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# Repercussões Metabólicas em Doenças Hepáticas Crônicas

**CAÍQUE SILVEIRA MARTINS DA FONSECA**

**Recife – 2015**

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# Repercussões Metabólicas em Doenças Hepáticas Crônicas

Tese apresentada como cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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# Repercussões Metabólicas em Doenças Hepáticas Crônicas

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À minha família, desde sempre no meu coração.

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Albert Camus

## RESUMO

O fígado é o centro executor metabólico mais importante do organismo, e, por esta razão, é alvo de uma miríade de agentes tóxicos, infecciosos ou não. Com isso, tanto distúrbios metabólicos externos ao órgão têm potencial para afetar o funcionamento do fígado, quanto injúria hepática pode afetar o metabolismo geral do organismo. Entretanto a forma como acontece esta interação de duas vias entre fígado é o resto do organismo não está clara sob diversos aspectos. A esquistossomose mansônica é uma doença infecto-parasitária crônica cujos efeitos mais graves têm gênese na injúria hepática, principalmente na fase hepatoesplênica. No entanto, sabe-se muito pouco sobre suas repercussões metabólicas. Do outro lado deste cenário, diversos distúrbios metabólicos crônicos, em boa parte relacionados ao equilíbrio energético do organismo, contribuem para o surgimento de doença hepática, das quais a mais prevalente é a Doença Hepática Gordurosa Não Alcoólica. Sendo assim, o objetivo deste trabalho foi avaliar as repercussões metabólicas dos distúrbios hepáticos causados por esquistossomose mansônica hepatoesplênica e pela doença hepática gordurosa não alcoólica. Para tanto, duas linhas de ação foram utilizadas. A primeira baseou-se na investigação da influência do polimorfismo da apolipoproteína E sobre o metabolismo lipídico/ lipoproteico e pela investigação da resistência à insulina e sua relação com os distúrbios nos lipídios plasmáticos de sujeitos com esquistossomose hepatoesplênica. Pacientes tiveram os genótipos da apolipoproteína E identificados e lipídios plasmáticos medidos. Foi observado que o referido polimorfismo poderia influenciar no metabolismo lipídico e que as modificações causadas pelos alelos ε2 e ε4 são contundentes, porém suprimidas na avaliação geral da população devido à alta frequência do alelo ε3. Outro coorte de pacientes com esquistossomose mansônica hepatoesplênica teve insulina, glicose e lipídeos plasmáticos quantificados e o resultados demonstraram que, nestes sujeitos, há evidente desregulação na ação da insulina, pela hiperinsulinemia observada. Interessantemente, distúrbios no metabolismo geralmente associados com o quadro de resistência à insulina não foram observados. A segunda linha de ação utilizou diferentes dietas hiperlipídicas, ricas em ácidos graxos, colesterol e/ou sacarose, em camundongos para investigar a interação entre obesidade, resistência à insulina e inflamação, e o desenvolvimento e progressão da doença hepática gordurosa não alcoólica. Foram utilizadas medições antropométricas, quantificação de lipídeos, glicose e insulina plasmáticos, teste de tolerância à glicose, histologia de fígado e tecido adiposo e análise da expressão de diversos genes relacionados à inflamação e metabolismo de fígado e tecido adiposo de forma a obter um panorama das interações metabólicas e da maneira como os diferentes compostos poderiam influenciar nestes processos. Foi observado que a dieta rica em ácidos graxos causou maior desregulação do tecido adiposo e resistência à insulina, enquanto que a dieta rica em sacarose induziu maior inflamação hepática e a dieta rica em colesterol provocou fibrose no fígado, mas sem o mesmo grau de inflamação. Nas diferentes abordagens foi possível observar que o comprometimento do funcionamento hepático esteve relacionado com a desregulação do metabolismo energético, pela observação de modificações no metabolismo lipídico e resistência à insulina. No caso específico do estudo com modelo animal, foi possível observar, adicionalmente, que efeitos específicos podem ser gerados em decorrência da utilização de moléculas diferentes.



**Palavras-chave:** Esquistossomose mansônica hepatoesplênica. Doença hepática gordurosa não alcoólica. Resistência à Insulina. Obesidade. Metabolismo lipídico.

## ABSTRACT

Liver is the most important metabolic executor center of the body, and, therefore, is the target of a myriad of toxic agents, infectious or not. In this scenario, the metabolic disorders outside of this organ have the potential to affect liver function. Similarly, but through the other way, liver injury can affect general metabolism of the organism. However, it is not clear in many aspects how this two-way interaction between liver is the rest of the body happens. Schistosomiasis mansoni is an infectious parasitic disease whose most serious chronic effects are generated by liver injury, especially in the hepatosplenic phase. Nevertheless, we know very little about its metabolic consequences. On the other side of this scenario, several chronic metabolic disorders, largely related to the energy balance of the body, contribute to the emergence of liver disease, of which the most prevalent is the non-alcoholic fatty liver disease. Thus, the aim of this study was to investigate the metabolic effects of chronic liver disorders caused by hepatosplenic mansonic schistosomiasis and for non-alcoholic fatty liver disease. For this, two lines of action were used. The first was based on the investigation of the influence of apolipoprotein E on lipid/lipoprotein metabolism and research of insulin resistance and its relation to the disturbances in plasma lipids of subjects with chronic hepatosplenic schistosomiasis. Patients had the apolipoprotein E genotypes identified and plasma lipid levels measured. It was observed that the referred polymorphism could influence lipid metabolism and that significant changes are caused by ε2 and ε4 alleles, but suppressed in the overall assessment of the population due to the high frequency of ε3 allele. Another cohort of patients with hepatosplenic mansonic schistosomiasis had insulin, glucose and lipid plasma levels quantified. The results showed that, in these subjects, there are obvious disruption in insulin action, by the observed hyperinsulinemia. Interestingly, metabolism disorders commonly associated with insulin resistance were not observed. The second line of action employed different hyperlipidemic diets, which were rich in fatty acids, cholesterol and / or sucrose, in mice to investigate the interaction between obesity, insulin resistance and inflammation and the development and progression of non- alcoholic fatty liver disease. Anthropometric measurements, quantification of lipids, glucose and insulin plasma levels, glucose tolerance test, histology of liver and adipose tissue, and analysis of the expression of various genes related to inflammation and metabolism on liver and adipose tissue were done in order to obtain an overview of the metabolic interactions and how the different compounds could influence these processes. It was observed that the diet rich in fatty acids caused more disruption of the adipose tissue metabolism and insulin resistance, while the sucrose-rich diet induced higher hepatic inflammation and the high cholesterol diet led to liver fibrosis, but without the same degree of inflammation. The different approaches showed that impaired liver function was related to the disruption of energy metabolism, by the observed changes in lipid metabolism and insulin resistance. In the specific case of the study with animal models, it was observed, additionally, specific effects that may be generated due to the use of different molecules.



**Key-words:** Hepatosplenic mansonic schistosomiasis. Non-alcoholic liver disease. Insulin resistance. Obesity. Lipid Metaolism.

# Sumário


| | |
|---|---|
| 1. Distúrbios Hepáticos Crônicos: Repercussões Metabólicas | 11 |
| 2. Esquistossomose mansônica | 13 |
| 2.1. Esquistossomose Crônica | 17 |
| 2.2. Repercussões Metabólicas da Esquistossomose | 18 |
| 3. Doença Hepática Gordurosa Não Alcoólica | 27 |
| 4. Objetivos | 37 |
| 4.1. Objetivo Geral | 37 |
| 4.2. Objetivos Específicos | 37 |
| 5. Referências | 38 |
| 6. Artigo 1 | 48 |
| 7. Artigo 2 | 56 |
| 8. Artigo 3 | 73 |
| 9. Conclusões | 100 |
| Apêndices | 101 |
| Apêndice A | 102 |
| Apêndice B | 107 |
| Apêndice C | 112 |
| Apêndice D | 122 |
| Anexos | 132 |
| Anexo A | 133 |
| Anexo B | 149 |

## 1. Repercussões Metabólicas em Doenças Hepáticas Crônicas

Ao longo da história do homem, o fígado aparece desde a antiguidade como órgão de extrema importância. A lenda grega de Prometeu sugere que os gregos já conheciam a capacidade de regeneração do fígado. Hipócrates (400 a.C.), descreve a existência de abcessos hepáticos. O anatomista romano Galen (aprox. 130-200 a.C) – que primeiro relatou as divisões do fígado em lóbulos – já acreditava que o fígado tinha grande importância na digestão, considerando o fígado o principal órgão do ser humano (RUTKAUSKAS et al., 2006).

> "Por que o estômago é circundado pelo fígado? É por que o fígado pode aquecê-lo e tornar a comida quente? Esta é, certamente, a razão pela qual está abraçado pelos lobos do fígado, como se fossem dedos."
>
> Galen, aprox. 200 a.C.

Com o passar dos séculos, as pesquisas caminharam no sentido do entendimento cada vez mais detalhado deste órgão. Com descrição de anatomia e fisiologia, identificação dos tipos celulares e das reações bioquímicas existentes.

O hepatócito, unidade celular formadora do fígado, representa 70% a 80% da massa citoplasmática hepática. Estas células estão envolvidas na síntese de proteínas, carboidratos e lipídeos como colesterol, sais biliares e fosfolipídeos. Os hepatócitos também são atores fundamentais na detoxificação, modificação e excreção de substâncias endógenas e exógenas. Além disso, os hepatócitos ainda têm a capacidade de sintetizar fatores de coagulação, proteínas complemento e tem participação na síntese de alguns hormônios (e.g. fator de crescimento semelhante à insulina, trombopoietina, eritropoietina) e estão diretamente envolvidos na imunidade inata, com produção de diversas interleucinas (LIM; OH; KOH, 2015).

Além de hepatócitos, outros tipos celulares coabitam o fígado: células imunes (principalmente macrófagos, chamados células de Kupffer), células estreladas e células endoteliais. Nos casos em que a capacidade de metabolização dos agentes tóxicos dos hepatócitos é superada, estas células entram em ação, muitas vezes provocando danos ao tecido na tentativa de eliminar os ativadores da disfunção (MCCLUSKY et al., 1997).

Em diversas enfermidades que acometem o fígado, observa-se um fluxo contínuo de componentes agressivos ao tecido hepático, durante períodos longos e

que são classificados como crônicos. Por outro lado, do ponto de vista imunológico, o processo de cronificação de uma doença passa pela modificação da forma de resposta inflamatória. Tendo em vista a importância do fígado em variados processos metabólicos, existe também um amplo espectro de possíveis agentes tóxicos que podem levar a distúrbios hepáticos crônicos. Estes agentes podem ter origem infecciosa ou não infecciosa. No contexto dos distúrbios hepáticos crônicos infecciosos destaca-se a forma hepatoesplênica da esquistossomose causada pelo parasita *Schistosoma mansoni*. Entre as afecções não infecciosas que atingem o fígado a doença hepática gordurosa não alcoólica (DHGNA) é a mais frequente, afeta de 20% a 30% da população mundial.

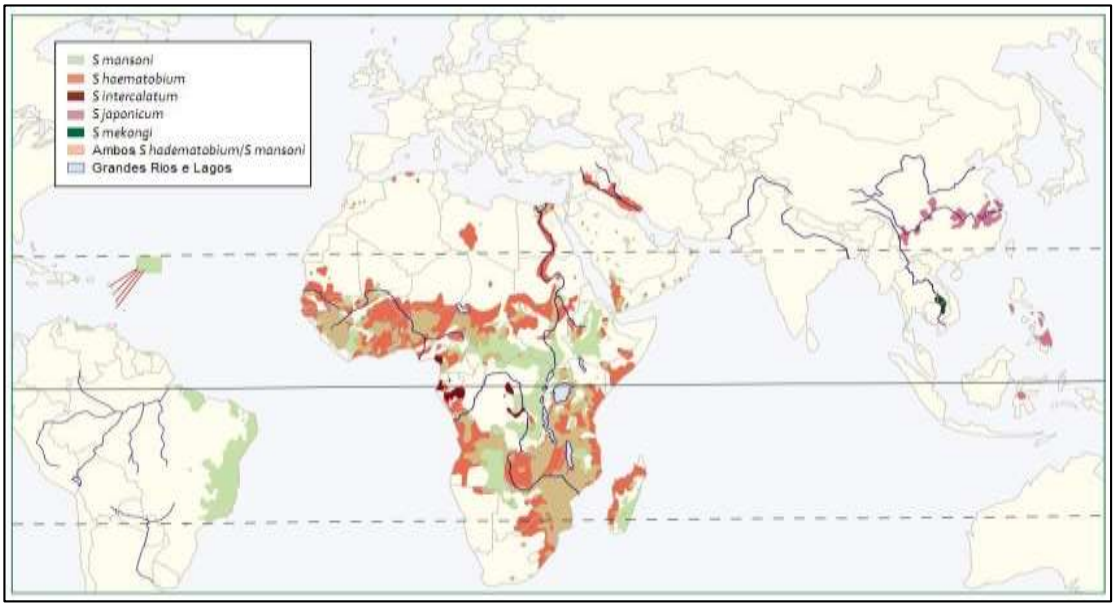
## 1. Esquistossomose mansônica

Esquistossomose (ou bilhardíase) é uma doença tropical causada pelos vermes digenéticos da classe Trematoda e do gênero *Schistosoma*. Diferentemente de outros trematódeos, o esquistossoma apresenta sexos separados. O macho é de cor esbranquiçada e mede de 6 a 13 mm de comprimento por 1,1 mm de largura. A fêmea é cilíndrica e mais fina e longa que o macho e mede de 10 a 20 mm de comprimento por 0,16 mm de largura. Como não apresentam órgão copulador, a cópula ocorre pela justaposição dos orifícios genitais feminino e masculino, quando a fêmea está alojada no canal ginecóforo (fenda longitudinal, no macho, para albergar a fêmea e fecundá-la). O casal permanece ligado ao longo de toda a vida do verme (GRYSEELS et al., 2006; ISNARD; CHEVILLARD, 2008).

Existem muitas espécies de vermes do gênero *Schistosoma*, a maior parte é parasita intestinal e infecta seres humanos apenas ocasionalmente. Entretanto, são conhecidas cinco espécies de valor clínico importante: *S. mansoni*, *S. japonicum*, *S. haematobium, S. intercalatum e S. mekongi* (GRYSEELS et al., 2006).

A esquistossomose está presente em 76 países, considerada endemia parasitária na América, África e Ásia. Estima-se que mais de 200 milhões de pessoas estejam infectadas pelos helmintos do gênero *Schistosoma* e aproximadamente 800 milhões estejam em áreas de risco de infecção, o que faz da esquistossomose a segunda doença parasitária de maior importância socioeconômica no mundo (**FIGURA 1**). Além das altas taxas de morbidade, estima-se que cerca de 280.000 pessoas morram por esquistossomose apenas na região da África subsaariana. (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2002; VAN DER WERF, 2003; KING; DICKMAN; TISCH, 2005; STEINMANN et al., 2006).

**FIGURA 1**. Distribuição global da esquistossomose




Fonte: Adaptado de GRYSEELS et al., 2006.

No Brasil, a esquistossomose está presente em 18 estados (Região Nordeste, Espírito Santo, Goiás, Mato Grosso, Minas Gerais, Pará, Paraná, Rio de Janeiro, Santa Catarina e São Paulo) e no Distrito Federal, com as mais altas prevalências nos estados de Pernambuco, Alagoas, Sergipe e Minas Gerais. A existência de focos de esquistossomose mansônica em estados fora da região Nordeste tem sido justificada pela mobilidade populacional a partir de zonas endêmicas existentes dentro do país. Estima-se que entre 4 e 6 milhões de pessoas estejam infectadas no Brasil e que 25 milhões residam em zona de risco de infecção, fazendo do Brasil o país com maior número de casos das Américas (CANTANHEDE; FERREIRA; MATOS, 2011).

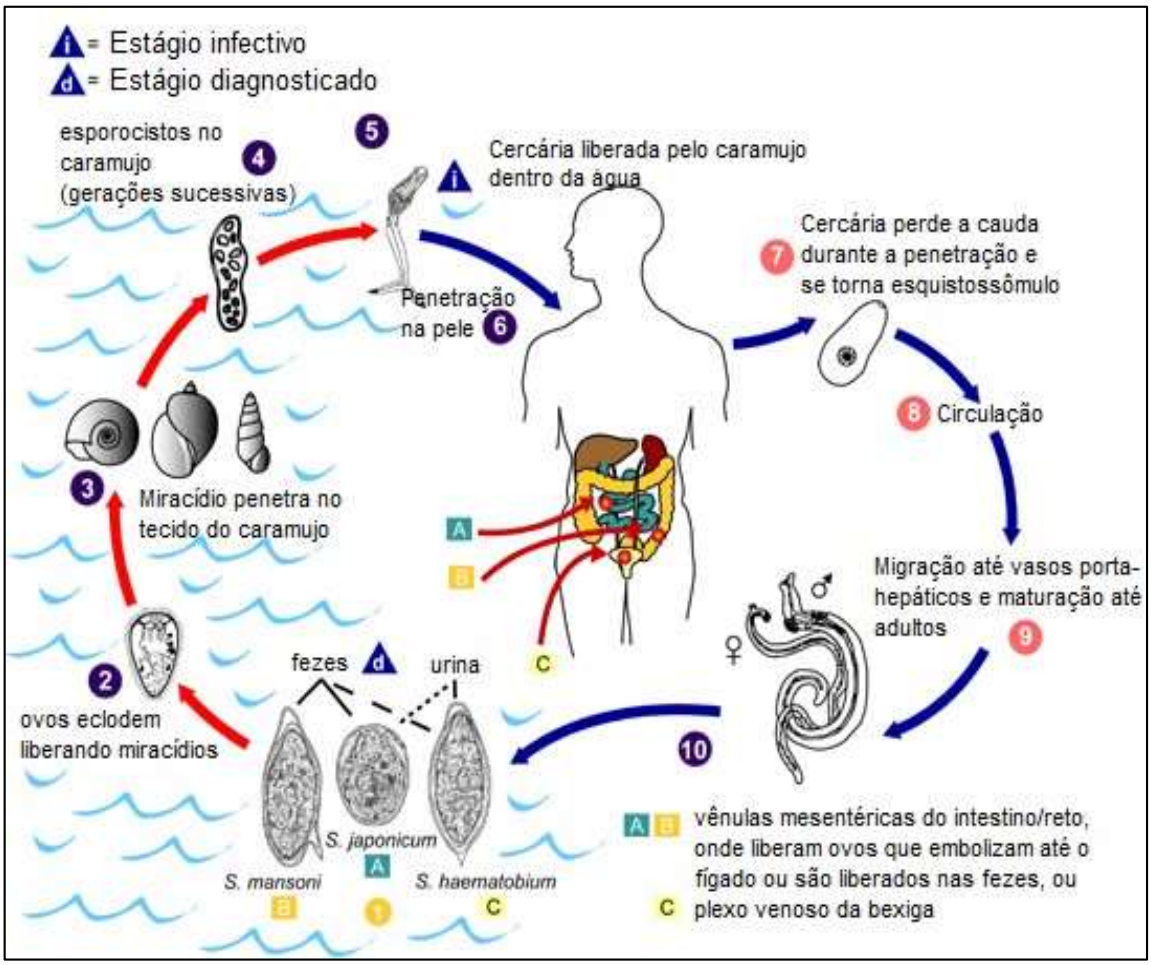
Existe apenas uma espécie de *Schistosoma* no Brasil, o *S. mansoni*, cujos hospedeiros intermediários são os caramujos do gênero *Biomphalaria*: *B. straminea, B. glabrata e B. tenagophila*. O ciclo evolutivo do *S. mansoni* é complexo, pois é formado por duas fases parasitárias: uma no hospedeiro intermediário (caramujo) e outra no hospedeiro definitivo (homem). Há ainda duas passagens larvárias no meio aquático, entre as fases parasitárias. O homem doente elimina os ovos do verme através de suas fezes. A postura ocorre nos vasos capilares intestinais, de onde os ovos atingem a luz do intestino e finalmente são liberados. Uma fêmea é capaz de liberar de 300 até 1000 ovos por dia, os quais amadurecem em uma semana (JORDAN, 1998; DA SILVA; CHIEFFIB; CARRILHO, 2005).

Em contato com a água, os ovos maduros incham, eclodem e liberam larvas ciliadas, os miracídios, os quais se locomovem rapidamente em busca do caramujo, seu hospedeiro intermediário. Neste estágio, a larva tem em média até 8 horas de vida. Durante a penetração no caramujo, o miracídio perde parte de suas estruturas. As partes remanescentes se juntam em até 48 horas, formando um saco alongado repleto de células germinativas, o esporocisto primário. Após este processo, há um segundo ciclo de transformação, desta vez em esporocisto secundário, os quais migram até o fígado e pâncreas do caramujo e se dividem em milhares de larvas chamadas de cercárias. O processo de desenvolvimento no interior do caramujo dura cerca de 40 dias (KATZ; ALMEIDA, 2003).

Após este período, as cercárias são liberadas na água, principalmente nos momentos de maior incidência solar. Cada caramujo pode liberar milhares de cercárias em um único dia. Uma vez dispersas na água, as cercárias nadam avidamente até encontrar a pele ou alguma mucosa do hospedeiro definitivo, por onde penetram. A penetração é consumada pela ação lítica de enzimas sobre proteínas da pele e pela ação mecânica da cauda da larva, que move intensamente (LAMBERTUCCI, 2010).

Durante este processo, o miracídio perde a cauda e se transforma em esquistossômulo, o qual é adaptado ao meio interno isotônico do hospedeiro. Os esquistossômulos permanecem na pele do homem por cerca de dois dias, quando penetram nos vasos sanguíneos ou linfáticos. O sistema imune do hospedeiro consegue eliminar boa parte dos vermes nesta fase, no entanto, alguns conseguem chegar até coração e pulmões, onde passam por modificações adicionais no desenvolvimento. Após isto, seguem para o fígado, onde completam seu amadurecimento sexual e onde ocorre o pareamento para acasalamento, em seguida seguem até o intestino para oviposição. Vermes adultos geralmente vivem 3 a 5 anos, muito embora possam permanecer até 20 anos no corpo humano (ISNARD; CHEVILLARD, 2008) (**FIGURA 2**).

