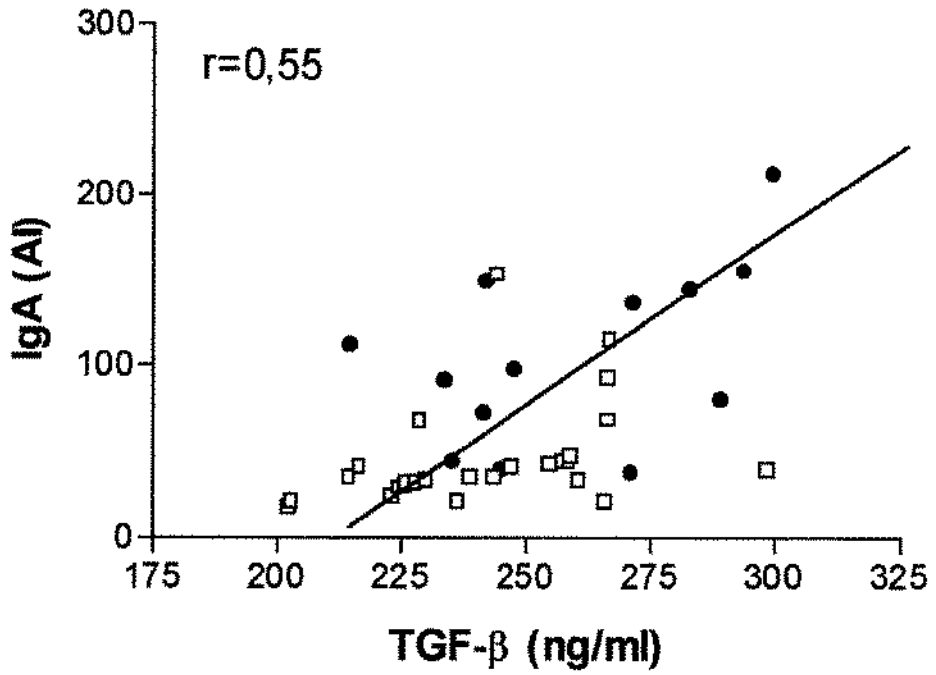


Figura 1 – Correlação entre níveis de IgA e TGF-β em pacientes com a Forma Adulta (□) e a FJ (●) da PCM. Análise estatística feita pelo método de Spearman: Valor de r=0,55 – $p<0,05$.