**FIGURA 2**. Ciclo biológico dos vermes do gênero *Schistosoma*




Fonte: Adaptado de GRAHAM et al., 2010.

Todos estes passos do desenvolvimento do verme no organismo do hospedeiro são caracterizados por reações específicas. A penetração da cercária pela pele pode gerar lesões com bastante prurido no local, chamada dermatite cercariana, principalmente em indivíduos em contato com o verme pela primeira vez, em sua maioria imigrantes e turistas. Esta reação pode permanecer por até uma semana, como lesões pápulo pruriginosas (BOTTIEAU et al., 2006; BOURÉE; CAUMES, 2010).

Após a penetração da cercária, agora como esquistossômulo, inicia-se a forma aguda da esquistossomose. Caracteriza-se por uma reação de hipersensibilidade sistêmica contra o esquistossômulo e ocorre poucas semanas após a infecção, no entanto frequentemente não é observada em indivíduos residentes em áreas endêmicas e sua intensidade pode variar de acordo com a intensidade da infecção e a resposta imune do indivíduo (ROCHA et al., 1996; DA SILVA; CHIEFFIB; CARRILHO, 2005).

## 2.1. Esquistossomose Crônica

Após as manifestações agudas da esquistossomose, a infecção geralmente progride para a forma crônica. Este é o estágio frequentemente observado em áreas endêmicas. As principais lesões não são provocadas pelos vermes adultos, mas sim pelos ovos que ficam presos nos tecidos. Os ovos secretam diversas moléculas, dentre as quais, enzimas proteolíticas que provocam forte atração de eosinófilos com reações inflamatórias e granulomatosas típicas, as quais são progressivamente substituídas por tecido fibrótico (CHEEVER; HOFFMANN; WYNN, 2000).

A esquistossomose mansônica crônica inicia-se no intestino, a migração dos ovos através da parede intestinal provoca uma intensa inflamação granulomatosa na mucosa, pseudopolipose, microulceração e sangramento superficial no intestino grosso distal. O paciente geralmente apresenta fraqueza, dor abdominal e trânsito intestinal irregular. Estas lesões são inespecíficas e podem ser confundidas com outras afecções do intestino, como as doenças inflamatórias do intestino, como Colite Ulcerativa e Doença de Chron (CHEEVER, 1968; CAO et al., 2010).

Contudo, parte dos ovos liberados nos vasos mesentéricos não atinge a luz do intestino, alguns são levados pela corrente sanguínea até outros órgãos, na maioria das vezes o fígado. Neste órgão, o ovo acaba preso no espaço periportal presinusoidal, local em que desencadeia uma forte reação inflamatória granulomatosa assim como no intestino (GRYSEELS; POLDERMAN, 1991; GRYSEELS et al., 2006).

Os mesmos mecanismos utilizados pelo sistema imune do hospedeiro para combater a infecção são os responsáveis pelas principais complicações da esquistossomose crônica. A reação granulomatosa completa-se com a criação de uma região de fibrose ao redor do ovo, os quais estão localizados nos vasos do fígado. Ao longo do tempo, a deposição de colágeno nos espaços periportais leva à fibrose de Symmers ou periportal. A qual pode ser observada por ultrassonografia, tomografia computadorizada ou ressonância magnética nuclear. Ultrassonografia tem sido utilizada associada ao exame clínico na detecção do grau e padrão de fibrose hepática baseado em critérios da organização mundial de saúde (SYMMERS, 1904 apud ISNARD; CHEVILLARD, 2008; HATZ; MURAKAMI; JENKINS, 1992; VOIETA et al., 2010).

A fibrose hepática periportal, por sua vez, resulta no aumento da resistência ao fluxo local e consequente hipertensão. Contudo, a circulação portal não apenas envolve intestino e fígado, também estão bastante próximos baço e esôfago. Com a progressão da doença, a hipertensão portal passa também a atingir o baço, o que provoca um aumento considerável no seu volume, a chamada esplenomegalia. Além disso, a hipertensão portal está associada ao surgimento de varizes esofágicas, cujo rompimento e sangramento são as principais causas de morte por *S. mansoni*. Além disso, continuados sangramentos ocultos podem levar à anemia, hipoalbuminemia e até mesmo ascite, quando associado à hipertensão portal (KING; DICKMAN; TISCH, 2005; WILSON et al., 2007).

### 2.2. Repercussões Metabólicas da Esquistossomose

Tendo em vista o papel central do fígado para o metabolismo humano, a esquistossomose tem a capacidade de promover graves manifestações, principalmente quando se consideram os longos períodos de infecção e reinfecção. Dois efeitos fundamentais devem ser considerados no entendimento da patologia hepática: inflamação e fibrose. Ambas causadas em resposta à deposição dos ovos de *S. mansoni*.

A inflamação na região provoca, ao longo do tempo, dano hepático tecidual, com morte de hepatócitos e diminuição da capacidade de síntese e secreção pelo fígado. Isto se reflete na avaliação laboratorial da função hepática no indivíduo com esquistossomose: Geralmente há aumento plasmático das enzimas alanina aminotransferase (ALT) e aspartato aminotransferase (AST), devido aos danos hepatocelulares, os quais também provocam diminuição da síntese de proteínas, refletidas na diminuição da albumina e de fatores de coagulação. A fibrose, consequência da inflamação crônica, provoca ainda aumento de gamma-glutamil transferase (GGT) e fosfatase alcalina (ALP) devido ao desenvolvimento de compressão biliar causada pela formação de granuloma na área ao redor do ovo. Além disso, ambas inflamação e fibrose podem provocar aumento da bilirrubina indireta, pelo aumento da hemólise (PEREIRA et al., 2010; LEITE et al., 2013, 2015).

Pesquisas utilizando modelos de experimentação animal observaram relação entre a esquistossomose com algumas alterações no metabolismo de lipídios e

lipoproteínas. Estudos com camundongos infectados relatam redução nos níveis plasmáticos de colesterol esterificado e elevação dos fosfolipídios plasmáticos. Experimentos, utilizando sagüis (*Callithrix jacchus*) como modelo de infecção em primatas, demonstraram haver alterações na composição das membranas lipídicas de eritrócitos dos animais que desenvolveram a infecção crônica e redução na atividade da enzima LCAT estando relacionada com alterações no metabolismo do colesterol, interferindo no seu transporte reverso. Outros estudos também relataram alterações no metabolismo de triglicerídeos e VLDL tendo sido observados níveis elevados em camundongos, que desenvolveram a infecção por *S. mansoni* (FEINGOLD et al.,1989; DIMENSTEIN, et al., 1992; LIMA et al.,1998; DOENHOFF et al., 2002, RAMOS et al., 2004; LA FLAMME et al., 2007).

Alguns poucos estudos realizados em humanos também reportaram que a esquistossomose crônica promove alterações no metabolismo lipídico como, por exemplo, a peroxidação de lipídios de membrana em eritrócitos e diminuição da atividade da LCAT, redução dos níveis plasmáticos de colesterol total, LDL-C, HDL-C, VLDL-C e triglicerídeos. Desta forma pode-se evidenciar que as alterações metabólicas observadas nestes trabalhos diferem de acordo com o tipo de hospedeiro e isto pode ser explicado pelas diferenças e peculiaridades existentes entre o metabolismo das espécies estudadas. (SILVA et al., 2002; FACUNDO et al., 2004).

Por outro lado, tanto os distúrbios na síntese de proteínas quanto as diversas interleucinas liberadas durante a inflamação independentemente do dano hepático podem provocar efeitos sobre o metabolismo de lipoproteínas. Como tem sido reportado em alguns estudos com relação aos níveis de apolipoproteína A, principal componente proteico da HDL, e a Apolipoproteína B, o principal constituinte proteico da VLDL, IDL e LDL (KHOVIDHUNKIT et al., 2000).

Além de ApoA e ApoB, diversas outras apolipoproteínas participam no metabolismo de lipídeos. Cada lipoproteína está associada a apolipoproteínas específicas que exercem diversas funções, como ativação/inibição das enzimas envolvidas no metabolismo lipídico e na ligação das lipoproteínas a receptores da superfície celular. Neste contexto, a ApoE apresenta função crucial, desde que intermedeia o reconhecimento e internalização hepática de lipoproteínas ricas em triglicerídeos, quilomícrons e VLDLs e seus remanescentes através de sua ação como

ligante de LDLR, LRP e HSPG (MAHLEY; HUANG, 1999; HAGBERG; WILUND; FERREL, 2000; HARRIS; EVANS; OWEN, 2006).

Além disso, a APOE também tem papel fundamental no metabolismo das lipoproteínas ricas em colesterol, como LDL, em que atua na distribuição de colesterol para os tecidos periféricos, ou ainda como constituinte de algumas frações de HDL, contribuindo com o transporte reverso de colesterol através da participação na internalização hepática destas lipoproteínas (MAHLEY, 1982; MAHLEY; INNERARITY, 1983).

Além de atuar como ligante de receptores, a ApoE ainda participa na ativação de diversas enzimas envolvidas no metabolismo de lipoproteínas, dentre as quais, pode-se mencionar as lipases hepáticas, a Proteína de Transferência de Colesterol Éster (CETP) e a LCAT (GREENOW; PEARCE; RAMJI, 2005).

A ApoE não se apresenta de maneira idêntica em todos os seres humanos, com consequentes repercussões metabólicas. De fato, apenas suas diferentes isoformas são responsáveis por 10% da variação dos níveis de colesterol plasmático na população geral. Estas isoformas existem devido a uma variação chamada de polimorfismo genético ou gênico, no qual um mesmo sítio apresenta diferentes versões ou alelos, neste caso, do tipo polimorfismo de único nucleotídeo (SNP, do inglês: *single nucleotide polymorphism*), em que a alteração de apenas um único nucleotídeo provoca modificação na sequência da proteína (MAHLEY; RALL, 2000; LI et al., 2011; FONSECA et al., 2014).

As três principais isoformas da ApoE, chamadas E2, E3 e E4, são produtos dos três alelos ε2 (lê-se épsilon), ε3, ε4. Três genótipos homozigotos (ε2/ε2, ε3/ε3 e ε4/ε4) e três heterozigotos (ε2/ε3, ε2/ε4, ε3/ε4) provêm da expressão de dois destes três alelos. As modificações que dão origem a estes três alelos acontecem em dois nucleotídeos presentes no éxon 3 e formam proteínas que diferem nos aminoácidos 112 e/ou 158. A ApoE3 possui um resíduo de cisteína na posição 112 e um resíduo de arginina na posição 158, enquanto a ApoE2 contém duas cisteínas (Arg158→Cys) e a ApoE4 contém duas argininas em ambos os sítios (Cys112→Arg) (UTERMANN et al., 1980; MAHLEY, 1988; MAHLEY; RALL, 2000).

Tem sido observado que o alelo ε3 é o mais comum, com frequências numa faixa de aproximadamente 51% a 80%. O alelo ε4 geralmente apresenta frequência maior do que ε2 variando entre 12% e 29%, enquanto que frequências do alelo ε2 têm sido encontradas variando entre 1% e 19%. Devido a prevalência majoritária do alelo ε3,

este tem sido utilizado como referência para a avaliação das diferenças entre as isoformas (MASEMOLA; ALBERTS; URDAL, 2007; MENDES-LANA et al., 2007).

Desde as primeiras investigações tem-se associado o alelo ε2 com a existência de disbetalipoproteinemia (ou hiperlipoproteinemia do tipo III, segundo classificação de Fredrickson) em que o indivíduo possui níveis muito elevados de triglicerídeos, frequentemente próximos e maiores do que 300 mg/dL. A hiperlipidemia é causada pelo acúmulo de quilomícrons remanescentes provenientes de lipídeos exógenos e de remanescentes de VLDL derivados do fígado, ambas chamadas de β-VLDL. Virtualmente todos os indivíduos com disbetalipoproteinemia são homozigóticos para o alelo ε2. A presença do alelo ε2 em heterozigose, entretanto, apresenta um efeito benéfico, pela associação com níveis diminuídos de colesterol e ApoB e níveis elevados de HDL e ApoA-I (DONG et al., 1996; MAHLEY; JI, 1999; FRIKKE-SCHMIDT et al., 2000).

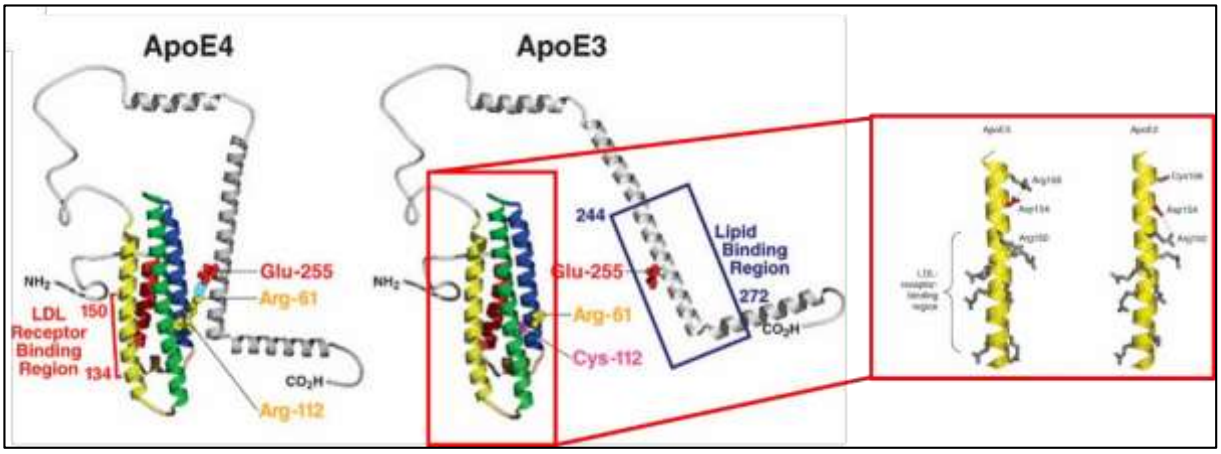
Um perfil lipídico-lipoprotéico diferente, no entanto, tem sido observado na presença do alelo ε4. Indivíduos portadores do alelo ε4 apresentam níveis aumentados de colesterol e ApoB enquanto apresentam valores bem menores de HDL e ApoA-I. Os níveis elevados de LDL-C presentes nos indivíduos portadores de ApoE4 estão fortemente associados com o desenvolvimento de aterosclerose e o aumento do risco de DCVs (DONG; WEISGRABER, 1996; GYLLING et al., 1997; GÓMEZ-CORONADO, 1999; HAAN; MAYEDA, 2010).

Estes diferentes padrões lipídico-lipoprotéicos observados entre os alelos da APOE são frequentemente justificados pelas diferenças estruturais causadas pelas modificações dos aminoácidos. A substituição do resíduo de cisteína (ApoE3) por Arginina (ApoE4) na posição 112 provoca modificações na região de ligação aos lipídeos de forma a fortalecer a afinidade da ApoE pelos mesmos. Com isso há maior concentração de ApoE nas lipoproteínas ricas em ApoB, como VLDL e LDL, do que na HDL. Por causa desta modificação na distribuição de ApoE há maior quantidade de ApoE ligando-se aos receptores de LDL (LDLR) e internalização de colesterol. Este processo, associado ao aumento da absorção de colesterol intestinal observada em indivíduos com APOE4, pode aumentar o conteúdo intra-hepático de colesterol e, consequentemente, diminuir a expressão de LDLR, levando ao aumento dos níveis de LDL-C observados em indivíduos com APOE4. Outros estudos ainda reportam que parte destas alterações pode ser justificada pela rápida depuração de quilomícrons remanescentes contendo APOE4 em relação aqueles contendo APOE3

(WEINTRAUB; EISENBERG; BRESLOW, 1987; KESÄNIEMI; EHNHOLM; MIETTINEN, 1987; DREON et al., 1995).

Por outro lado, a substituição do resíduo de arginina (ApoE3) por cisteína (ApoE2) na posição 154 provoca modificações na região de ligação a receptores, provocando uma afinidade de apenas 2% em comparação com ApoE3 ou ApoE4. Assim, há uma diminuição acentuada da depuração hepática de lipoproteínas e consequente hiperlipidemia (WEISGRABER, INNERARITY & MAHLEY, 1982; WEINTRAUB, EISENBERG & BRESLOW, 1987) (**FIGURA 3**).

**FIGURA 3**: Diferenças observadas nas estruturas de ApoE3, ApoE4 e ApoE2.




Fonte: Adaptado de MAHLEY e RALL, 2000.

Desta forma, o polimorfismo da ApoE poderia estar relacionado às dislipidemias observadas durante a esquistossomose mansônica tanto pelas diferenças intrínsecas das isoformas, quanto pelas disfunções hepáticas, que poderiam provocar mudanças na síntese, secreção e depuração de ApoE, e consequentemente de lipoproteínas, pelo fígado.

Por outro lado, as inter-relações de ApoE e esquistossomose mansônica poderiam se dar devido à influência da ApoE sobre processos imunológicos. Embora ainda escassos em relação a ampla quantidade de possibilidades de ação, alguns estudos têm observado efeitos da ApoE em diferentes situações. Há evidências de que as isoformas da APOE podem ter um papel importante na infecção e desenvolvimento de doenças causadas por bactérias, vírus, protozoários e fungos (WOZNIAK, 2007; TURSEN et al., 2004; BURT et al., 2008; WANG et al., 2009).

Tem sido relatado que a progressão de infecções bacterianas pode ser influenciada pelo genótipo presente em cada caso. Camundongos expressando

ApoE3 humana apresentaram maior sobrevivência à sepse do que aqueles expressando ApoE4. Resultados semelhantes também foram observados em humanos, portadores do alelo ε3 apresentaram menor incidência de sepse severa e menor duração em Unidade de Terapia Intensiva após cirurgia do que aqueles com o alelo ε4 (MORETTI et al., 2005; WANG et al., 2009).

As infecções virais também têm sido alvo de estudos em que se avalia a influência do polimorfismo da APOE. Tem sido observado que a presença do genótipo ε4/ε4 está associada a um curso mais rápido da doença e progressão para morte em portadores do Vírus da Imunodeficiência Humana, ao menos parcialmente explicado pela inibição da expressão da proteína Tat – um transativador de expressão do gene do HIV – e consequente inibição da formação do sarcoma de Kaposi, pela APOE3 (BROWNING et al., 1994; BURT et al., 2008).

O alelo ε4 também tem sido associado ao herpes labial, provocado pelo Herpes Simplex Vírus (HSV) tipo I. O HSV-I está presente no cérebro de um alto percentual da população e quando associado com a APOE4 confere um risco de desenvolvimento de doença de Alzheimer muito maior do que o alelo ε4 de maneira isolada. No entanto, o alelo ε4 conferiu proteção em indivíduos infectados com o vírus tipo C da Hepatite (HCV) contra doença hepática severa. Ademais, indivíduos portadores de APOE2 apresentaram um maior risco de desenvolvimento de encefalite causada por HSV-I (ITZHAKI et al., 1997; LIN et al., 2001; WOZNIAK et al., 2002; TONIUTTO et al., 2004).

A importância da APOE durante a infecção não se limita a vírus ou bactérias, foi observado que o genótipo ε2/ε2 esteve associado a uma infecção precoce pelo protozoário causador da malária, o *Plasmodium falciparum.* O parasita utiliza, através de proteínas de superfície, moléculas HSPG/LRP para invadir os hepatócitos. De certo que outras proteínas, como a ApoE, utilizam esta rota de internalização celular, pode existir competição por estes receptores. Além disso, foi relatado maior risco de desenvolvimento de micoses superficiais, principalmente por fungos dermatófitos, em indivíduos com o genótipo ε3/ε2, ou mesmo na ausência de ε3/ε3 (SHAKIBAEI; FREVERT, 1996; SINNIS et al., 1995; WOZNIAK et al., 2007; TURSEN et al., 2004).

Estes patógenos (vírus, bactérias, protozoários e fungos) e a ApoE utilizam-se das mesmas moléculas para se ligar e penetrar na célula, receptores da família do LDLR e HSPG. Como a afinidade de ligação é específica para cada isoforma, a ApoE poderia determinar o grau de competição pelo receptor e consequentemente a entrada

do patógeno na célula e sua subsequente propagação e doença. As afinidades de ligação das isoformas da APOE podem resultar em uma proteção específica de um alelo para uma doença e conferir risco para outra (ITZHAKI et al, 1997).

Em adição à influência das isoformas da ApoE sobre a infecção em si, a ApoE poderia influenciar a resposta imune do hospedeiro. Estudos com modelos animais observaram que a ApoE pode atuar como um inibidor da resposta Th1, provocando uma modificação do balanço Th1/Th2 na direção do padrão Th2. Também foi observado que camundongos $APOE^{-/-}$ apresentavam inibição da resposta inflamatória Th2 após administração de peptídeo derivado da ApoE. Em humanos foi observado que o a presença do alelo ε4 aumenta consideravelmente o risco de desenvolvimento de Colite Ulcerativa (ZHENG et al, 2003; LI et al, 2009).

Interessantemente, os efeitos da ApoE sobre o metabolismo lipídico e sobre a resposta imune do indivíduo infectado por *S. mansoni* poderiam estar associados. Acredita-se que o verme, que não sintetiza lipídeos, os absorve através da ligação de partículas de LDL a uma proteína de seu tegumento semelhante ao LDLR. Este processo de ligação à LDL, assim como a outras moléculas, teria um papel secundário muito importante como um mecanismo de evasão do verme à resposta imune do hospedeiro, no qual o parasita camufla-se com as lipoproteínas e passa a não ser mais detectado pelo sistema imune do indivíduo infectado (SMITHERS; TERRY; HOCKEY, 1969; RUMJANEK; CAMPOS; AFONSO, 1988; TEMPONE et al., 1997).

Enfim, a investigação das dislipidemias no contexto da esquistossomose se torna ainda mais importante pelo reconhecimento da participação de lipídeos e lipoproteínas na gênese de diversos distúrbios associados às doenças crônicas não- transmissíveis (DCNTs), como aterosclerose, obesidade, resistência à insulina e hipertensão arterial sistêmica. Estes distúrbios são fortemente associados entre si e observados concomitantemente na síndrome metabólica, a qual é considerada um transtorno complexo formado por um conjunto de fatores de risco cardiovascular. Tais fatores são usualmente relacionados à resistência à insulina (DOENHOFF et al., 2002; SOCIEDADE BRASILEIRA DE CARDIOLOGIA, 2005).

A resistência insulínica é um distúrbio metabólico, caracterizado pela diminuição da capacidade dos receptores de insulina teciduais em interagir com a insulina, e consequente diminuição na captação da glicose plasmática. Com isso há um aumento na concentração de glicose no sangue, o qual reforça o estímulo de secreção de insulina – comumente chamada compensatória – levando à hiperinsulinemia. A

resistência à insulina pode promover várias repercussões metabólicas, tais como hiperglicemia/diabetes mellitus do tipo 2, dislipidemias/ dislipoproteinemias, obesidade/obesidade visceral, hiperuricemia, aumento dos fatores pró-trombóticos e antifibrinolíticos, elevação do fator de necrose tumoral (TNF-α) e de algumas interleucinas, bem como hipertensão arterial sistêmica. Os principais órgãos e tecidos afetados são o tecido adiposo, o tecido muscular, o fígado e os rins (SIMONE et al., 2003).

No metabolismo lipídico/lipoproteico, a resistência insulínica está relacionada com a diminuição dos níveis de HDL-C, na formação de uma LDL pequena e densa (mais aterogênica), aumento nos níveis plasmáticos de triglicerídeos e de VLDL-C. Estas alterações lipídicas e lipoprotéicas podem ocorrer devido à estimulação da lipase lipoprotéica nos capilares do tecido adiposo, liberando com isso, grandes quantidades de ácidos graxos livres provenientes das VLDLs, que seguem pela circulação para o fígado, aumentando a síntese hepática de triglicerídeos e que pode provocar, consequentemente, desordens metabólicas como a esteatose hepática (HOLZL et al., 1998; OLIVEIRA; MIRABEAU; LIMA, 2004; CUSSONS; BRONWYN; GERALD, 2005).

As LDLs são afetadas em sua composição, pois são produzidas a partir de VLDLs anormais, incorporando em suas moléculas grandes quantidades de triglicerídeos, tornando-se desse modo, mais suscetíveis a hidrólises pelas lipases hepáticas e lipoprotéicas, resultando em LDLs de partículas menores, mais densas e aterogênicas, que são fagocitadas pelos macrófagos dando origem às “células espumosas”, que são as principais responsáveis pelo conteúdo de colesterol das placas de ateroma (BORGGREVE et al., 2003).

Paralelamente, ocorre uma redução na concentração das HDLs, por mecanismos que ainda não estão bem elucidados, tais como: o bloqueio da transferência de apolipoproteínas e de fosfolipídios das lipoproteínas ricas em triglicerídeos para a HDL; a troca entre colesterol que foi esterificado pela atuação da LCAT na HDL e o conteúdo triglicerídico na VLDL; elevada atividade da lipase hepática, a qual facilita a depuração da HDL e alterações na função hepática, tais como a inibição da produção da Apo A-I, a principal apolipoproteína da HDL, e/ou a inibição da secreção de HDL nascente (GINSBERG; ZHANG; HERNANDEZ-ONO, 2005).

Nos últimos anos, a resistência à insulina – frequentemente associada à obesidade e, consequentemente, a dietas hipercalóricas – passou a atingir também populações de menor poder econômico, as quais classicamente apresentavam maiores prevalências de doenças parasitárias, como a esquistossomose mansônica. A intercessão destas duas enfermidades promoveu um fenômeno muito interessante do ponto de vista imunológico. Enquanto a esquistossomose promove uma forte reação inflamatória do tipo Th2, obesidade e resistência à insulina estão relacionados à resposta do tipo Th1.

A forma como estas interações acontecem ainda parece longe de ser elucidada. No entanto, recentemente foi observado que os ovos do verme produzem antígenos solúveis contendo glicoproteínas e glicanos com a capacidade de promover a melhora da tolerância à glicose e da sensibilidade à insulina. Este efeito se daria através da estimulação de macrófagos no tecido adiposo para produzirem interleucina-10, a qual promoveria diminuição da inflamação e melhora da captação de glicose no tecido adiposo. Além disso, o composto lacto-N-fucopentaose III, especificamente, tem a capacidade de promover a diminuição da síntese de lipídeos no fígado (BHARGAVA et al., 2012; CHEN et al., 2013).

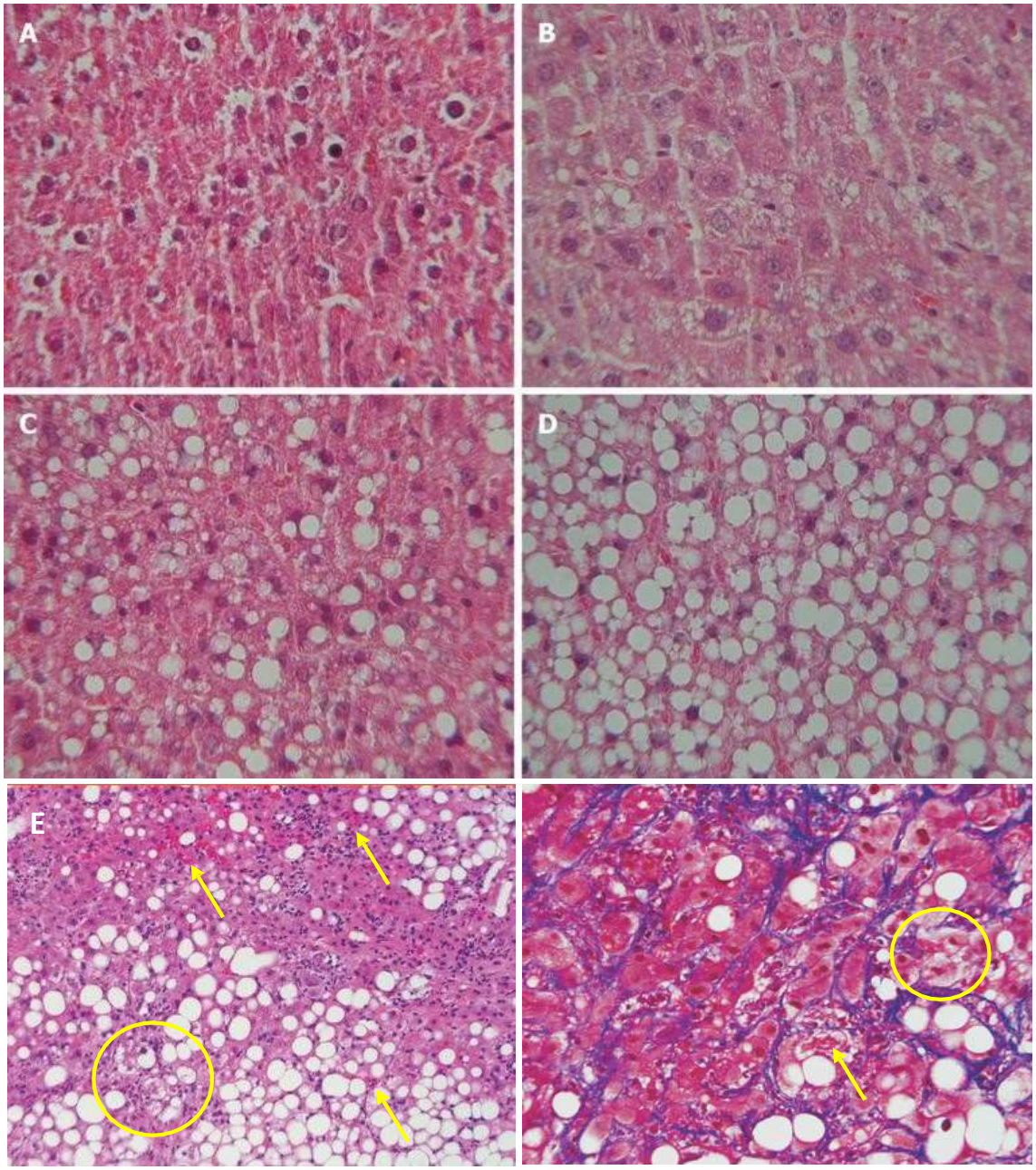
Assim, é possível perceber as várias comunicações entre o distúrbio hepático provocado pela esquistossomose mansônica e diversas rotas metabólicas que, integralmente, levam ao comprometimento geral do organismo do indivíduo afetado. Contudo, além dos problemas hepáticos advindos das infecções, representados aqui pela esquistossomose mansônica crônica, diversos distúrbios não infecciosos podem provocar sério comprometimento da função hepática. Entre eles se destaca a Doença Hepática Gordurosa Não Alcoólica (DHGNA).

## 2. Doença Hepática Gordurosa Não Alcoólica

A DHGNA é definida por alterações hepáticas que se assemelham a doença hepática induzida por álcool, mas que ocorre em pacientes que não apresentem consumo de álcool significativo. A doença inclui amplo espectro de alterações histológicas desde esteatose simples, passando pela esteato-hepatite podendo evoluir até fibrose e cirrose, para o diagnóstico é necessária correlação anátomo-clínica e exclusão de causas secundárias. A definição pressupõe a abstenção de álcool, existindo grande discussão na literatura sobre qual seria o ponto de corte aceitável para não considerar o álcool como o responsável por este processo. A maioria dos autores fala em consumo de até 20g de álcool para homens e 10g para mulheres (ASSOCIAÇÃO AMERICANA DE GASTROENTEROLOGIA, 2002).

A definição patológica da presença de esteatose é gordura hepática excedendo 5% do total do peso do órgão. Quando em avaliação microscópica, o critério é usualmente a presença de gordura em mais de 5% dos hepatócitos. A presença de esteato-hepatite não alcoólica (EHNA) é definida pela concomitância de processo inflamatório, que costuma ser misto, envolvendo tanto células mononucleares como polimorfonucleares. Outros achados característicos em humanos são a degeneração dos hepatócitos em balão (balonamento) e o aparecimento dos corpos de Mallory-Denk; posteriormente, ocorre o aparecimento de fibrose, que inicialmente é perivenular, mas depois envolve outras regiões (OH; WINN; POODARD, 2008) (**FIGURA 4**).

**Figura 4:** Cortes histológicos de fígado de camundongo corados com hematoxilina e eosina (A-E) ou Tricromo de Masson (F) demonstrando o desenvolvimento de DHGNA. **A:** Fígado Normal; **B**: Esteatose de grau 1 (5% a 33%); **C**: Esteatose de grau 2 (34% a 66%); **D**: Esteatose de grau 3 (67% a 100%). **E:** Esteato-hepatite; **F:** Esteato-hepatite com fibrose. Círculos destacam células em balonamento e setas destacam Corpos de Mallory-Denk.




FONTE: Adaptado de OH; WINN; POODARD, 2008 e HIJONA et al., 2010.

O entendimento da patogênese da DHGNA passa pelo reconhecimento da interação metabólica que o fígado mantém com os diversos tecidos corporais. De maneira que o acúmulo de lipídeos no fígado, característica conceitual da DHGNA,

deriva-se fundamentalmente do excesso de energia disponível no organismo. Este excesso é frequentemente causado por ingestão excessiva de calorias, as quais causam obesidade e outros distúrbios associados.

Dietas ricas em gordura têm sido utilizadas muito eficazmente em modelos animais, principalmente camundongos, como forma de mimetizar os efeitos observados em humanos – obesidade, dislipidemias, resistência insulínica etc – apesar das diferenças metabólicas existentes. Camundongos não expressam CETP, enzima responsável pela transferência de éster de colesterol da HDL para a LDL. Além disso, camundongos também produzem ApoB-48 no fígado, a qual apresenta uma maior depuração hepática que a ApoB-100 em humanos. Roedores em geral apresentam, ainda, maior taxa metabólica, a qual influencia nos níveis de ácidos graxos circulantes e desenvolvimento de obesidade. Por estas diferenças, há aumento considerável de HDL-c em relação aos níveis de LDL-c em camundongos, diferentemente dos humanos, nos quais há bem mais colesterol nas partículas de LDL. (KIM; YOUNG, 1998; LIMA et al., 1998).

Inúmeros modelos animais de investigação de obesidade e morbidades associadas têm sido desenvolvidos ao longo das últimas décadas. Como resultado, três classes de modelos animais são utilizadas hoje: modelos dietéticos, modelos genéticos e modelos que utilizam tanto modificações genéticas quanto na dieta. Nos modelos dietéticos, são utilizadas dietas hipercalóricas, a maioria baseada em lipídeos. Neste campo há uma ampla gama de formulações, com vasta variação nas fontes animais (banha de porco, pele de frango, manteiga, gema de ovo e cérebro bovino) ou diferentes óleos vegetais. Os modelos genéticos se baseiam no silenciamento ou mutação de genes reguladores metabólicos, como o modelo ob/ob para a leptina (ARAÚJO et al., 2011, 2012).

Além disso, nos últimos anos, tem-se aumentado o número de modelos que buscam investigar o efeito de componentes específicos nas dietas. Como as dietas deficientes em metionina e colina, ricas em sacarose ou frutose, ou ainda, ricas em colesterol. Estes modelos, assim como os mais tradicionais, apresentam vantagens e desvantagens que devem ser levados em consideração de acordo com o objetivo de investigação. Além disso, variações como arcabouço genético dos animais, tempo de

dieta, idade de início e sexo dos animais também podem levar a diferentes respostas (BUETTNER et al., 2007; PIERCE et al., 2015).

A obesidade é caracterizada pela presença de maior peso do que é considerado saudável para a altura do indivíduo, geralmente avaliado pelo índice de massa corpórea (IMC), o qual é calculado utilizando o peso corporal em quilogramas dividido pela altura em metros, ao quadrado (Peso [Kg] / Altura$^2$ [m]). Valores elevados de IMC geralmente refletem expansão do tecido adiposo corporal. Esta avaliação pode ser traduzida para os modelos experimentais pela medição da razão entre o peso e o tamanho do animal, ou mais eficientemente, pela determinação da gordura corporal (KUHEL et al., 2013).

A principal função do tecido adiposo é armazenar e liberar ácidos graxos, os quais são incorporados nos triglicerídeos de adipócitos de acordo com demandas de energia a todo o organismo. A massa adiposa corporal é determinada pelo equilíbrio entre armazenamento e remoção de triglicerídeos em adipócitos. A expansão do tecido adiposo em resposta ao excesso de ingestão calórica é uma resposta sistêmica importante para evitar os efeitos colaterais lipotóxicos exercidos pelo excesso de deposição de lipídeos em outras células (ASTERHOLM et al., 2014).

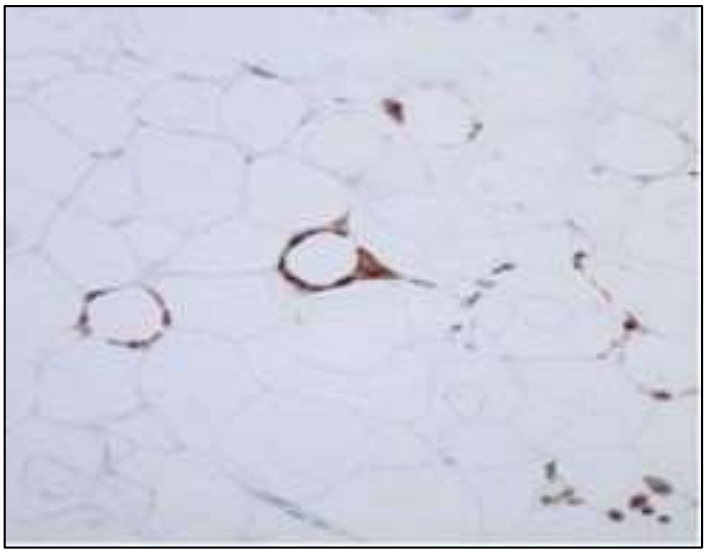
Nos estados de aumento da demanda de ácidos graxos, como nas dietas hiperlipídicas, o tecido adiposo responde primeiramente com aumento do número de células – devido à multiplicação e diferenciação de pré-adipócitos, angiogênese e remodelamento da matriz extracelular. Diversos fatores parecem estar envolvidos neste processo. No entanto, tem sido observado que a expressão de diversos genes envolvidos na maturação é dependente da ativação do *peroxisome proliferator-activator receptor gamma* (PPARy), um fator de transcrição ativado por ácidos graxos. Entretanto, a continuidade do influxo aumentado de ácidos graxos sobrecarrega o adipócito e gera dano ao tecido, provavelmente causado pela incapacidade tecidual de manter um aporte adequado de oxigênio. (KUBOTA et al., 1999; ARNER et al., 2010; CUMMINS et al., 2014; SHULMAN, 2014).

O estresse celular no tecido adiposo provoca então apoptose de adipócitos e infiltração de macrófagos. Este processo de sobrecarga de ácidos graxos, estresse, apoptose e inflamação no tecido adiposo se torna contínuo. Este estado é conhecido como inflamação crônica de baixo grau. Contudo, é importante salientar que o termo

"crônica" se reflete ao tempo prolongado e caráter persistente da inflamação e não a uma inflamação do tipo Th2, como comumente descrita em imunologia.

Muitos fatores pró-inflamatórios são produzidos no tecido adiposo do indivíduo obeso. Há aumento da expressão de TNF-α, interleucina-6, interleucina 1 beta (IL-1β), proteína quimiotática de monócitos 1 (MCP-1), sintase de óxido nítrico induzível (iNOS), fator de crescimento transformante beta 1 (TGF-β1), etc. Boa parte destes fatores é produzido por macrófagos infiltrados no tecido adiposo, os quais atuam em conjunto na fagocitose de adipócitos mortos, formando estruturas com formato semelhante ao de uma coroa, chamadas *crown-like structures* (GREENBERG; OBIN, 2006) (**FIGURA 5**).

**FIGURA 5**: Adipócitos rodeados por macrófagos identificados por imuno-histoquímica de CD68.



**FONTE:** Adaptado de BIGORNIA et al., 2012.

Durante a expansão do tecido adiposo, observada na obesidade, a função endócrina dos adipócitos também entra em desequilíbrio. No estado normal de funcionamento, tanto adipócitos quanto outras células do tecido produzem diversas substâncias com atividade endócrina, como leptina e adiponectina.

A leptina é produzida proporcionalmente ao volume do tecido adiposo e em resposta a uma miríade de fatores estimulatórios como insulina, glicocorticoides, ativadores de PPARy e hormônio do crescimento. Os principais efeitos da leptina estão envolvidos com a homeostase energética, em que atua em nível hipotalâmico e também periférico na regulação do apetite e do gasto energético. Este hormônio atua

como um termômetro do nível de reserva energética do organismo, em que reduz rapidamente quando há diminuição das reservas de forma a induzir aumento do apetite e redução do gasto energético corporal. Por outro lado, durante a obesidade há desequilíbrio desse sistema, apesar dos níveis elevados de leptina é observada resistência aos seus efeitos. A adiponectina, diferentemente da leptina, apresenta secreção relacionada com o número de adipócitos diferenciados e não com o volume do tecido adiposo. Receptores para adiponectina são encontrados principalmente em músculos e no fígado e atuam de maneira a melhorar a eficiência do uso de ácidos graxos nestes tecidos (YAMAUCHI et al., 2001; KERSHAW; FLIER, 2004).

A partir do funcionamento endócrino do tecido adiposo é possível compreender como este órgão atua de maneira a proteger o organismo da lipotoxicidade. Entretanto, na expansão descontrolada observada durante a obesidade, este mesmo tecido não consegue mais proteger os outros órgãos do excesso de ácidos graxos e acaba por se tornar fonte de produção de diversos fatores deletérios para o funcionamento normal do organismo. Enfim, a obesidade pode ser vista como um desequilíbrio metabólico do tecido adiposo (incapacidade de absorção adicional dos ácidos graxos ingeridos, desenvolvimento de processos inflamatórios e desregulação endócrina) que repercutem no funcionamento geral do organismo. Neste ponto, a obesidade, através das dislipidemias e da resistência à insulina, pode ser considerada o elo entre as dietas hiperlipídicas e a DHGNA.

No final dos anos 1990 foi desenvolvida a primeira teoria para o desenvolvimento de DHGNA, baseada na hipótese de que primeiro haveria acúmulo hepático de triglicerídeos (esteatose), o qual aumentaria a susceptibilidade para um segundo passo ou “golpe” (*hit*, em inglês) o qual desencadearia a cascata de progressão da DHGNA. Este efeito seria mediado por citocinas e adipocinas inflamatórias, disfunção mitocondrial e estresse oxidativo, que promoveriam inflamação no fígado. A progressão do processo inflamatório levaria à fibrose, perda de função hepática e, nos casos mais graves, poderia levar à cirrose e carcinoma hepatocelular. Por causa da organização em dois passos, acúmulo de lipídeos e depois inflamação, esta hipótese foi chamada de *two-hit theory* (DAY; JAMES, 1998; DAY, 2006).

O acúmulo de triglicerídeos no fígado acontece, basicamente, pelo aumento do influxo de triglicerídeos e/ou diminuição da capacidade de metabolizá-los. Isto pode

acontecer devido ao aumento do fornecimento de ácidos graxos livres provenientes do tecido adiposo, aumento da lipogênese *de novo*, diminuição da oxidação de ácidos graxos, ou ainda pela diminuição da secreção de triglicerídeos através das VLDLs (BROWNING; HORTON, 2004; ANSTEE; GOLDIN, 2006; MALAGUARNERA et al., 2009).

A teoria desenvolvida pelos pesquisadores britânicos Day e James em 1998 foi muito importante pois possibilitou uma estratificação e classificação das fases do ponto de vista do tempo da doença. Entretanto, com o passar dos anos foi sendo observado que o acúmulo de triglicerídeos hepáticos não detinha uma correlação fisiopatológica com a progressão da DHGNA. Alguns estudos demonstraram que, pelo contrário, pacientes com maior capacidade de armazenar lipídeos no fígado na forma de triglicerídeos teriam relativa proteção para o desenvolvimento de EHNA. Outros estudos também observaram que a inflamação grave poderia acontecer mesmo com esteatose moderada ou leve (DAY; JAMES, 1998; FELDSTEIN et al., 2004; YAMAGUCHI et al., 2007; TILG; MOSCHEN, 2010; TINIAKOS et al., 2010).