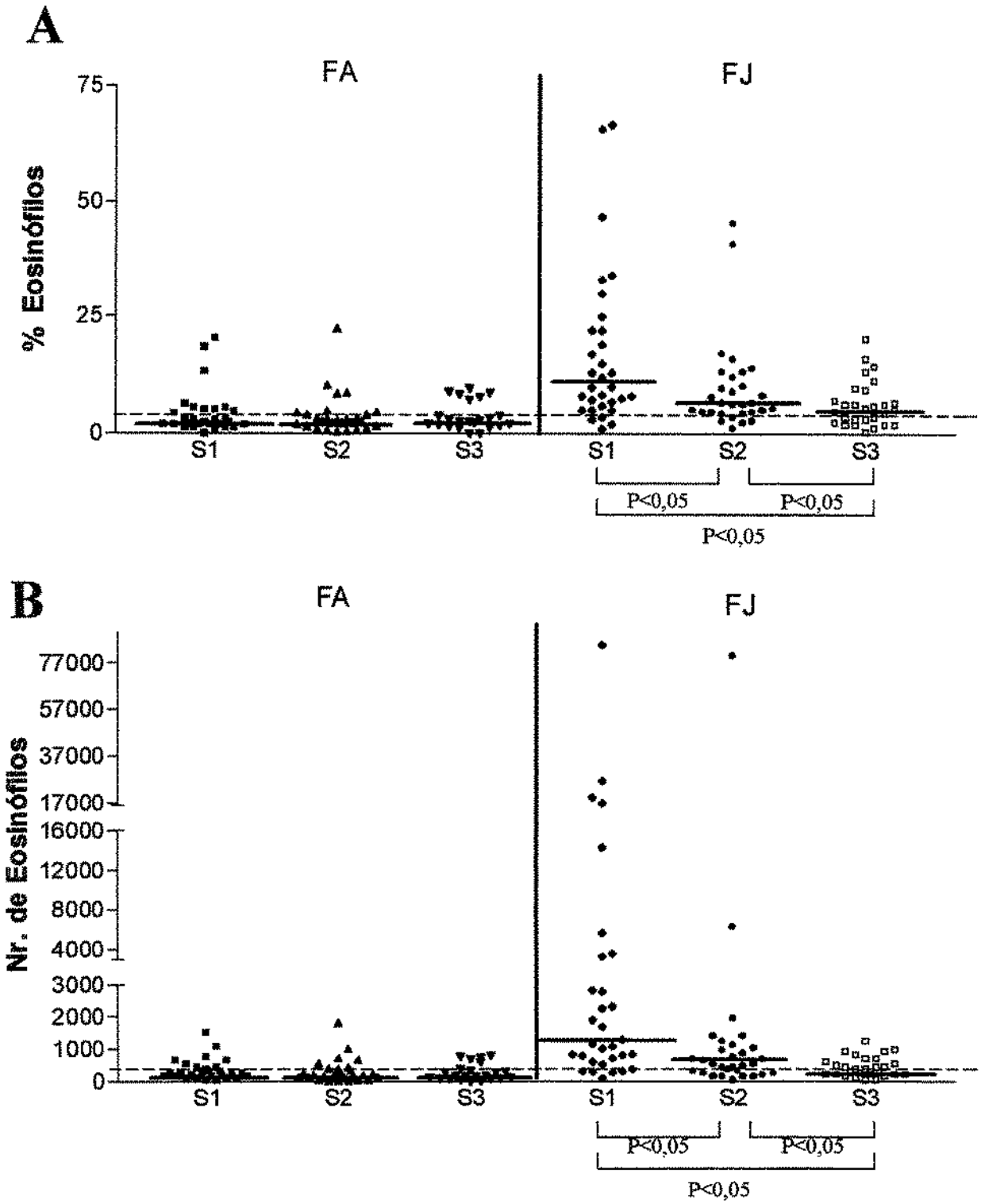


Figura 2 – A) Porcentagem de eosinófilos no sangue periférico de pacientes com a Forma Adulta (FA) e a Forma Juvenil (FJ), antes (S1), durante (S2) e após o tratamento (S3). B) Número de eosinófilos na sangue periférico de pacientes com a FA e a FJ da PCM. As barras horizontais representam a mediana.. A linha tracejada representa o valor normal (A=4%, B=450). A comparação entre as duas formas clínicas apresentou valor de $p<0,05$, em todos os tempos (S1, S2 e S3 - Teste de Mann-Whitney). A comparação entre os soros de fases diferentes só foi significante na FJ representado na figura pelos colchetes (método de Wilcoxon).

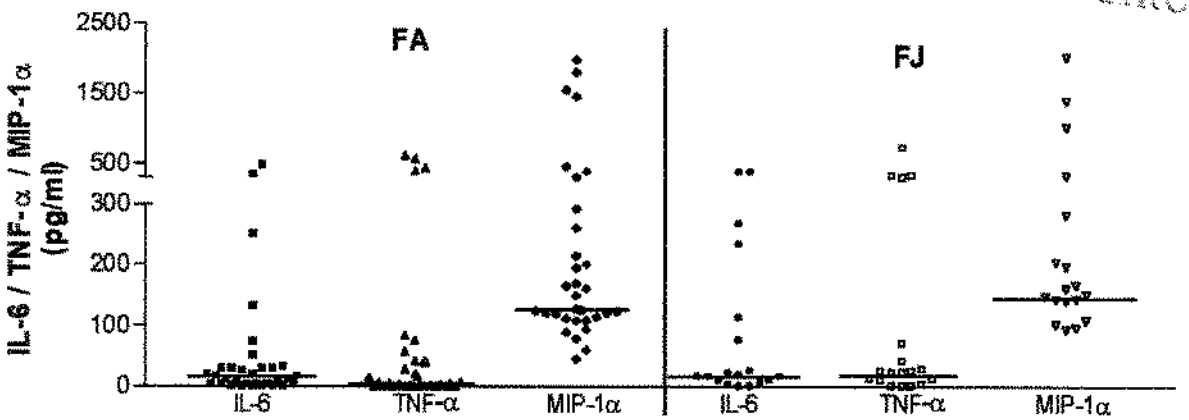


Figura 3 – Níveis de IL-6, TNF-α e MIP-1α, detectados em soros de pacientes com a FJ e a FA da PCM. Não foram observadas diferenças significantes entre os dois grupos estudados. As barras horizontais representam a mediana. Análise estatística feita com o teste de Mann-Whitney.

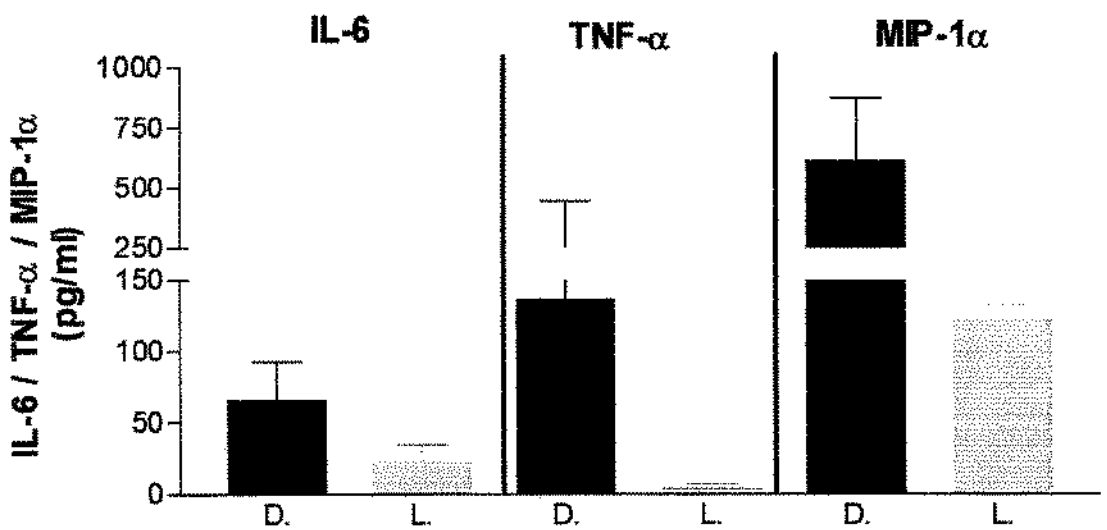


Figura 4 – Níveis de IL-6, TNF-α e MIP-1α, detectados em soros de pacientes com a forma FA da PCM, disseminada (D) e localizada (L). Foram observadas diferenças significantes entre os dois grupos estudados nas três citocinas estudadas ($p<0,05$). Análise estatística feita com o teste de *t* de Student.