Dessa forma, a pesquisa da DHGNA progrediu para a identificação de agentes responsáveis pela progressão da doença. Diversas moléculas lipídicas se mostraram capazes de induzir inflamação no tecido hepático: diferentes tipos de ácidos graxos, ceramidas, diacilglicerois, lisofosfatidilcolina, colesterol e seus metabólitos oxidados atuam como indutores da formação de espécies reativas de oxigênio (PEVERILL; POWELL; SKOIEN, 2014).

Além de lipídeos, carboidratos também têm sido associados ao desenvolvimento de DHGNA. Sacarose tem a capacidade de induzir lipogênese *de novo* e provocar rápida progressão pra EHNA em animais submetidos à dieta deficiente em metionina e colina, sendo a frutose mais citotóxica do que a glicose para este modelo. Adicionalmente, alto consumo de frutose causa esteatose hepática acompanhada de depleção de fosfato intracelular. A frutose é metabolizada principalmente por frutocinase, que existe em duas isoformas: frutocinase C e A. A frutocinase C é a principal isoforma no fígado e metaboliza frutose a frutose-1-fosfato rapidamente, resultando na depleção de fosfato e adenosina trifosfato (ATP) intracelular temporariamente. Em contraste, a frutocinase A metaboliza frutose lentamente, sem consumo de ATP significativo. Estudos utilizando comundongos *knockout* têm

mostrado que a atividade de frutocinase C é responsável pela esteatose hepática. Adicionalmente, animais *knockout* para a frutocinase A apresentaram aumento dos níveis de lipídeos hepáticos devido ao aumento do metabolismo da frutose através da frutocinase C. O metabolismo de frutose resulta no aumento da rotatividade de nucleotídeos e geração de ácido úrico que podem ter um papel na indução de estresse oxidativo mitocondrial e acumulação de gordura (PICKENS et al., 2009; ISHIMOTO et al., 2012).

Entretanto, os mecanismos pelos quais tais compostos, lipídeos e carboidratos, induziriam a inflamação ainda não são completamente conhecidos. Parte da dificuldade acontece por que a maioria dos estudos têm se dedicado à investigação de uma molécula ou processo por vez. Como muitas destas moléculas apresentam lipotoxicidade, têm-se a ideia de que há um efeito redundante sobre a progressão da DHGNA, no entanto, com o aprofundamento do conhecimento das vias mecanísticas envolvidas tem sido observado meios de atuação diferentes. Por outro lado, há ainda a possibilidade de que os diferentes processos convirjam para uma mesma rota, ainda não esclarecida.

A inflamação característica da EHNA causada por dietas ricas em gorduras ou carboidratos é desencadeada, em parte, pela ativação das vias NF-κB, o qual está associada a uma elevada expressão de citocinas inflamatórias, como TNF-α, IL-6, IL-1β e ativação das células de Kupffer. No paciente com EHNA, os níveis séricos e hepáticos de TNF-α e IL-6 estão elevados e se correlacionam com a severidade histológica, além de promover a resistência à insulina (CRESPO et al., 2001; YUAN et al., 2001; HUI et al., 2004; KLOVER; CLEMENTI; MOONEY; 2005; CAI et al., 2005).

Por causa do dano hepático, o sistema de reparo tecidual é ativado. Células mortas são substituídas por matriz extracelular e subsequentemente por novos hepatócitos, através da proliferação das células sobreviventes ou de células progenitoras. Entretanto, com o prolongamento da injúria, como observado na DHGNA, a formação de matriz extracelular é mais rápida do que a capacidade do tecido de regeneração. Isto provoca uma distorção na arquitetura tecidual que culmina maior dano tecidual. Histologicamente, é observada a formação das chamadas “pontes” de fibrose entre a veia central e a tríade portal no lóbulo hepático. O processo de reparo tecidual / fibrose como um todo é promovido pela atuação de diversas

citocinas sintetizadas localmente em resposta ao dano celular, como TGF-β, TNF-α, fator de crescimento derivado de plaquetas beta e fator de crescimento semelhante à insulina 1 (RAMADORI; SAILE, 2004; RAMADORI et al., 2007).

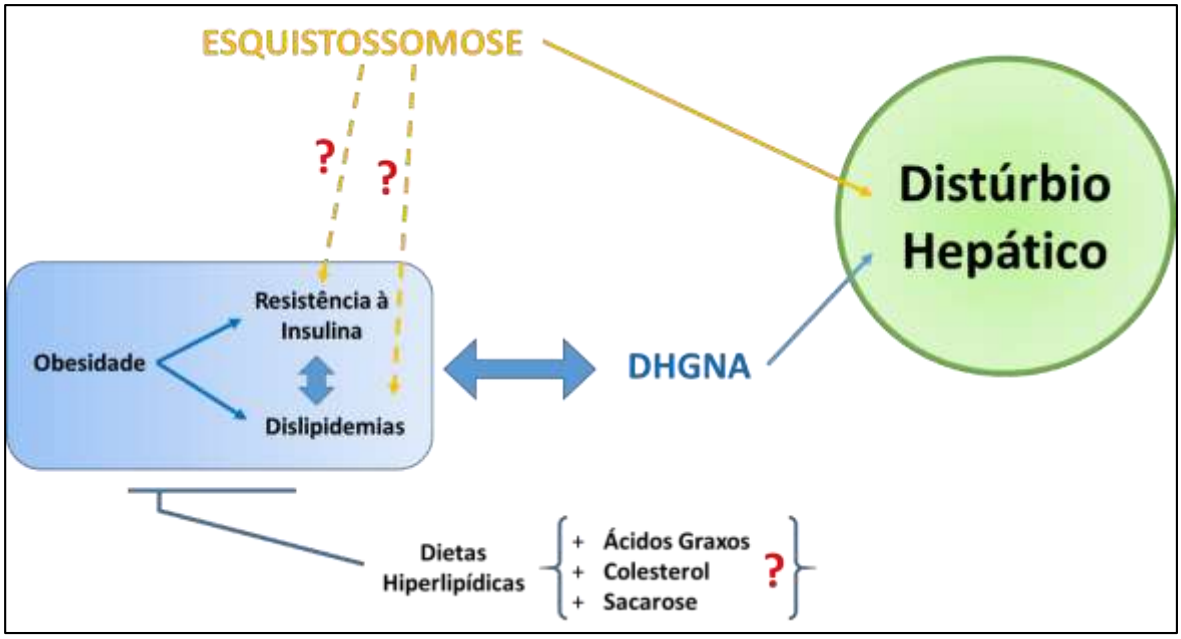
O dano fibrótico acumulado pode levar a cirrose. Caracterizada pela formação de nódulos regenerativos no parênquima hepático separados pelo septo fibrótico, resultado de morte celular, deposição de matriz extracelular desregulada e reorganização vascular. Neste estágio observa-se frequentemente insuficiência hepática e hipertensão portal. Ciclos sustentados de destruição hepatocelular e proliferação compensatória em resposta a toxicidade metabólica e oxidativa, inflamação, imunidade inata e adaptativa, e fibrose criar um ambiente propício à carcinogênese. Aberrações cromossômicas acumulam com a evolução da DHGNA através de cirrose em HCC. Inicialmente, mecanismos epigenéticos podem levar a hipo- ou hipermetilação aberrante de DNA em grupos de CpG nas regiões de promotores e outros segmentos cromossômicos além de induzir cis- e de transativação e acetilação da cromatina. Posteriormente, estas modificações epigenéticas podem levar a lesões genômicas estruturais, tais como mutações pontuais, deleções alélicas múltiplas, ganhos cromossômicos, a erosão dos telômeros e reativação da telomerase. Um passo crítico nestes processos é a seleção de populações monoclonais de hepatócitos pré-malignas ou células progenitoras a partir da qual emergem HCC (PARADIS et al., 1998; KONDO et al., 2000; THORGEIRSSON; GRISHAM, 2002; BAFFY; BRUNT; CALDWELL, 2012).

A complexidade da DHGNA tem se mostrado um desafio instigante para os pesquisadores do mundo todo. Inúmeros fatores estão presentes e o entendimento da maneira como cada um atua é um alvo difícil de ser atingido, principalmente pela grande interação existente. A DHGNA surge como face hepática de obesidade, resistência insulínica e dislipidemias em uma indissociável relação de causa e efeito simultâneos e progressivos. Neste cenário, a inflamação atua de maneira muito importante e adiciona ainda mais complexidade ao esclarecimento dos mecanismos envolvidos. Deste modo, é de fundamental importância para o entendimento da DHGNA que sejam feitas pesquisas que investiguem de maneira integrada os distúrbios metabólicos envolvidos de maneira a formar um quadro mais completo da fisiopatologia da doença. Ademais, para que isso aconteça, é necessária uma clara

delimitação dos processos iniciais da doença, o que inclui a identificação dos mecanismos pelos quais as moléculas causadoras da sobrecarga metabólica atuam.

Por outro lado, a identificação destes diversos processos envolvidos com a doença hepática transporta a problemática da investigação para outras doenças causadoras de distúrbios no fígado e, neste caso, promove o retorno para o ponto inicial da discussão, a esquistossomose mansônica. Deste modo, as diferenças evidentes entre esquistossomose e DHGNA do ponto de vista imunopatológico podem, ao invés de serem ponto negativo, proverem um ponto de conexão para o entendimento de possíveis distúrbios metabólicos presentes na esquistossomose, como dislipidemias e resistência à insulina (**FIGURA 6**).

**FIGURA 6**: Representação esquemática do estudo.




FONTE: Fonseca, 2015.

## 4. Objetivos

### 4.1 Objetivo Geral

Investigar as repercussões metabólicas dos Distúrbios Hepáticos Crônicos causados por Esquistossomose Mansônica Hepatoesplênica e pela Doença Hepática Gordurosa Não Alcoólica.

### 4.2 Objetivos Específicos

- Avaliar as repercussões da esquistossomose mansônica hepatoesplênica sobre os níveis de lipídeos plasmáticos e sua relação com o polimorfismo de ApoE.

- Investigar a influência dos genótipos de ApoE sobre a chance de infecção e o desenvolvimento de esquistossomose mansônica hepatoesplênica.

- Pesquisar a existência de resistência à insulina em pacientes com esquistossomose mansônica hepatoesplênica.

- Correlacionar os níveis de lipídeos plasmáticos e índices lipídicos com a resistência à insulina em sujeitos com esquistossomose mansônica hepatoesplênica.

- Investigar a influência de dietas ricas em ácidos graxos, colesterol e sacarose sobre o desenvolvimento de obesidade, resistência à insulina, inflamação e dislipidemias.

- Avaliar a influência de dietas ricas em ácidos graxos, colesterol e sacarose sobre o desenvolvimento de Doença Hepática Gordurosa Não Alcoólica.

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## 6. Artigo 1

Human Plasma Lipid Modulation in schistosomiasis Mansoni Depends on Apolipoprotein E Polymorphism

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# Human Plasma Lipid Modulation in Schistosomiasis Mansoni Depends on Apolipoprotein E Polymorphism

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## Abstract

***Background:*** Schistosomiasis mansoni is a parasitic liver disease, which causes several metabolic disturbances. Here, we evaluate the influence of Apolipoprotein E (*APOE*) gene polymorphism, a known modulator of lipid metabolism, on plasma lipid levels in patients with hepatosplenic schistosomiasis.

***Methodology/Principal Findings:*** Blood samples were used for *APOE* genotyping and to measure total cholesterol (TC), LDL-C, HDL-C and triglycerides. Schistosomiasis patients had reduced TC, LDL-C and triglycerides (25%, 38% and 32% lower, respectively; $P<0.0001$) compared to control individuals, whereas HDL-C was increased (10% higher; $P=0.0136$). Frequency of the common alleles, ε2, ε3 and ε4, was similar ($P=0.3568$) between controls (n = 108) and patients (n = 84), implying that *APOE* genotype did not affect susceptibility to the advanced stage of schistosomiasis. Nevertheless, while patient TC and LDL-C levels were significantly reduced for each allele (except TC in ε2 patients), changes in HDL-C and triglycerides were noted only for the less common ε2 and ε4 alleles. The most striking finding, however, was that accepted regulation of plasma lipid levels by *APOE* genotype was disrupted by schistosomiasis. Thus, while ε2 controls had higher TC and LDL-C than ε3 carriers, these parameters were lower in ε2 versus ε3 patients. Similarly, the inverse relationship of TG levels in controls (ε2>ε3>ε4) was absent in patients (ε2 or ε4>ε3), and the increase in HDL-C of ε2 or ε4 patients compared to ε3 patients was not seen in the control groups.

***Conclusion/Significance:*** We confirm that human schistosomiasis causes dyslipidemia and report for the first time that certain changes in plasma lipid and lipoprotein levels depend on *APOE* gene polymorphism. Importantly, we also concluded that *S. mansoni* disrupts the expected regulation of plasma lipids by the different ApoE isoforms. This finding suggests ways to identify new metabolic pathways affected by schistosomiasis and also potential molecular targets to treat associated morbidities.

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## Introduction

Schistosomiasis, caused by *Schistosoma mansoni* worms, is one of the most prevalent parasitic diseases. More than 200 million people are infected and worldwide at least 280,000 people die because of schistosomiasis every year, most in developing countries [1,2]. *S. mansoni* infections progress to hepatic fibrosis associated with portal blood hypertension [3] and 5–10% of patients present with the most severe form, hepatosplenic schistosomiasis [4,5].

Previous studies have reported that human schistosomiasis alters plasma lipid composition [6–9] and metabolism [10]. From animal model studies, it is generally agreed that *S. mansoni* infection reduces levels of plasma cholesterol and triglycerides in both rodents [11,12] and non-human primates [13,14]. Nevertheless, the mechanisms behind these changes and the possible consequences for human health are not well understood.

One factor known to affect human plasma lipid concentrations is Apolipoprotein E (*APOE*, gene; ApoE, protein), which distributes between triglyceride-rich lipoproteins (very-low-density lipoproteins, VLDL and postprandial chylomicrons) and high-density lipoproteins (HDL), helping to regulate their metabolism and the plasma levels of cholesterol and triglyceride. The *APOE* gene is polymorphic with three major alleles, ε2, ε3 and ε4, arising from point mutations at a single gene locus to produce three common protein isoforms, ApoE2, E3, and E4. The parent form, ApoE3 has cysteine and arginine residues at positions 112 and 158, respectively, while ApoE2 (Arg158Cys) and ApoE4 (Cys112Arg) have single amino acid substitutions [15,16]. These variant ApoE isoforms have different receptor binding activities, which affect lipoprotein clearance, while their differential affinity for triglyceride-rich lipoproteins influences lipolysis [17,18].

due to inefficient recycling of the ApoE4 protein into the Space of Disse [71]. Consistent with the data from meta-analyses [56,69,70], we found that the mean TG values for control individuals with ε4- and ε2-alleles were higher than the ε3/ε3 group (though $P<0.05$ only for ε4-carriers; Figure 1D). Unexpectedly, the mean TG (252±48 mg/dL) of this ε4 control group was much higher than seen in other studies, including different Brazilian populations [30–32,72], an unexpected finding for which we have no immediate explanation. Of greater interest, however, was that the mean TG level in the ε3/ε3 patients was higher, albeit not significant, than levels in patients carrying ε2- or ε4-alleles (Figure 1D). These data suggest that the mechanism(s) which promotes increased plasma TG in healthy ε2- and ε4-carriers is either inoperative or ineffective in schistosomiasis patients.

One limitation of this study is that it was conducted only at a single hospital, the Hospital das Clínicas, UFPE, which is the reference hospital for schistosomiasis in Pernambuco State, Brazil. Here, the Gastroenterology Outpatient Department receives the most severe cases of schistosomiasis, usually patients with a history of one or more episodes of gastrointestinal bleeding and hence most of the patients have the hepatosplenic form of the disease. Moreover, we had no information on plasma lipid levels before infection to compare with levels after the patients had developed the hepatosplenic form of schistosomiasis. Therefore, the findings from the present study may not be extrapolated to all patients from other endemic areas who present with the hepatosplenic form of the disease.

In summary, we confirm that human schistosomiasis causes dyslipidemia and, for the first time, report that certain changes in plasma lipid levels and lipoprotein profiles are dependent on patient *APOE* gene polymorphism. Importantly, we also conclude that the normal regulation of plasma lipid levels by *APOE* genotype is disrupted by schistosomiasis mansoni. This finding merits further investigation; it may uncover new metabolic pathways and pathological processes associated with human schistosomiasis. In turn, these may identify molecular targets to aid treatment of schistosomiasis morbidity, and perhaps also inform other lipid-associated diseases, including atherosclerosis and diabetes.

## Author Contributions

Conceived and designed the experiments: CSMF CAS VLML. Performed the experiments: CSMF AAPF BSS ALCD. Analyzed the data: CSMF BSS AAPF VLML. Contributed reagents/materials/analysis tools: VLML. Wrote the paper: CSMF AAPF BSS CAS ALCD JSO VLML.

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effect of schistosomiasis. We, and others, have previously reported low plasma total cholesterol in human studies [6,7] and in infected animals [11–13,41]. Doenhoff et al. [11] have shown that when fat-fed ApoE-deficient mice are infected with *S. mansoni* the decrease in plasma cholesterol is associated with a 30% reduction in atherosclerotic plaque progression, consistent with the low frequency of atherosclerosis noted in schistosomiasis patients [6,42].

Schistosomes need, but do not synthesize, cholesterol and one explanation for reduced host plasma cholesterol is that adult worms internalize LDL, via tegumental proteins analogous to mammalian LDL receptors [43]. Another suggestion is that the worms shed antigenic glycosyl-phosphatidylinositol (GPI)-anchored proteins into the circulation, which are sequestered by host lipoprotein particles [44]. Subsequent, antibody attack leads to lipoprotein removal by neutrophil endocytosis, although any plasma cholesterol-lowering effect in vivo has yet to be assessed. Against both these mechanisms is the failure of same-sex worms to lower cholesterol during mouse infections [41], implying that adult worms alone are not responsible and that the parasite's eggs are hypocholesterolemic. This concept is supported by La Flamme et al. [12] who noted reduced plasma cholesterol in mice chronically exposed to schistosome eggs, while Stanley et al. [41] found that soluble factors released from *S. mansoni* eggs were responsible.

Although TC and LDL-C were decreased in SM patients, we noted increased levels of HDL-C, consistent with an early report that alpha-lipoproteins were significantly higher in patients with Bilharzial hepatic fibrosis [6]. By contrast, infection of ApoE-deficient mice with *S. mansoni* cercariae resulted in reduced HDL-C [11], although levels do not change during chronic exposure to schistosome eggs [12]. However, direct comparisons of mouse and human plasma lipoprotein metabolism are complex, as there is a marked difference in the LDL-C to HDL-C ratio [13]. Absence of cholesteryl ester transfer protein (CETP) in mice increases HDL levels compared to humans [45], while much of their LDL is cleared rapidly using ApoE as ligand rather than the slow ApoB100 pathway used by human LDL [46].

Effects of infection and inflammation on host lipoprotein metabolism are multi-faceted. Although the acute-phase response inhibits ApoAI synthesis and lowers HDL-C [47,48], the most profound changes are in structure and composition, which transform the HDL from anti-inflammatory to proinflammatory particles [49–51]. Such pathological changes in HDL have yet to be studied in hepatosplenic schistosomiasis, although chronic inflammation is known to impair reverse cholesterol transport and the antioxidant capacity of HDL [52–54]. Indirect evidence suggests that HDL in schistosomiasis patients is a poor antioxidant, as we previously found elevated levels of erythrocyte lipid peroxidation [55].

In humans, *APOE* polymorphism is well-documented to affect plasma TC and LDL-C; for example, meta-analyses by Bennet et al. [56] found differences between ε2/ε3 and ε3/ε4 carriers, the most common genotypes after ε3/ε3, of 8% and 14%, respectively. As indicated earlier, differential binding affinities of the individual ApoE isoforms to receptors and for surfaces of triglyceride-rich lipoprotein particles underlie such variation [16,18]. We also noted effects of *APOE* genotype on TC and LDL-C in our controls as mean values were significantly lower in ε2-carriers compared to the ε3/ε3 group ($P = 0.0052$ and $P = 0.0157$, respectively), though unchanged for the ε4-allele. Interestingly, schistosomiasis abolished, and indeed reversed, this relationship; ε2 patients had higher mean TC and LDL-C than ε3/ε3 patients, although only the TC difference reached significance ($P = 0.0458$).

The relation of *APOE* genotypes with HDL-C was reported by Bennet et al. [56] to be inverse and weak with a 5% difference between the ε2/ε3 and ε3/ε4 carriers. Despite small numbers, we also noted a slight but significant fall of HDL-C in control ε4 carriers compared to their ε3/ε3 counterparts. The small (10%) HDL-C increase in our schistosomiasis patients (Table 1) was due to higher levels in ε2- and ε4-carriers, as the HDL-C of ε3/ε3 genotypes was near-identical for controls and patients. Can these findings be explained? One difficulty is the complexity of HDL formation, maturation and clearance, namely reverse cholesterol transport, which though involving the major HDL protein, ApoAI, is influenced and assisted at each step by ApoE [57]. Thus, initial sequestration of excess cellular cholesterol [58], activation of the cholesterol esterifying enzyme, plasma lecithin-cholesterol acyltransferase (LCAT) [59] and cholesterol ester delivery to the liver [17,60] are all processes that involve ApoE in an isoform-dependent manner. To this complexity, we can overlay HDL metabolic changes due to *S. mansoni* infection and associated inflammatory responses and fibrogenesis. For example, we have reported LCAT deficiency in human [7] and animal [13] schistosomiasis, while decreased CETP activity is a feature of the acute-phase response [47] and raises HDL levels, particularly ApoE-rich HDL [18,61].

We can speculate, therefore, that HDL-C increases in schistosomiasis are a multi-step process, promoted by low CETP activity and enhanced further by the ε2-allele: ApoE2 has a higher affinity than ApoE3 or ApoE4 for HDL [62], allowing the particles to expand in size [18,63], while defective ApoE2 receptor binding delays their clearance from plasma via hepatic LDL-receptors [17,60]. A different scenario is required to explain the HDL-C increase in the ε4-carrying patients, since ApoE4 associates poorly with HDL and has high affinity for LDL receptors, properties predicted to reduce HDL-C. One tentative possibility is that the poor antioxidant capacity of cysteine-negative ApoE4 [64] allows excessive formation of oxidized HDL, particularly as *S. mansoni* infections markedly increase oxidative stresses [65]. As oxidized HDL impedes normal reverse cholesterol transport [51–53], this delay in maturation may increase HDL-C in patients with ε4-alleles.

The plasma triglycerides change has inconsistent results in humans, as seen in mice earlier reports [9,11,12]. We observed 30% reduction in plasma triglycerides in SM patients. The mechanism(s) causing reduced plasma TG is uncertain. It may simply reflect lower levels of non-HDL lipoproteins since the acquired LCAT deficiency of human schistosomiasis increases the TG:CE ratio of core lipids [9], or be independent of infection-related responses, as pulmonary fibrosis with non-infectious origins results in low plasma TG [64]. A direct effect is also possible, as *S. mansoni* infected mice had reduced hepatic expression of acetyl coenzyme A acyltransferase, an enzyme involved in fatty acid metabolism [67]. Nevertheless, data from animal studies are inconsistent. Infection of non-human primates resulted in TG rises >10% after 30 or 60 days, whereas in mice TG levels were reported to rise two-fold [11] or be unchanged 7–10 weeks post-infection [66], or to significantly decline from the 4$^{th}$ week [68].

Meta-analyses to assess association of *APOE* genotypes with plasma triglycerides report non-linear relationships, the ε2- and ε4-carriers having higher levels than those with the ε3/ε3 genotype [56,69,70]. For ApoE2 the simplest explanation is reduced binding and delayed hepatic clearance of VLDL remnants, whereas a dual mechanism is invoked for ApoE4: impaired lipolysis because ApoE4 has higher affinity for VLDL [17] and, paradoxically as it has high receptor binding, by the failure of ApoE4 to accelerate hepatic removal of VLDL remnants

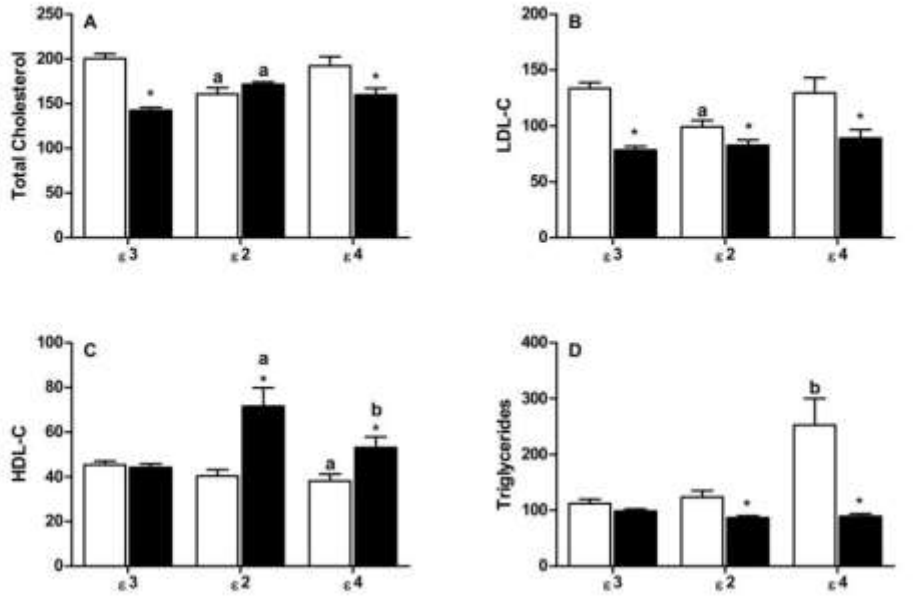


**Figure 1. Effect of *APOE* gene polymorphism on plasma levels of Total Cholesterol (A), LDL-C (B), HDL-C (C), and Triglycerides (D) in Control subjects (open bars) and SM patients (filled bars).** Plasma lipids are expressed in mg/dL. * = $P<0.05$ for group of SM patients vs. Control of the same allele. Comparisons between the different alleles of the same SM patient group, or of the same Control group, are indicated as follows: **a** = $P<0.05$ vs. ε3; **b** = $P<0.05$ vs. ε2 and ε3. Exact *P* values are given in Table 3.
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that the different ApoE isoforms do not affect progression of schistosomiasis to the chronic hepatosplenic condition. Conceivably, this conclusion may not hold for the earlier, less severe hepatointestinal stage or for hepatosplenic patients subdivided on the extent of liver fibrosis [3]. Though of interest, as *APOE* alleles are suggested to affect fibrosis progression in hepatitis C infection [26,37], a much larger patient population would be needed to ensure adequate power for subtle genotype effects [35]. Gene studies of individuals infected with schistosomiasis have found significant associations of cytokines related to the immune response [38–40]. However, to date and similar to our result, no study has reported a link between the *APOE* gene polymorphism and schistosomiasis prevalence or severity.

The changes we report in plasma lipoprotein profiles, reductions in TC and LDL-C and an increase in HDL-C, is considered cardioprotective and hence can be regarded as a beneficial side-

**Table 3.** Values of *P* from comparisons showed in Figure 1.

| | | TC | LDL-C | HDL-C | TG |
|---|---|---|---|---|---|
| Control vs SM | ε3 | <0.0001 | <0.0001 | 0.6337 | 0.1422 |
| | ε2 | 0.8494 | 0.0098 | 0.0005 | 0.0166 |
| | ε4 | 0.0403 | 0.0219 | 0.0113 | 0.0152 |
| Control | ε2 vs ε3 | 0.0052 | 0.0157 | 0.2533 | 0.7348 |
| | ε2 vs ε4 | 0.0547 | 0.0842 | 0.6825 | 0.0018 |
| | ε3 vs ε4 | 0.4590 | 0.7536 | 0.0431 | <0.0001 |
| SM | ε2 vs ε3 | 0.0458 | 0.9933 | <0.0001 | 0.3366 |
| | ε2 vs ε4 | 0.8342 | 0.3445 | 0.0316 | 0.9945 |
| | ε3 vs ε4 | 0.0586 | 0.1883 | 0.0413 | 0.2727 |

ANOVA followed by Fisher's PLSD test.
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**Table 1.** Participants, genotype and lipid parameters of participants.

| Parameters* | Control | SM | *P*-value |
|---|---|---|---|
| Age (years) | 47.0 ± 3.2 | 55.0 ± 2.3 | 0.0541 |
| Gender | | | |
| Male | 30 | 21 | - |
| Female | 78 | 63 | - |
| *N* total | 108 | 84 | 0.7309 |
| ε2 | 14 (13.0) | 8 (9.5) | - |
| ε3 | 73 (67.6) | 64 (76.2) | - |
| ε4 | 21 (19.4) | 12 (14.3) | - |
| TC | 194.4 ± 4.3 | 146.4 ± 3.0 | <0.0001 |
| LDL-C | 129.0 ± 4.5 | 79.8 ± 2.7 | <0.0001 |
| HDL-C | 43.4 ± 1.4 | 47.9 ± 2.7 | 0.0136 |
| TG | 140.6 ± 11.9 | 95.8 ± 2.8 | <0.0007 |

SM, schistosomiasis mansoni patients; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; Continuous variables are presented as mean ± standard error and were compared by unpaired t-test, whereas categorical variables are presented as absolute (percentage) frequencies and were compared by the Chi-square test.
*Plasma lipids are expressed in mg/dL.
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tion of HDL-C was significantly increased in the patients (10% higher; $P = 0.0136$) (Table 1).

To assess the influence of *APOE* gene polymorphism on plasma lipid parameters, we repeated the analyses after subdividing each group on the basis of *APOE* alleles. Lower TC and LDL-C levels were found in the ε3 subgroup (ε3/ε3 genotype) of SM patients, as observed without allele differentiation (Figure 1A and 1B). However, the increases in HDL-C and reductions in TG noted for all SM patients were not seen, even though the ε3/ε3 genotype is carried by 70% of participants (Figure 1C and D). This analysis of the ε3/ε3 genotype allows the influence of schistosomiasis on human lipid metabolism to be evaluated without possible interfering factors from the inclusion of ε2 and ε4 alleles. All the values of *P* from comparisons showed in Figure 1 are shown in Table 3.

Despite this significantly lower plasma TC in ε3 patients, the TC levels were similar ($P = 0.5360$) in ε2 patient and control groups. This reflected a marked HDL-C increase (77%) and a positive correlation between TC and HDL-C ($R = 0.724$; $P = 0.0250$) for patient ε2-carriers. By contrast, LDL-C was reduced in ε2 patients (as it was in ε3 patients; Figure 1A) and unrelated to TC levels ($R = 0.225$; $P = 0.5750$).

Plasma cholesterol changes associated with schistosomiasis were also noted for ε4-carriers. As with the ε2 allele, the ε4 SM patients had increased HDL-C (39% higher) compared to their control counterparts (Figure 1C); and like ε3-carriers they had reduced TC and LDL-C (Figure 1A and 1B).

Decreased plasma TG concentrations were seen in SM patients with the ε2 or ε4 variant alleles, but not for the ε3/ε3 genotype. However, the most striking difference in TG was noted in healthy ε4-carriers; their TG concentration was two-fold higher than the five other subgroups (Figure 1D).

## Discussion

Our report is the first to identify a host genetic factor, *APOE* polymorphism, which influences the extent and nature of plasma lipid changes associated with schistosomiasis mansoni. In future studies, this finding will help in understanding how the parasite affects particular steps in host lipid metabolism and how host genetic background modifies disease progression and morbidity.

Several studies have shown the *APOE* genotype to influence infection susceptibility and damage in certain diseases caused by viruses, including human immunodeficiency virus [18] and hepatitis C [26,35] and B [36], protozoa [23] and fungi [24]. As allele frequencies were similar for patients and controls, we infer

**Table 2.** *APOE* genotype frequencies among patients with hepatosplenic schistosomiasis mansoni and controls.

| Genotype | Control | SM |
|---|---|---|
| ε2/ε2 | 2 | 0 |
| ε2/ε3 | 12 | 8 |
| ε2/ε4 | 2 | 4 |
| ε3/ε3 | 73 | 64 |
| ε3/ε4 | 18 | 11 |
| ε4/ε4 | 1 | 1 |
| Total | 108 | 84 |

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In addition, ApoE has several biological functions not directly related to lipid transport, including roles in inflammation and the immune response [19,20], which may be modulated in an isoform-dependent manner [21,22]. Susceptibility and variable outcome of some infectious diseases is also linked to *APOE* gene polymorphism [23–26]. However, it remains unclear whether the plasma lipid changes induced by schistosomiasis depend on *APOE* genotype. Thus, the aim of our study was to determine whether the different *APOE* alleles influence plasma lipid levels and lipoprotein profiles in patients with hepatosplenic schistosomiasis mansoni.

## Methods

### Ethical Statement

The whole study was planned and executed following the Ethical Guidelines of the Helsinki Declaration. Participants were volunteers and all signed an informed consent statement after a full explanation about the scope of the study, including its objectives, procedures and potential risks. Ethical approval for all procedures was granted by the Human Research Ethics Committee, Center for Health Sciences, UFPE (Protocol No. 359/08).

### Study Area and Subjects

Eighty-four patients diagnosed with hepatosplenic schistosomiasis and attending the Gastroenterology Outpatient Department at the "Hospital das Clínicas - UFPE" were recruited during 2009 and 2010. The control group comprised 108 individuals with an epidemiological history incompatible with schistosomiasis and were drawn from the same age group (18–65 years) and socioeconomic background, as judged by a standardized questionnaire that enabled family budget, education level and lifestyle to be matched with those of the patients. Three stool samples from all individuals in both groups were also analyzed for parasitological infections. Subjects were excluded from the study if there was any evidence of parasitic infections, hepatitis B or C virus infections, cardiovascular or chronic kidney diseases, thyroid dysfunction or cancer. Individuals who had taken lipid-lowering drugs at anytime within the previous year were also excluded.

All participants lived in *Zona da Mata*, an endemic area in the state of Pernambuco, northeast Brazil, and their grandparents and parents were also born in this same region. The study population comprised unrelated individuals. Hepatosplenic schistosomiasis was diagnosed by physical examination and upper abdominal ultrasound, conducted by a qualified and experienced professional according to the WHO protocol for ultrasound of schistosomiasis-related morbidity [27]. The patients with hepatosplenic schistosomiasis mansoni (SM) had typical hepatosplenomegaly and portal hypertension, and at least 6 months prior to the study had been treated with praziquantel (50 mg/Kg).

### Sample Collection and Processing

Venous blood samples were drawn into evacuated tubes containing EDTA (0.362 M) after a 12 h fasting period. Plasma was separated within 2 h by centrifugation at 1500×*g* (10 min at 4°C), stored at −20°C and used for lipid analyses within 24 h. Whole blood samples were stored at 2–8°C and *APOE* genotype determined within 7 days.

### Biochemical Measurement

Plasma total cholesterol (TC) and triglyceride (TG) concentrations were assayed by routine enzymatic methods. HDL cholesterol (HDL-C) was measured after precipitation of ApoB-containing lipoproteins from plasma with phosphotungstic acid in the presence of magnesium ions [28]. Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula in subjects whose TG levels were ≤400 mg/dL [29]. Individuals whose TG levels were >400 mg/dL (six controls with the ε4-allele) were excluded from LDL-C analysis.

### Determination of *APOE* Genotype

Genomic DNA was extracted from leukocytes in whole blood, following a standard salting-out technique [30]. Single nucleotide *APOE* polymorphisms (rs7412 and rs429358) were detected by polymerase chain reaction (PCR) [31]. Amplified sequences were digested with the enzyme *Hha*I (5 units/mL) for 3 h and the restriction fragments were separated by 4% agarose gel electrophoresis and stained with ethidium bromide (0.5 mg/L).

Genotyping was performed with blinding to subject identity. Sequence-proven controls were run with each PCR. A random 1/24 of samples were genotyped again on another day; no discrepancies were observed.

### Statistical Analysis

The chi-square ($\chi^2$) goodness-of-fit test was used to assess deviation from Hardy-Weinberg equilibrium for each polymorphism and to compare categorical parameters among groups. All continuous variables were checked for normality and present a Gaussian distribution. Unpaired *t*-test was used to compare differences among continuous variables of SM patients and control individuals, while *APOE* allele groups were analyzed by one-way ANOVA followed by Fisher's Protected Least Significant Difference (PLSD). Lipid levels were adjusted for potentially confounding variables of age and gender. Pearson's Correlation test was used to estimate association between continuous parameters. Quantitative variables were expressed as mean ± standard error of media, while qualitative variables were expressed as absolute frequencies (percentage). *P*-values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using StatView SAS Inc. (1998; NC, USA).

To evaluate *APOE* genotype effects on schistosomiasis mansoni, subjects were categorized into three groups: ε2 carriers (ε2/ε2+ ε2/ε3 genotypes), ε3 carriers (ε3/ε3 genotype) and ε4 carriers (ε4/ ε4+ε4/ε3 genotypes). In each model, the homozygous ε3/ε3 genotypes formed the reference group. Six individuals (ε2/ε4; 3.13%) were excluded from the analyses because of the putative opposing effects of these two alleles.

## Results

For this cross-sectional study, the two groups were matched by age and gender, as shown in Table 1. The frequency of *APOE* alleles among all participants were: ε2 – 11.46%, ε3 – 71.35%, and ε4 – 17.19%, similar to other studies in Brazilian populations [32–34]; the detailed genotype frequency is given in Table 2. All SNPs were in accordance with Hardy-Weinberg equilibrium for both SM patients ($\chi^2 = 3.4164$, $\varphi = 3$, $p = 0.3318$) and controls ($\chi^2 = 3.2518$, $\varphi = 3$, $p = 0.3544$). Both control and patient groups showed similar mean age (control: $P = 0.3803$; SM: $P = 0.4125$) and gender frequency (control: $\chi^2 = 2.960$, $\varphi = 2$, $P = 0.2776$; SM: $\chi^2 = 2.439$, $\varphi = 2$, $P = 0.2953$) among the three different alleles. The allele frequencies were not statistically different between control and SM groups ($P = 0.3568$), indicating that *APOE* polymorphism was not able to affect the chance or course of schistosomiasis in this population.

When compared to healthy controls, the SM patients showed significant reductions ($P<0.0001$) in the plasma levels of TC (25%), LDL-C (38%) and TG (32%). By contrast, the concentra-

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## 7. Artigo 2

Insulin Resistance in Chronic Human Schistosomiasis mansoni

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# Insulin Resistance in Chronic Human Schistosomiasis mansoni

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## ABSTRACT

**Background:** Schistosomiasis is a parasitic disease that causes serious liver injuries. In some subjects with impaired fasting glucose, hepatic and extrahepatic insulin resistance seems to be responsible for postprandial glucose intolerance even in absence of dyslipidemia feature of the state of insulin resistance. The main objective was to investigate insulin resistance in patients with hepatosplenic schistosomiasis mansoni without spleen surgery (HS) and with splenectomy (HSS).
**Methodology/Principal Findings:** A cross-sectional study was conducted in 83 HS and 46 HSS Brazilian patients, which were characterized by ultrasonography, and 111 non-infected individuals, in 2010-2011. Total Cholesterol (TC), HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), VLDL-cholesterol (VLDL-c), Triglycerides (TG), plasma glucose, insulin, Homeostasis Model Assessment Insulin Resistance (HOMA-IR), TC/HDL-cl, LDL-cl/HDL-c and TG/HDL-c were determined. $\chi^2$ test, ANOVA and Pearson's correlation ($p<0.05$) were used for statistical analysis.
**Results:** The lipid profile was abnormal in HS and HSS in comparison to controls. TC and LDL-c were significantly reduced only in HS patients, whilst HDL-c was increased in HS and HSS. On the other hand, both patient groups showed lower levels of TG and VLDL-c, as well as the ratios TC/HDL-c, LDL-c/HDL-c and TG/HDL-c. Furthermore, as indicated by HOMA-IR, hyperinsulinemia/insulin resistance was present in HS and HSS patients, but its correlation with TG, VLDL-c, and TG/HDL-c was absent or negative respectively. On the other hand, as expected for the control group, the correlations between insulin resistance with these lipid levels and this ratio were positive.
**Conclusion/Significance:** Hyperinsulinemia/insulin resistance state was present in HS and HSS patients, despite its nonexistent or negative correlations with TG and with TG/HDL-c which are considered usual lipid markers for insulin resistance.

**Short title:** Insulin Resistance in Schistosomiasis

***Key words:*** Hyperinsulinemia, Insulin Resistance, Hepatosplenic Schistosomiasis mansoni, Absence of Dyslipidemia, Lipid Ratios.

**Author Summary**

Schistosomiasis mansoni is a parasitic disease which may cause serious injuries to liver function. Although hepatic damage being a classical factor for disruption in insulin acting, there are not known evidences of impaired insulin action in schistosomiasis patients. Furthermore, schistosomiasis also causes significant disturbances on lipid metabolism, which in turn may contribute to the genesis of insulin resistance. The present study aim to investigate insulin resistance in patients with hepatosplenic schistosomiasis mansoni who underwent splenectomy (HSS) and without splenectomy (HS), and its relation with lipid metabolism. Analyze of blood sample from patients indicated that although glucose levels were normal, a significant increase in insulin concentration was found in both group of patients. Also, HOMA-IR analysis demonstrated that hyperinsulinemia/insulin resistance was present in HS and HSS patients, despite its usual lipid markers for insulin resistance, triglycerides (TG) and TG/HDL-cholesterol were absent or negative. These results demonstrate that chronic schistosomiasis causes a condition of insulin resistance independent of lipid disorders. Therefore, we suggest that plasma insulin levels of patients with schistosomiasis should be monitored in order to prevent the development of insulin resistance and its consequences, and thus, ameliorate the morbity associated with schistosomiasis mansoni.

## Introduction

Liver exerts an important role in the homeostasis of carbohydrates promoting the balance of the plasma glucose concentration. Hepatic diseases that promote liver dysfunction are potential factors to trigger alterations in glycemic metabolism. Some studies have indicated the possibility that these changes have a close association with insulin homeostasis disorders (1).

Insulin resistance is the major and/or the prior feature of the diabetes, metabolic syndrome, cardiovascular diseases, i. e., a series of chronic degenerative diseases most prevalent around the world (2, 3). Furthermore, the state of hyperinsulinemia/insulin resistance in liver chronic diseases is emerging as a very important metabolic disturbance of host with viruses, as for example hepatitis C, host with cirrhosis, or with some kind of parasites, and this alteration in the response of the organism to the insulin has been related to steatosis development, onset and progression of fibrosis, and alterations in the response of the host to the conventional treatments (4, 5).

Schistosomiasis is a parasitic disease present in regions of tropical and subtropical climates and, approximately, 200 million people in world are infected by the disease. In Brazil, the etiologic agent of schistosomiasis is *Schistosoma mansoni* that infects about 2,5 million people (6, 7). The pathology of the schistosomiasis is composed of two stages, acute and chronic. The chronic phase is characterized by commitment of organs and tissues caused by granulomatous reactions and fibrosis, which are activated by the deposition of parasite eggs. The chronic phase is subdivided into three sub-phases or forms of the disease: Intestinal Schistosomiasis (IS); Hepatointestinal Schistosomiasis (HI); and Hepatosplenic Schistosomiasis (HS). HS stage is the most advanced in which

the greatest damage occurring in the hepatic parenchyma, portal hypertension and liver congestion, and sometimes the patients require splenectomy surgery for removing the spleen (HSS) (8, 9).

Schistosomiasis mansoni still affects a good part of the population, and the possibility of its presence is related to the framework of hyperinsulinemia and insulin resistance, concomitantly to the liver damage, makes to see that it still has many issues to be studied and understood about this pathology, including the possible link endocrine and metabolic disorders and the infectious and parasitic diseases. Besides, the presence of impaired glucose tolerance was observed in hepatic schistosomiasis, in 1974, but in the following decades there was an apparent lack of studies relating diabetes, for example, and schistosomiasis (10). However in the last years, this hypothesis has been raised, and new studies have been made, and the results have been presented discordant of this thematic, emphasized by the fact that the lipid profile from patients with schistosomiasis mansoni is better than in individuals without the contact with this parasite, since that one of the major abnormalities seen in individuals with insulin resistance is the dyslipidemia, apart from glicose, another one of the major abnormalities seen in individuals with insulin resistance is the dyslipidemia, which is characterized by a significant increase in plasma triglycerides and decrease in HDL-cholesterol levels (11-14). It was suggested that infection by *Schistosoma mansoni*, in turn, may prevents insulin-dependent diabetes mellitus in experimental animals, and it was observed an association between previous schistosome infection and a lower prevalence of diabetes and a better metabolic profile in Chinese (14, 15). On the other hand, it has been reported a relation between insulin and *Schistosoma*. It was highlited the role of the insulin on development of schistosomula, pointing to the fact that this hormone greatly increases both the rate and the extent of resistance of schistosomula to antibodies and complement system. Besides, insulin receptors have been identified in *Schistosoma japonicum* that can bind to human insulin, and the insulin regulates the glucose uptake in *Schistosoma mansoni* (16-18).

Thus, depending on the species of *Schistosoma*, the stage of the disease, the degree of liver involvement, the presence of treatment, splen surgery, the population studied, and all this taken together with the damage that hyperinsulinemia/insulin resistance may cause, it is important investigate the dysfunction in glucose metabolism and insulinemic profile in patients with schistosomiasis.

Therefore, the present study aim to investigate insulin resistance in patients with hepatosplenic form of schistosomiasis mansoni (splenectomized and non-splenectomized), and to access the lipid and lipoprotein profile, to investigate possible correlations between insulin resistance and lipid concentrations. Besides, the present study evaluated the lipid ratios known as Castelli indexes, I (Total Cholesterol/LDL-cholesterol) and II (LDL-cholesterol/HDL-cholesterol), which are indexes to access the probability of occurrence and development of cardiovascular diseases; and the lipid ratio Triglycerides/HDL-cholesterol, which is a possible marker to insulin resistance and to access cardiovascular risk.

## Materials and Methods

### Ethical Statement

The whole study was planned and executed following the Ethical Guidelines of the Helsinki Declaration and fulfilled recommendations 196/1996 from the Brazilian

National Health Council for research involving humans. A written informed consent was obtained from all the participants after a full explanation about the scope of the study, such as objectives, procedures and potential risks. Ethical approval for all procedures was obtained from the Ethics Committee of Human Research of the Health Sciences Center of the Federal University of Pernambuco (Protocol number CEP/CCS/UFPE N$^0$ 359/08).

## Study subjects

A hundred twenty nine patients that were diagnosed with the HS form of schistosomiasis mansoni attended into the Gastroenterology Outpatient at the *Hospital das Clínicas* of the *Universidade Federal de Pernambuco* were recruited from 2009 to 2011. These patients were divided into two groups – HS without splenectomy surgery (n = 83; 34.6% of the total of participants); and HSS patients who underwent splenectomy surgery (n = 46; 19.2% of the sample). All patients had a history of contact with water within an endemic area. The diagnosis of schistosomiasis was based on clinical history, physical examination and on upper abdominal ultrasonography conducted by a qualified and experienced examiner, according to the WHO protocol for ultrasound assessment of schistosomiasis-related morbidity (19). Moreover, 111 (46.2%) non-infected individuals from same socioeconomic conditions without epidemiological history compatible with schistosomiasis enrolled this study and composed the control group. Three stool examinations were taken in control group, in order to exclude infection, by the Kato-Katz method. The subjects were excluded if they presented the following clinical conditions: Hepatitis B or C infection, chronic kidney disease, thyroid dysfunction, collagenosis, blood diseases or cancer. Patients also were excluded if they reported alcohol abuse (>60 g ethanol/day for men and >40 g/day for women) or use of lipid-lowering drugs. The hepatosplenic schistosomiasis patients were treated with praziquantel (50 mg/Kg) prior to the study.

## Processing of samples and measurement of biochemical parameters

Blood samples were collected into three vacuum tubes in aseptic conditions (Vacutainer; Becton Dickinson, USA). The first tube containing EDTA-K3 (1 mg/mL) at a 1:9 ratio was used for determination of insulin levels, and the second tube with Sodium Fluoride (1 mg/mL) was used for glucose determination, while the third blood collection tube, without anticoagulant, was used for liver function and lipid profile analyses. Besides, the plasma and serum were isolated after centrifugation (Sorvall, USA) at 1,500 x g for 15 minutes and the all samples were stored in 0.5 ml aliquots at -80º C.

Plasma concentration of insulin was measured by electrochemiluminescence "ECLIA" (ROCHE-USA) using Cobas C501 analyzer (ROCHE-USA). Plasma levels of glucose, total cholesterol (TC), triglycerides (TG) and HDL-cholesterol (HDL-c) were determined by enzymatic spectrophotometry (Roche, Diamond Diagnostics, USA). LDL-cholesterol (LDL-c) and VLDL-cholesterol (VLDL-c) were determined by Friedewald equation [LDL-c = TC – HDL-c – VLDL-c; VLDL-c = TG/5]. Lipid ratios of cardiovascular risk, known as (?)Castelli indexes I and II, were assessed through TC/HDL-c and LDL-c/HDL-c.

Insulin resistance was accessed by Homeostasis Model Assessment Insulin Resistance – HOMA-IR [fasting glucose (mmol/L) x fasting insulin (µU/ml)/22.5] and TG/HDL-c ratio (20, 21).

Hepatic tests were also evaluated using hepatic enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT) and albumin levels were quantified by automated spectrophotometry (Cobas C501, Roche, Diamond Diagnostics, USA).

## Statistical analysis

The results were expressed as mean ± Standard Error of Mean. One-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) was used to compare continuous variables among groups. Pearson's correlation test was used to estimate the association between continuous parameters and $\chi^2$ test to compare categorical parameters, as gender, to investigate this possible confusion variable. Statistical significance for all comparisons was assigned at $p < 0.05$. All tests were performed using Statview SAS Inc. (1998, NC, USA).

## Results

Among the 240 subjects that participated of this study, the means of age (± S.E.M.) in the three groups were: 49.9 ± 1.4 years in HS patients; 48.1 ± 1.3 years in HSS; and 48.0 ± 1.6 in control group. Thus, statistical differences related to age were not found among the groups. The same was observed to gender ($\chi^2 = 4.180$; $p = 0.1237$).

Biochemical parameters that reflect liver function are demonstrated in Table 1. In summary, patients with schistosomiasis mansoni presented lower serum levels of albumin, and increased levels of the liver enzymes, when compared to the levels obtained from the control group. HSS patients had significantly higher levels of GGT than HS patients.

Serum lipid concentrations and lipid ratios were also determined and their values are in Table 2. HSS patients presented concentrations of TC and LDL-c similar to those found in the control individuals, unlike what happened in the HS patients. This group had lower concentrations of TC and LDL-c when compared to the control group, and the levels of LDL-c were also significantly lower in HS group than those found in HSS. On the other hand, concentrations of HDL-c were significantly higher in subjects with schistosomiasis mansoni than in non-infected individuals. However, concentrations of VLDL-c, triglycerides, and the lipid ratios (TC/HDL-c, LDL-c/HDL-c, and Triglycerides/HDL-c) were significantly decreased in the patients with hepatosplenic schistosomiasis mansoni, regardless of splenectomy.

HS and HSS patients had plasma levels of glucose statistically identical, as shown in Figure 1. However, the plasma levels of insulin were significantly higher in the patients with hepatosplenic form of the schistosomiasis mansoni, with or without removal of the spleen, when compared with the levels found in individuals who have never come into contact with the parasite, also as shown in Figure 1.

HOMA-IR values were significantly increased in HS (increase of approximately 45%) and HSS (increase of almost 70%) patients when compared to values obtained in the control group. Values of HOMA-IR and statistical differences among the groups are in Figure 2.

Table 3 shows the correlations between the values of HOMA-IR and the levels of lipids as well as between the values of HOMA-IR and the values of the lipid ratios, in HS

and HSS patients and in non-infected individuals. The HOMA-IR values correlated positively and significantly to levels of TC, LDL-c, VLDL-c, Triglycerides, and to values of TC/HDL-c, and Triglycerides/HDL-c, in normal individuals, without schistosomiasis mansoni. HS patients did not present significant correlation between HOMA-IR values and lipid concentrations and lipid ratios. In turn, HSS patients showed significant correlations, however negative correlations, between HOMA-IR values and levels of Triglycerides, VLDL-c, and the lipid ratio TG/HDL-c.

## Discussion

Bloodworth (1961) already highlited the association of several disturbances of carbohydrate metabolism with chronic liver diseases (22). However, nevertheless this association remains under discussion. Fartoux et al. (2005) reported a possible relationship between insulin resistance and hepatitis C, a chronic liver disease, and their study showed that insulin resistance is the cause than the consequence of fibrosis and steatosis in individuals with hepatitis (23). Kruszynska et al. (1991) observed that cirrhotic individuals, after taking oral glucose, responded with a hypersecretion of insulin, 4 to 6 times longer than the response obtained by the control group (24). Though Wang et al. (2008) did not find association between insulin resistance and hepatitis B, another chronic liver disease (25).

In our study, the patients with hepatosplenic schistosomiasis mansoni, regardless the splenectomy, presented normal glycemia, however these patients also presented increase in the insulin plasma levels and higher HOMA-IR values, showing a tendency for hyperinsulinemia/insulin resistance. In turn, Chen et al. (2013) have suggested a protecting effect of the schistosoma infection against diabetes, since rural Chinese presented a lower prevalence of diabetes when had a history of previous schistosome infection (14).

On the other hand, the findings of Sukkar, Omer, and El Din Ahmed (1974) already indicated that a degree of glucose intolerance occurs in hepatic schistosomiasis even before the development of ascites (10). However insulin resistance in hepatic schistosomiasis is still discussed, considering that individuals with schistosomiasis mansoni present a better lipid profile, as reported by Chen et al. (2013) (14), and since hyperinsulinemia/insulin resistance are directly related to certain lipid disorders – hypertriglyceridemia and decrease of the levels of HDL-c, as defined by Reaven (2005) (3).

In the present study, it was observed that patients with hepatosplenic schistosomiasis mansoni, even without changes in the glucose levels, may have insulin resistance despite the absence of dyslipidemia feature. We found that the patients with schistosomiasis mansoni in the hepatosplenic form, regardless of whether these patients made splenectomy surgery, presented a significant reduction in the levels of triglycerides and in the cholesterol content of their correlated lipoprotein, VLDL-c, which corroborates with Stanley et al. (2009) (26). Besides, HS and HSS patients presented lower values of the lipid ratios, TC/HDL-c, LDL-c/HDL-c, and Triglycerides/HDL-c; and HS patients showed no correlation between their HOMA-IR values and lipid concentrations or lipid ratios values, though TC/HDL-c and LDL-c/HDL-c are two important predictors of cardiovascular risk, as described by Castelli (1983) (27), and though Triglycerides/HDL-c is a good surrogate marker for insulin resistance and a candidate to estimate the cardiovascular risk, as suggested by Reaven (2005) (03). It is still unclear the role of

schistosomiasis in the genesis of atherosclerotic cardiovascular disease, as can be seen in Doenhoff et al. (2002) (11) when contrasted with La Flame et al. (2007) (12).

Our results also demonstrated a negative correlation between insulin resistance, through HOMA-IR values, with Triglycerides and Triglycerides/HDL-c, in splenectomized patients, i.e., even with lower levels of triglycerides and Triglycerides/HDL-c, the values of HOMA-IR increased considerably. This tendency for insulin resistance may contribute to increased hepatic and systemic injury, as warned by Dandona et al. (2003) (28), Fartoux et al. (2005) (23). Another study, of Kostandi et al. (2011), also warms against the presence of insulin resistance in chronic liver disease. Kostandi et al. (2011) (05) reported an increased HOMA-IR score in subjects with chronic hepatitis C reflecting the existence of IR irrespective of treatment response. In the schistosomiasis, the increase of HOMA-IR is based in the increase of insulin plasma levels, perhaps the parasite causes stimulation of this production, trough inflammatory response. High levels of IL-17, as observed in schistosomiasis patients by Mbow et al. (2013) (29), were associated with insulin resistance by Ohshima et al. (2012) (30). It also was shown that fibrosis was associated with hyperinsulinemia in non-diabetic patients, as reported Kimura et al. (2011) (31). Possibly hyperinsulinemia occurs due to diminished hepatic insulin degradation rate in patients with hepatosplenic schistosomiasis mansoni, as suggested by Pimenta et al. (32), to the hyperinsulinemia in individuals with hepatitis C.

HS patients also presented lower levels of TC and LDL-c, which corroborates with Lima et al. (1998) (33) who have suggested that the cholesterol content in the plasma decreases with concomitant increases in erythrocyte membranes; with Doenhoff et al. (2002) (11), who proposed a reduction in blood total cholesterol concentrations by modulating host lipid metabolism; and with La Flamme et al. (2007) (12), who emphasize that the chronic exposure to *Schistosoma mansoni* eggs causes a reduction in the concentrations of TC and LDL-c, possibly due to a possible increase in uptake by macrophages. However, splenectomized patients presented concentrations of TC and LDL-c similar to those found in the control individuals. These findings corroborate with results of another study conducted in our laboratory, Silva et al. (2002) (34), in which it was observed that individuals undergoing splenectomy also showed plasma concentrations of TC and LDL-c similar to the control group. However, regardless splenectomy, hepatosplenic patients had levels of HDL-c, in this study, higher than control individuals, disagreeing with Doenhoff et al. (2002) (11) and corroborating with Chen et al. (2013) (14). Our patients also had decrease in albumin concentration and increase in hepatic enzymes, demonstrating an impairment of liver dysfunction, according to Mansour et al. (1982) (35).

Thus, despite a better lipid profile with lower concentrations of TC, LDL-c and Triglycerides, higher concentrations of HDL-c, and lower values of lipid ratios, which predict a lower cardiovascular risk, this risk, as well as the risk to diabetes, and to metabolic syndrome, regardless lipid alterations, may be increased, since the hyperinsulinemia/insulin resistance state is installed in hepatosplenic schistosomiasis mansoni, splenectomized or non-splenectomized, remembering that this state of insulin resistance can in turn trigger many other diseases and metabolic disorders. Hence individuals with chronic schistosomiasis should be monitored, even after treatment to schistosomiasis, in order to prevent the development of insulin resistance and its consequences.

## Author Contributions

Pimenta Filho AA, Santos BS, Fonseca CSM and Lima VLM designed the study protocol; Pimenta Filho AA, Santos BS, Fonseca CSM, Leite LAC, Domingues ALC and Lima VLM realized biochemical assays and were involved in analysis and interpretation of all the data; Pimenta Filho AA, Santos BS and Fonseca CSM did statistical analysis; Pimenta Filho AA, Santos BS, Fonseca CSM and Lima VLM contributed to drafting the manuscript and/ or critically revising the paper and intellectual content. All authors read and approved the final manuscript.

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**TABLE 1.** Biochemical Parameters of Liver Function of Patients with Hepatosplenic Schistosomiasis Non-splenectomized (HS) and Splenectomized (HSS) and Non-infected Controls.

| Characteristics | Controls | Hepatosplenic Patients | | *p*-value | | |
|---|---|---|---|---|---|---|
| | | HS | HSS | C vs. HS | C vs. HSS | HS vs HSS |
| Albumin (g/dL) | 4.46 ± 0.03 | 4.05 ± 0.05 | 4.01 ± 0.05 | <0.0001 | <0.0001 | ns |
| AST (U/L) | 23.8 ± 0.5 | 45.2 ± 2.5 | 46.9 ± 1.9 | <0.0001 | <0.0001 | ns |
| ALT (U/L) | 21.8 ± 0.4 | 38.8 ± 2.2 | 43.1 ± 2.6 | <0.0001 | <0.0001 | ns |
| ALP (U/L) | 68.1 ± 4.9 | 137.0 ± 5.6 | 132.6 ± 8.1 | <0.0001 | <0.0001 | ns |
| GGT (U/L) | 58.5 ± 7.0 | 120.4 ± 8.9 | 237.4 ± 8.9 | <0.0001 | 0.0006 | ns |

Abbreviations: AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; AP, Alkaline Phosphatase; GGT, Gamma Glutamyl Transferase; ns, no significance. Values expressed as mean±Standard Error (SE). One-way ANOVA followed by Fisher's PLSD post test.

**TABLE 2.** Serum Lipid Concentrations and Values of Lipid Ratios in Patients with Hepatosplenic Form of Schistosomiasis, Non-splenectomized (HS) and Splenectomized (HSS), and Non-infected Controls.

| Characteristics | Controls | Hepatosplenic Patients | | *p*-value | | |
|---|---|---|---|---|---|---|
| | | HS | HSS | C vs. HS | C vs. HSS | HS vs HSS |
| TC (mg/dL) | 197.4 ± 3.3 | 178.8 ± 4.8 | 189.5 ± 6.0 | 0.0012 | ns | ns |
| LDL-c (mg/dL) | 123.9 ± 2.7 | 102.8 ± 4.4 | 121.6 ± 5.1 | p<0.0001 | ns | 0.0026 |
| HDL-c (mg/dL) | 45.3 ± 0.8 | 56.0 ± 1.4 | 57.3 ± 1.6 | p<0.0001 | p<0.0001 | ns |
| VLDL-c (mg/dL) | 25.4 ± 1.0 | 17.8 ± 0.7 | 15.4 ± 0.7 | p<0.0001 | p<0.0001 | ns |
| TG (mg/dL) | 140.1 ± 7.7 | 88.0 ± 3.4 | 81.8 ± 4.5 | p<0.0001 | p<0.0001 | ns |
| TC/HDL-c | 4.61 ± 0.12 | 3.36 ± 0.11 | 3.45 ± 0.11 | p<0.0001 | p<0.0001 | ns |
| LDL-c/ HDL-c | 3.06 ± 0.08 | 1.98 ± 0.10 | 2.27 ± 0.11 | p<0.0001 | p<0.0001 | ns |
| TG/HDL-c | 3.37 ± 0.19 | 1.76 ± 0.09 | 1.58 ± 0.11 | p<0.0001 | p<0.0001 | ns |

Abbreviations: TC, Total Cholesterol; TG, Triglycerides; ns, no significance. Values expressed as mean±Standard Error (SE). One-way ANOVA followed by Fisher's PLSD post test.

**Table 3.** Pearson's Correlation (r) Between HOMA-IR Values and Lipids in Chronic Schistosomiasis.

| Parameters | Groups | r | 95% CI | *p* value |
|---|---|---|---|---|
| | Control | 0.219 | 0.090 to 0.341 | 0.0010 |
| TC (mg/dL) | HS | - 0.128 | -0.320 to 0.075 | 0.2148 |
| | HSS | 0.035 | -0.246 to 0.310 | 0.8113 |
| | Control | 0.152 | 0.019 to 0.279 | 0.0257 |
| LDL-c (mg/dL) | HS | -0.092 | -0.287 to 0.110 | 0.3727 |
| | HSS | 0.070 | -0.213 to 0.341 | 0.6322 |
| | Control | -0.031 | -0.162 to 0.101 | 0.6457 |
| HDL-c (mg/dL) | HS | -0.185 | -0.367 to 0.009 | 0.0619 |
| | HSS | -0.018 | -0.295 to 0.262 | 0.9030 |
| | Control | 0.173 | 0.040 to 0.300 | 0.0107 |
| VLDL-c (mg/dL) | HS | -0.008 | -0.206 to 0.191 | 0.9392 |
| | HSS | -0.340 | -0.565 to -0.068 | 0.0152 |
| | Control | 0.192 | 0.062 to 0.316 | 0.0039 |
| TG (mg/dL) | HS | -0.007 | -0.109 to 0.096 | 0.8971 |
| | HSS | -0.317 | -0.547 to -0.042 | 0.0246 |
| | Control | 0.168 | 0.037 to 0.293 | 0.0123 |
| TC/HDL-c | HS | 0.002 | -0.198 to 0.203 | 0.9818 |
| | HSS | 0.018 | -0.262 to 0.295 | 0.9026 |
| | Control | -0.081 | -0.213 to 0.053 | 0.2334 |
| LDL-c/ HDL-c | HS | -0.013 | -0.213 to 0.188 | 0.9021 |
| | HSS | 0.062 | -0.220 to 0.335 | 0.6704 |
| | Control | 0.187 | 0.057 to 0.311 | 0.0050 |
| TG/HDL-c | HS | 0.102 | -0.100 to 0.297 | 0.3229 |
| | HSS | -0.279 | -0.517 to -0.001 | 0.0492 |

TC, Total Cholesterol; TG, Triglycerides; Non-Splenectomized (HS) and Splenectomized (HSS).

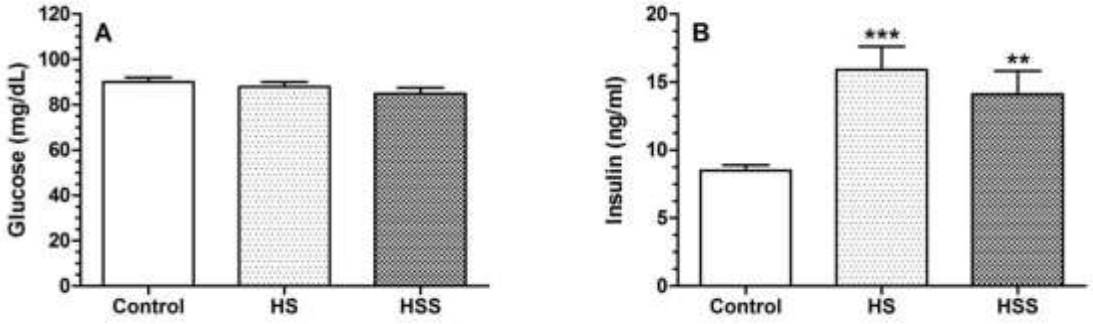


**FIGURE 1**. Plasma Levels of Glucose (A) and Insulin (B) in Individuals with Hepatosplenic form of Schistosomiasis Mansoni, Non- splenectomized (HS) and Splenectomized (HSS), and in Non-infected Controls. Values expressed as mean±Standard Error (SE). One-way ANOVA followed by Fisher's PLSD post test. **$p \leq 0.001$ and ***$p \leq 0.0001$ vs Control.

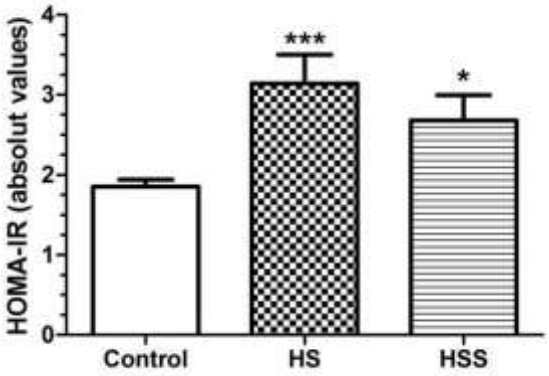


**FIGURE 2**. Values of the Homeostatic Model Assesment – Insulin Resistance (HOMA-IR) in Patients with Hepatosplenic Form of Schistosomiasis Mansoni, Non-splenectomized (HS) and Splenectomized (HSS), and in Non-infected Controls. Values expressed as mean±Standard Error (SE). One-way ANOVA followed by Fisher's PLSD post test. *$p \leq 0.05$ and ***$p \leq 0.0001$ vs Control.

## 8. Artigo 3

Distinct Adipose Tissue and Liver phenotypes in response to very high dietary fat, cholesterol or sucrose.

**Artigo à ser submetido ao periódico *The Journal of Lipid Research***

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**Title:** Distinct Adipose Tissue and Liver phenotypes in response to very high dietary fat, cholesterol or sucrose.

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**Running title:** Fat, cholesterol and sucrose on NAFLD and Obesity development**.**

**Abbreviations:**

VHF: very-high fat

HF/HC: high-fat/high-cholesterol

HF/HS: high-fat/high-sucrose

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

HOMA: homeostasis model assessment

EAT: epididymal adipose tissue

ipGTT: intraperitoneal glucose tolerance test

ALT: alanine aminotransferase

H&E: hematoxylin & eosin

MCP-1: monocyte chemoatractant factor-

IL: interleukin

GLUT4: glucose transporter type 4

**ABSTRACT**

High-fat diet feeding is an established factor for metabolic syndrome development, by inducing obesity, insulin resistance and NAFLD. However, other dietary stressors are also related. Herein, to evaluate how such triggers could act on liver and adipose tissue metabolism, we added extra fat, cholesterol or sucrose to high fat diets – VHF, HF/HC, and HF/HS diets, respectively – to C57BL/6 mice during 16 weeks. We observed that VHF diet developed a more pronounced adipose tissue inflammatory response and insulin resistance than mice under HF/HC or HF/HS diets. On the other hand, high content of fructose provoked the worst liver inflammatory scenario. In turn, even not producing similar levels of liver inflammation than HF/HS, HF/HC diet fed mice showed increased presence of liver fibrosis. Thus, our results suggest that adipose and liver tissues respond in different, and possibly opposite, manners to the different diets used.

**KEYWORDS**

Nutrition; Obesity, Diet and Dietary Lipids; Inflammation; Insulin.

## INTRODUCTION

Prolonged nutrient overfeeding can cause metabolic imbalance, which leads to an overwhelming of the biological capacity and consequent vast cellular and tissue dysfunction and disease. A wide range of dietary factors are known to be important on the development of obesity (1). Worldwide, it is estimated that are 1.46 billion overweight adults and more than 500 million obese (2). Such pandemic is associated with several comorbidities, having a crucial causative role in insulin resistance, diabetes mellitus, metabolic syndrome and non-alcoholic liver disease (NAFLD) (3,4).

Obesity is associated with expansion of the metabolic active adipose tissue leading to chronic low-grade inflammation and insulin resistance, which, in turn can affect hepatic metabolism leading to hepatic injury (5,6). Both obesity and NAFLD are key components of the metabolic syndrome and, given their interplay, an integrated approach is of great interest as a means for identification of the complex pathology of chronic inflammatory diseases.

NAFLD affects 10-35% of the general population (7), and is recognized as the most common cause of liver dysfunction globally (8). The spectrum of NAFLD ranges from simple benign hepatic steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (9). Its prevalence is strongly increasing together with obesity and type 2 diabetes and it is therefore present in up to 85% in extremely obese individuals (10). Despite the clear relationship among central obesity, insulin resistance and NAFLD, the cause of progression to advanced forms remains elusive.

A key issue in understanding the pathogenesis of the NAFLD concerns the identification of the mechanisms responsible for switching from simples steatosis to NASH. Additionally to liver lipid accumulation itself, it seems that further stressors are also involved. In the past few years, lipotoxicity, or the ability of specific lipid molecules (e.g. fatty acids, cholesterol, and ceramides) to cause liver inflammation, has been emerged as a possible causative factor for NAFLD progression (11).

Recently, diet containing high content of carbohydrates were added to this list. Sucrose and fructose intake, mainly, have been associated with liver disease (12).

Nonetheless, this heterogeneity of related disorders and possible causes, each with distinct phenotypes, likely contributes to the difficult in identifying effective and long-term strategies of treatment and suggests that a wider approach of the NAFLD research is needed. Herein, we aimed to investigate the metabolic response within liver and adipose tissue to the strong stressor state of overfeeding caused by high-fat diets with additional content of fat, cholesterol or sucrose.

## METHODS

### Animals and Diets

All experiments were conducted in accordance with the guidelines of the University of Cincinnati Institutional Animal Use and Care Committee. Mice were maintained in a temperature- and light-controlled facility and had free access to food and water during the period of study. Adult male C57BL/6 mice were fed a rodent chow, very high-fat (VHF, Research Diets D12492), high-fat high-cholesterol (HF/HC, Research Diets D12108C), or high-fat high-sucrose (HF/HS, Teklad TD08811) diets for 16 weeks (Table 1).

### Weight and Adiposity Measurements

Age-matched (10-week-old) male mice were housed with one to four mice per cage. Food intake was monitored daily over a 1-week period. Body weight and adiposity measurements were performed every 4 weeks. Body weight was measured using a Denver 300 K scale and adiposity levels were determined by echo magnetic resonance imaging (MRI) using a Whole-Body Composition Analyzer (Echo Medical, Houston, TX).

### Analytical Procedures

Blood was collected after overnight fast. Blood glucose obtained using an Accu-Check Active Glucometer (Roche Applied Science, Indianapolis, IN). Plasma and liver cholesterol and triglycerides were measured using colorimetric kits. Plasma insulin (Crystal Chem, Chicago, IL), leptin (R&D Systems, Minneapolis, MN) and adiponectin (R&D Systems, Minneapolis, MN) were measured using ELISA Kits. Alanine aminotransferase (ALT) and non-esterified fatty acids (NEFA) were measured using colorimetric kits (Thermo Scientific). Homeostasis Model Assesment (HOMA) index was calculated according to the formula: fasting plasma glucose (mM) x fasting plasma insulin (ng/mL) / 22.5. Folch technique was used to perform liver lipid extraction.

**Glucose Tolerance Test**

Overnight fasted mice received a solution of glucose (1g/Kg of weight) intraperitoneally and had their blood glucose levels measured before and 15, 30, 60, 120, and 180 minutes afterward.

**Tissue Histology and Immunohistochemistry/fluorescence**

Liver and epididymal adipose tissue (EAT) were excised at the time of euthanization and wet weight was recorded. Parts of liver left lateral lobe and EAT were carefully isolated, fixed in isotonic neutral 4% paraformaldehyde (3 days), embedded in paraffin, and cross-sectioned (7um). Sections from both organs were stained with Hematoxylin and Eosin. Liver sections were also stained with Masson's Trichrome. Adipocyte sizes were evaluated by the quantification of 120 areas of random cells per animal. Crown-like structures were identified by the presence of nucleated cells surrounding individual adipocytes. Each stain was carried out on at least four mice per group and the images in the figures are representative of each group.

**Real-time quantitative PCR analysis**

Consistent regions of liver and EAT were collected, snap-frozen and stored at -80◦C. Total RNA (N=5 to 7 per group) was extracted with TRIzol reagent (Invitrogen) followed by a further isolation using RNeasy plus mini kit (Qiagen) and treatment with Turbo DNase (Applied Biosystems/Ambiom,

Austin, TX). cDNA was made using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time quantitative PCR was performed using a StepOnePlus Fast Thermocycler using Fast SYBR Green Mix (Applied Biosystems, Carlsbad, CA) with primer sequences as shown in Table 2. Gene expression values were normalized to the value of the housekeeping gene *CycA* (Cyclophilin A) and calculated based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$).

**Statistical Analyses**

Data were analyzed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) and are presented as means and standard errors. Analysis of Variance (ANOVA) followed by Bonferroni multiple comparison post hoc test was used to detect differences among groups that had normal distribution. When data distribution did not reach those parameters, the non-parametrical test Kruskal-Wallis followed by Dunn's Multiple comparison test was used. P-value <0.05 was considered statistically significant and indicated by an asterisk (*) or different letters.

## RESULTS

**Resistance to diet-induced obesity on mice under HF/HC diet.**

Mice were fed VHF, HF/HC, or HF/HS diets for up to 16 weeks and body composition was monitored. All animals had similar baseline weight and showed body weight gain over the period of study, but mice under HF/HC diet developed a lesser increase (Figure1A). The HF/HS group showed increased daily calorie intake compared with the HF/HC diet fed group (Figure1B). In line, whole-body MRI showed higher levels of body fat mass in both VHF and HF/HS diets fed mice compared to HF/HC, although all groups showed significantly increase along the study (Figure1C). Quantification of liver and EAT weights revealed no differences among groups (Figure1C-D). It is well known that high calorie intake reflects on a higher body weight and body fat mass (13–15). However, even with the higher calorie intake observed on mice fed HF/HS diet, the VHF diet group

showed higher body fat mass during all period of the study, with tendency of catch up only after 16 weeks when they reach body weights close to 50g, an upper limit for wild type C57BL/6 mice.

**Similar plasma lipid levels of mice under the different diets.**

Lipid serum analyses showed minor changes in their lipid profile. Mice showed increased total cholesterol levels after 16 weeks but similar serum triglycerides and NEFA levels (Figure 2). We and others have previously reported that wild type rodent plasma lipids are mostly on HDL lipoproteins (16) and it takes longer to observe differences in lipid levels, with frequent no difference in plasma triglycerides (17,18).

**VHF diet feeding exarcebates glucose dysmetabolism.**

All groups subjected do high-fat diets showed similar fasting blood glucose, but the VHF animals displayed worse glucose tolerance, demonstrated by the increased ipGTT values (Figure 3A). Similarly, they also showed markedly insulin resistance, which can be noted by the higher HOMA index (Figure 3B). Interestingly, HF/HS feeding induce an intermediate state of glucose intolerance and insulin resistance compared to VHF and HF/HC diets.

**Intraabdominal adipose tissue histology.**

To determine changes in adipose tissue architecture that might occur with the experimental diet feeding, epididymal adipose tissues harvested at the time of sacrifice were studied (Figure4A). Histological analysis revealed a significant increase of adipocyte size in mice under HF/HS diet whereas VHF and HF/HC diet feeding induced similar hypertrophy (Figure 4B). Adipose tissue from mice under VHF also showed remarkable increased number of crown-like structures compared with both HF/HC and HF/HS (Figure 4C).

**VHF diet induced endocrine and inflammatory disbalance at adipose tissue.**

In line with the high frequency of CLSs, levels of inflammatory markers TNF-a, MCP-1 and IL-1b were elevated in VHF mice (Figure 5A-C, respectively). A marker of adipose tissue maturity state, PPARy, was decreased in VHF mice compared to all other groups (Figure 5D).

All diets induced disrupted levels of the adipose tissue-derived cytokines leptin and adiponectin. Consistent with the exaggerated VHF-induced obesity, VHF group showed increased levels of leptin compared with HF/HC (Figure 5E). Interestingly, mice under HF/HC diet demonstrated a lesser increase of leptin levels and a higher adiponectin to leptin ratio when compared with both VHF and HF/HS feeding groups (Figure 5F-G).

**HF/HS provoked the worst liver damage of all diets.**

All diets provoked wide hepatocyte damage, evaluated by similar increased levels of serum ALT (Figure 6A). In line, liver cholesterol and triglycerides were also similar (Figure 6B-C). Hematoxylin and eosin (H&E) staining of liver sections revealed an increasing content of steatosis VHF < HF/HC ≤ HF/HS (Figure 6D). Liver of mice under HF/HS diet also showed a higher degree of steatosis, balooning degeneration and microgranuloma (Table 2). Furthermore, Masson's trichrome staining displayed a 2 to 3-fold increase in fibrotic bridges among livers of HF/HC and HF/HS groups compared to VHF (Table 3, Figure 6E).

**Increased levels of pro-inflammatory markers in liver of HF/HS fed mice.**

Another distinctive feature of the HF/HS hepatic tissue observed was the altered pattern of gene expression. High sucrose feeding provoked a stronger inflammatory reaction within liver than high cholesterol or fat. We observed increased expression of the chemotactic factor, MCP-1 and also markers of macrophages: F4/80, CD68 and CD11b (Figure 7A-D). In addition, It was also observed increased levels of the pro-inflammatory citokynes TNF-α, IL-1β, and IL-6 (Figure 7E-G).

## DISCUSSION

Previous studies have reported that high fat diets induce hepatic steatosis, however, lipid accumulation itself is not enough to provoke liver damage and NAFLD progression, usually another stressor is also necessary to trigger NAFLD development (19). In order to evaluate how such triggers could act on liver metabolism we added extra fat, cholesterol or sucrose to high fat diets and analyzed the inflammatory response of the liver. Besides that, given the importance of the adipose tissue for lipid metabolism, we evaluated how the adipose tissue reacted to those dietary stressors.

We observed that high content of fructose provoked the worst liver inflammatory scenario. On the other hand, VHF diet developed a more pronounced adipose tissue inflammatory response and insulin resistance than mice under HF/HC or HF/HS diets. In turn, even not producing similar levels of liver inflammation than HF/HS, HF/HC diet fed mice showed increased presence of liver fibrosis.

In our study, we observed that mice subjected to high fat diet with extra content of fatty acids, instead of cholesterol or sucrose, were more susceptible to develop obesity and insulin resistance. The precise physiological events leading to the initiation of the inflammatory response in adipose tissue remains to be understood. During high fat feeding conditions, the adipose issue is overload by lipids and often respond with hypertrophy and hyperplasia, as a mechanism to store more triglycerides (6). Excessive lipid intake trigger cellular stress pathways, which in turn causes cell autonomous inflammation and release of cytokines and other pro-inflammatory signals (19). Part of these signals, such as the MCP-1, induce migration and infiltration of monocytes/macrophages to adipose tissue, where they accumulate around necrotic adipocytes, forming crown-like structures, and release further pro-inflammatory cytokines, as TNF-a and IL-1β (20). In the meanwhile, multiple intracellular inflammatory pathways are ongoing leading to blunted insulin signaling, thereby potentially increasing insulin resistance (21).

Interestingly, adipocytes from mice under VHF diet had lower size than HF/HS. Insulin resistance has been correlated with adipocyte size but also with their degree of maturation. In fact, McLaughlin et al, 2007 matched insulin resistant and sensitive individuals by body mass index and find that were no differences in adipocyte size. Despite that, they find decreased expression of differentiation markers, suggesting that impairment in adipose cell differentiation rather than cell area contribute to obesity-associated insulin resistance (22). Similarly, we observed reduced levels of PPARγ gene expression only on adipose tissue of VHF group, compared with controls.

A possible cause for the differences observed among the studied groups could be the excess of fatty acid content of the VHF diet. In this case, the approximately 50% extra fat would cross the equilibrium threshold of the adipose tissue lipid storage capacity and induce adipocyte apoptosis, which may trigger the inflammatory responses observed, with high macrophage infiltration. Another possibility is that the addition of cholesterol or sucrose could change the mechanism of the diet- induced obesity leading to different phenotypes.

With regards to the HF/HC diet, several studies have observed the effects of the addition of cholesterol to a high fat diet, but none of them had compared them in the way we did, adding extra amounts of cholesterol but also of fatty acids. In those studies, added cholesterol usually causes worse effects than high fat alone (17,18,23). Nonetheless, the opposite effect, decreasing the content of cholesterol by the using of cholesterol lowering drugs has been demonstrated to cause impairment of adipose tissue metabolism (24,25).

HMG-CoA inhibitors has been reported to inhibit adipocyte differentiation. Nakata et al, 2006 reported decreased GLUT4 and caveolin-1 expression in adipocytes upon treatment with statins (24). Because caveolin-1 is find in lipid rafts – in which cholesterol has a crucial role – it is possible that the VHF produces an overload of fatty acids, which secondary decrease of cholesterol below a critical threshold. Such cholesterol depletion could lead to a disruption in the lipid droplet membrane fluidity, culminating in dysfunctional adipocyte (26,27). These effects would not being seen in mice under

HF/HS diet because fructose is mostly absorved by the liver, during fed states while fat comes through lymphatic circulation and goes to periferic tissues first (28,29).

As an organ where the most different components from circulation and gut are metabolized, the liver is subjected to very different sources of injury and has to have a well-organized system of tissue repair. Scarring is an essential component of the remodeling response during the replacement of the damaged parenchyma for a fully functional tissue (30). However, in some circumstances, this wound-healing response is disrupted, resulting in misrepair and consequent progressive scarring, ultimately leading to cirrhosis and hepatocellular carcinoma (31).

During NAFLD development, it has been recently hypothesized the occurrence of multi-parallel hits forcing the liver towards a more progressive inflammatory disease phenotype that could induce later fibrosis as a mechanism of tissue repair (19). Among these stressors, cholesterol has arisen as crucial factor for disease progression not only to NASH but mainly to fibrosis (32,33). In fact, high dietary consumption of cholesterol was associated with a higher risk of cirrhosis and liver cancer (34).

Our data of the liver analysis revealed that mice under HF/HC diet developed increased fibrosis bridging compared to VHF and at same levels of HF/HS, but without severe signals of inflammation as in the latter group.

In spite of the confidence on the role of cholesterol in NAFLD, contradictory data has been published regarding weather those effects would be on inflammation and/or fibrosis. Some reports, where mice were fed a high fat high cholesterol diet reported that cholesterol would induce a stronger pro-inflammatory effect, which culminate with a more prominent fibrosis (17,18,23). However, other studies have seen that high-cholesterol diet could induce fibrosis without stimulating inflammation. Teratani et al, 2012 and Tomita et al, 2013 observed increased fibrosis in mice under high cholesterol diet submitted to bile-duct ligation compared to control diet fed mice. Similarly to us, they did not

find differences among inflammation or hepatocellular injury. The authors argue that the presence of fat on diet could shadow the cause of fibrosis (32,33).

Nevertheless, Nanji et al, 1997 reported decreased necrosis and TNF-a but enhanced fibrosis after cholesterol supplementation in alcoholic liver disease (35). Recently, Ichimura et al, 2015 observed diet-induced fibrosis in mice under high-fat high-cholesterol diets, but not when mice were fed with high-fat diet only (36). Then, our data suggests that cholesterol itself has a major role on liver fibrosis than inflammation.

A number of animal models have been developed to study NAFLD (37,38). Among these are nutritional models based on complete diets containing high content of carbohydrates, usually sucrose and/or fructose. In mammals, the liver is the principal organ responsible for the conversion of excess dietary carbohydrate into triglycerides. Both glucose and fructose are metabolized into liver to provide the precursor, acetyl CoA, for fatty acid synthesis. Fatty acids are then incorporated into triglycerides that function as long-term energy reservoirs (29).

Recently, Chung et al. (2014) reported that the apparent association between high sucrose or fructose intake and liver damage is confounded by increased calorie intake (39). On the other hand, Oliveira et al. (2014) reported increased lipid liver accumulation in mice fed isocaloric high-sucrose diet compared to high-fat diet (40). Ishimoto et al, 2013 observed that the combination of high-fat and high-sucrose diet was able to induce NASH. Interestingly, such effects were blunted in the absence of the liver fructokinase, which phosphorylates fructose into fructose-1-6-phosphate, strengthening the role of fructose in NAFLD development. In their study, mice had similar daily energy intake (41). We observed that HF/HS feeding induced a remarkable liver inflammation, even when compared with VHF or HF/HC diets. Indeed, there were no significant differences between daily calorie consumption of mice under HF/HS and VHF.

In Summary, our study showed that in addition to simple lipid accumulation within liver caused by high fat diet, extra dietary factor acts in specific ways inducing metabolic abnormalities. Besides the extra fat added to diet had caused the more pronounced adipose tissue dysregulation, high sucrose and cholesterol were more related to liver disease. And, between them, high cholesterol intake caused liver fibrosis without provoking major inflammation. In fact, our results suggest that adipose and liver tissues respond in different, and possibly opposite, manners to the different diets used.

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**TABLES**

**Table 1. Diets Components**

| Diet | VHF | HF/HC | HF/HS |
|---|---|---|---|
| **Fat (kcal%)** | 60 | 40 | 44.6 |
| **Fat (gm%)** | 35 | 20 | 23.2 |
| **Fat Source (wt%)** | lard (31.65%)<br>soybean oil (3.23%) | cocoa butter (17.27%)<br>soybean oil (2.78%) | anhy milkfat (21%)<br>soybean oil (2%) |
| **Added Cholesterol** | 0% | 1.25% | 0% |
| **Sucrose (gm%)** | 8.89 | 12.59 | 34 |
| **Calories (Kcal/g)** | 5.2 | 4.5 | 4.7 |

**Table 2: Primer sequences used for RT-PCR amplification of RNA.**

| Gene | Sense Primer | Anti-sense Primer |
|---|---|---|
| **Cyclophilin A** | TCATGTGCCAGGGTGGTGAC | CCATTCAGTCTTGGCAGTGC |
| **TNF-α** | ATCCGCGACGTGGAACTG | ACCGCCTGGAGTTCTGGAA |
| **MCP-1** | CCTCCTCCACCACCATGCA | CCAGCCGGCAACTGTGA |
| **IL-1β** | CTACAGGCTCCGAGATGAACAAC | TCCATTGAGGTGGAGAGCTTTC |
| **PPAR-γ** | CTGCAGGCCCTGGAACTG | CGATCTGCCTGAGGTCTGTCA |
| **F4/80** | TGTCTGACAATTGGGATCTGCCCT | ATACGTTCCGAGAGTGTTGTGGCA |
| **CD68** | TTTCTCCAGCTGTTCACCTTGA | CCCGAAGTGTCCCTTGTCA |
| **CD11b** | GCTCAGAGGTTCTTCACAGCTATG | CAAGCCCATGGCACTCATG |
| **IL-6** | TTCCATCCAGTTGCCTTCTTG | GGGAGTGGTATCCTCTGTGAAGTC |

**Table 3. Histological Characteristics of Liver According to the Different Diets Studied.**

| | **VHF** | **HF/HC** | **HF/HS** |
|---|---|---|---|
| Steatosis | 1 | 2 | 2.5 |
| Balooning degeneration | 1.5 | 1 | 2 |
| Microgranuloma | 1 | 1 | 2 |
| Bridging Fibrosis* | 2.9 | 11.1 | 12.9 |

Values showed as median of at least 3 pictures of each animal. *percentage relative to positive pictures among total taken.

## FIGURES

**Figure 1: Resistance to diet-induced obesity on mice under HF/HC diet.** C57BL/6 mice were fed VHF, HF/HC, or HF/HS diet for 16 weeks. Body weight (A), food intake (B), and body fat mass (C) were evaluated along the study. Liver to body weight (D) and EAT weight to body lean mass (E) ratios were measured after sacrifice (16 weeks). *P<0.05 versus HF/HC at the same time point; $^{+}$P<0.05 versus HF/HS at the same time point (A and C). Different letters represents P<0.05 (B, D and E). All data are expressed as mean ± SEM.

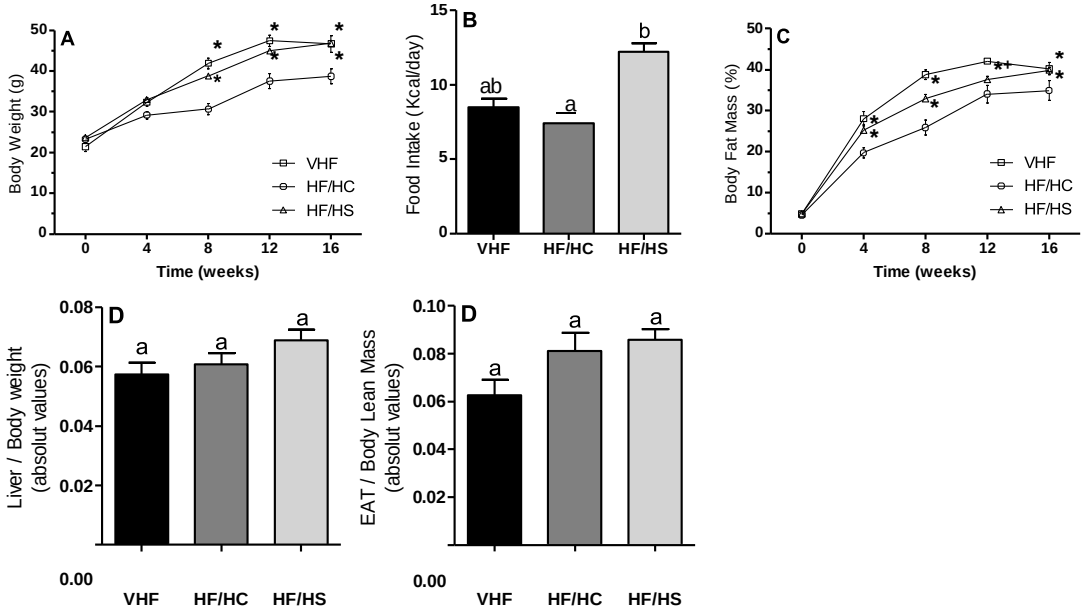


**Figure 2: Similar plasma lipid levels of mice under the different diets.** Plasma total cholesterol (A), triglycerides (B), and NEFA (C) of C57BL/6 mice fed VHF, HF/HC, or HF/HS diet during 16 weeks. *P<0.05 versus HF/HC and HF/HS at the same time point (A and B). Different letters represents P<0.05 (C). All data are expressed as mean ± SEM.

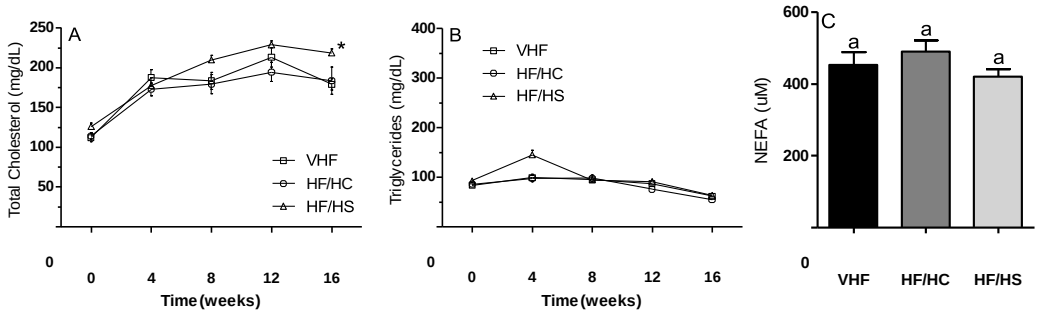


**Figure3: VHF diet feeding exarcebates glucose dysmetabolism.** C57BL/6 mice fed VHF, HF/HC, or HF/HS diet for 16 weeks were fasted overnight and submitted to ipGTT (A); inset graph represents area under curve for the different groups glucose values during the experiment. Overnight fasted plasma glucose and insulin levels of the same mice (16 weeks under diet) were also measured to assess the HOMA index (B). Different letters represents P<0.05. All data are expressed as mean ± SEM.

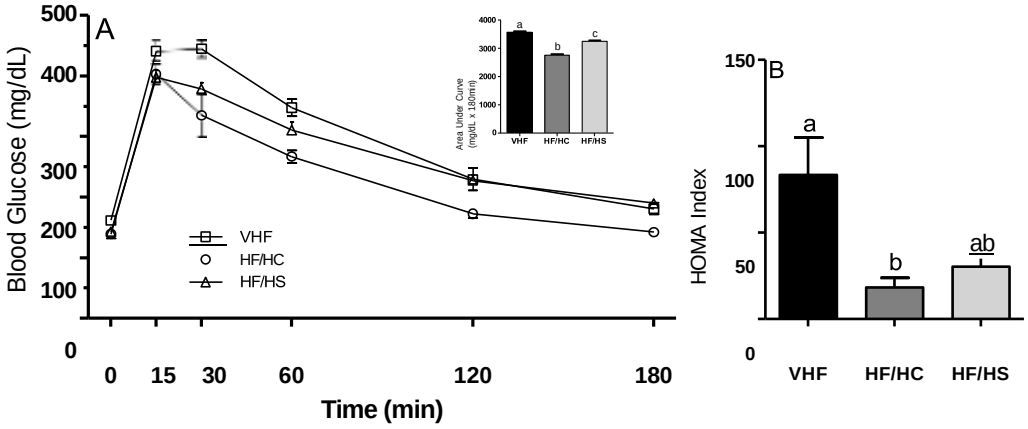


**Figure 4: Intraabdominal adipose tissue histology.** Epididymal adipose tissue (EAT) from C57BL/6 mice fed VHF, HF/HC, or HF/HS diet for 16 weeks were collected, processed, stained with Hematoxylin & Eosin (A) and had the adipocyte size measured (B). Crown-like structures (CLSs) were also quantified (C). Different letters represents P<0.05. Pictures at magnification 20x, arrows indicate CLSs. Data are expressed as relative frequency (B) or mean ± SEM (C).

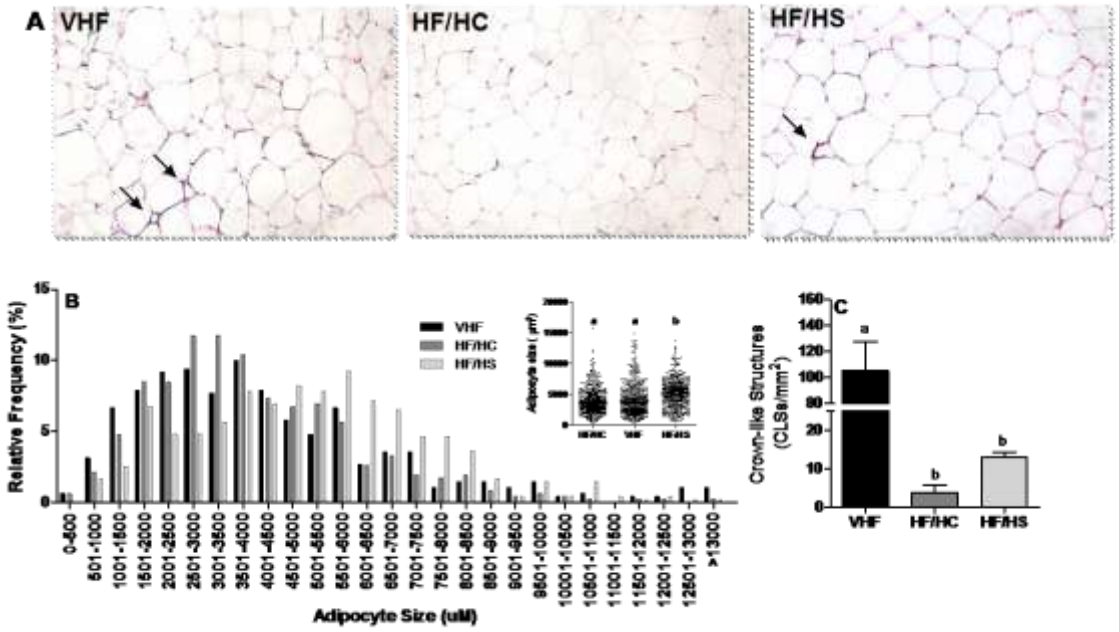


**Figure 5: VHF diet induced endocrine and inflammatory disbalance at adipose tissue.** Plasma and epididymal adipose tissue (EAT) from C57BL/6 mice fed VHF, HF/HC, or HF/HS diet for 16 weeks were collected. mRNA expression of TNF-α (A), MCP-1 (B), IL-1β (C), and PPAR-γ (D) was evaluated. Plasma levels of adiponectin (E), Leptin (F), and the adiponectin to leptin ratio (G) were also measured. Different letters represents $P<0.05$. Data are expressed as mean ± SEM.

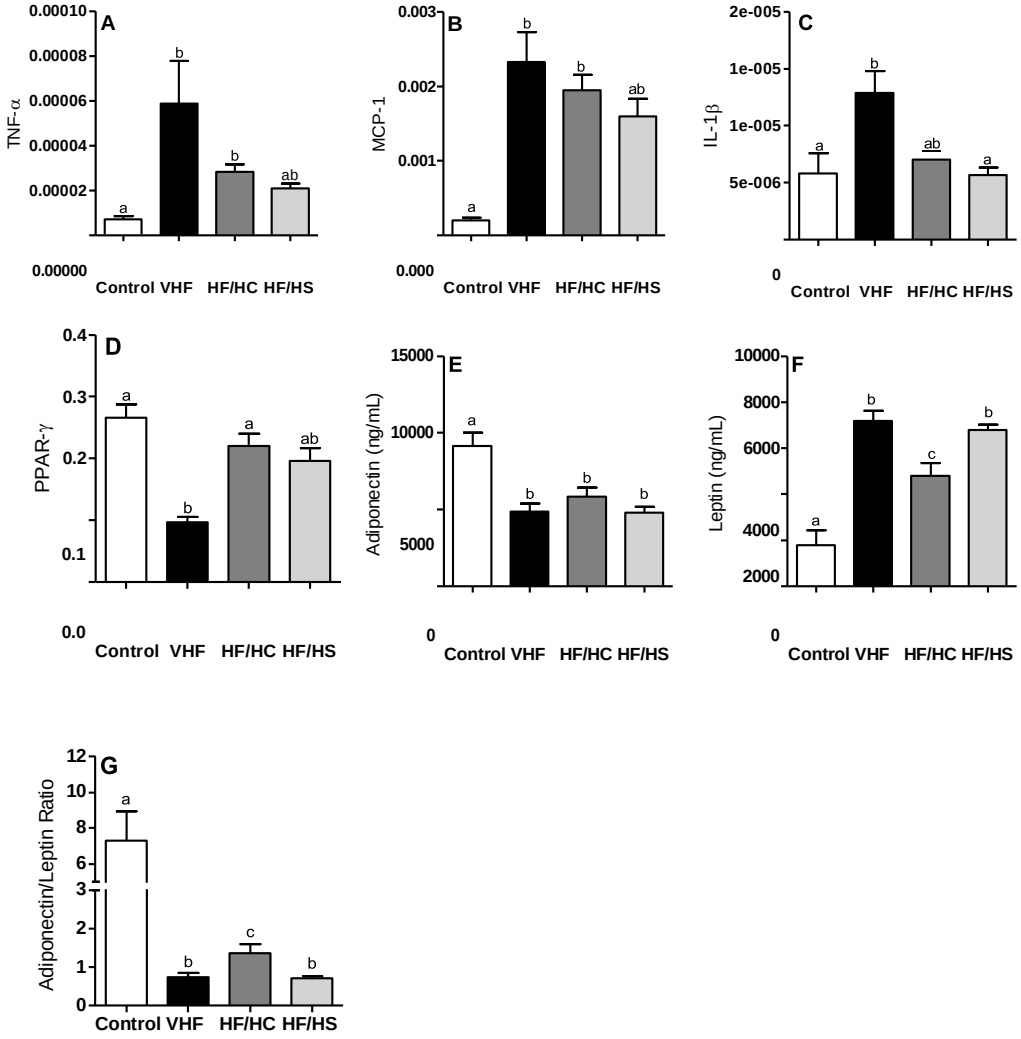


**Figure 6: HF/HS provoked the worst liver damage of all diets.** Liver tissue and plasma from C57BL/6 mice fed VHF, HF/HC, or HF/HS diet for 16 weeks were collected. Liver damage was evaluated by the measurements of plasma alanine aminotransferase (ALT) (A). Liver tissue was used to quantify the levels of cholesterol (B) and triglycerides (C) and to evaluate tissue architecture (D) and fibrosis (E). Pictures at magnification 20x. Different letters represents $P<0.05$. Data are expressed as mean ± SEM.

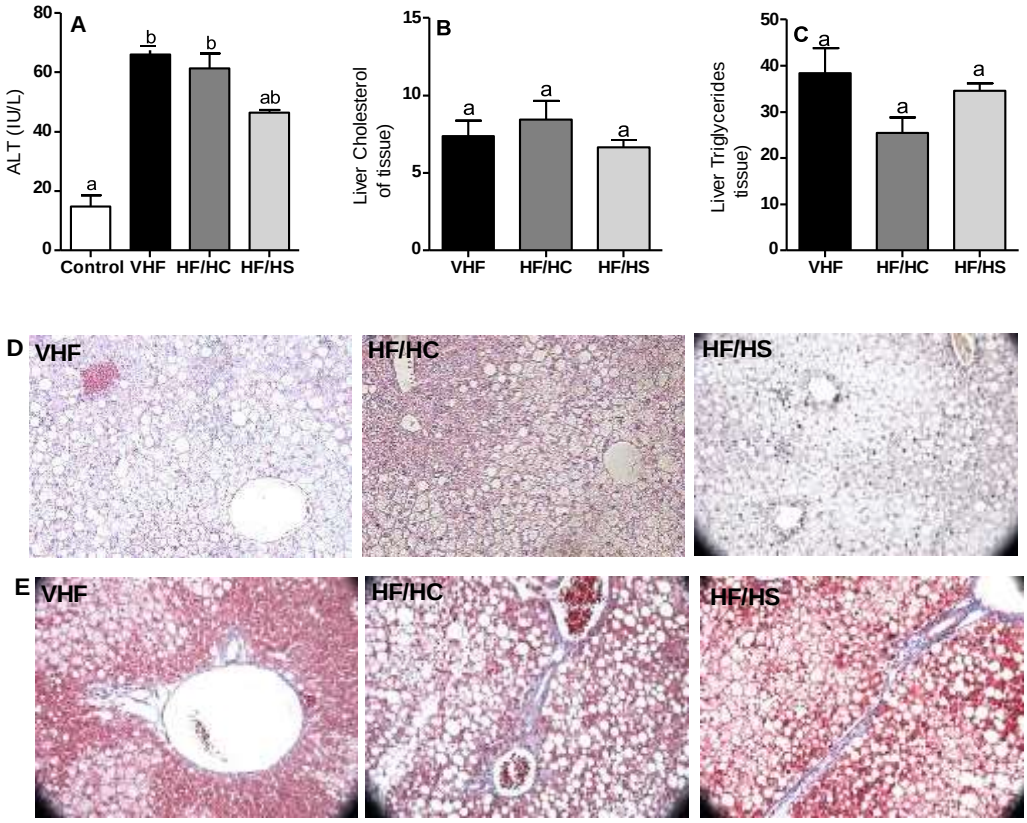
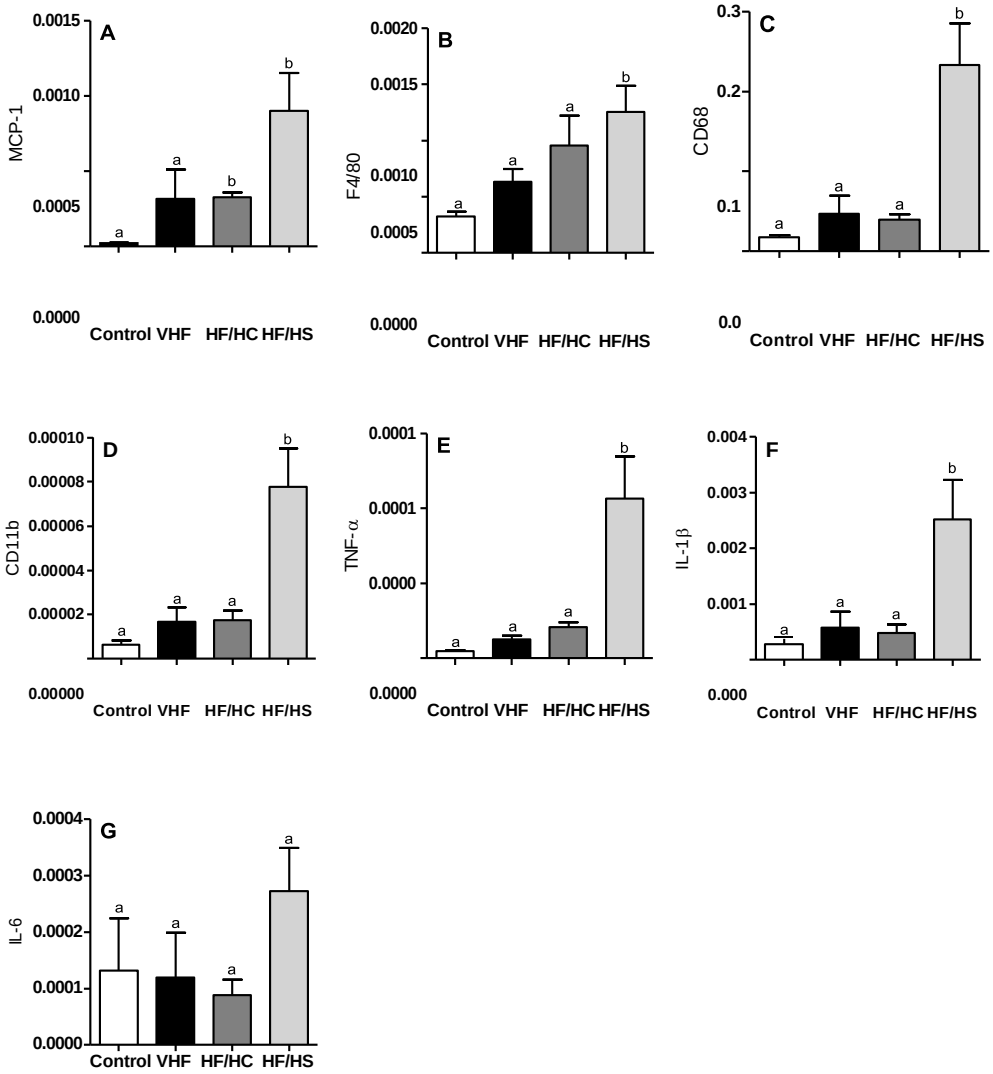


**Figure 7: Increased levels of pro-inflammatory markers in liver of HF/HS fed mice.** Livers from C57BL/6 mice fed VHF, HF/HC, or HF/HS diet for 16 weeks were collected. mRNA expression levels of MCP-1 (A), F4/80 (B), CD68 (C), CD11b (D), TNF-α (E), IL-1β (F), and IL-6 (G) were evaluated. Different letters represents P<0.05. Data are expressed as mean ± SEM.



## 9. Conclusões

- Os diferentes genótipos de ApoE não influenciaram a chance de infecção por *S. mansoni* como também o desenvolvimento da doença para a fase hepatoesplênica nesta população.
- A presença dos alelos ε2 e ε4 provocou modificações na forma como a esquistossomose mansônica hepatoesplênica repercute no metabolismo lipídico.
- Pacientes com esquistossomose mansônica hepatoesplênica, esplenectomizados ou não esplenectomizados, apresentam resistência à insulina.
- A resistência à insulina nos sujeitos com esquistossomose mansônica hepatoesplênica apresentou correlação negativa ou inexistente com marcadores lipídicos usuais de resistência insulínica.
- A dieta rica em ácidos graxos causou maior desregulação do tecido adiposo e resistência à insulina, enquanto que a dieta rica em sacarose induziu maior inflamação hepática e a dieta rica em colesterol provocou fibrose no fígado, mas sem o mesmo grau de inflamação.
- Distúrbios hepáticos de origem bastante diferentes, como esquistossomose mansônica e DHGNA, se mostraram semelhantes no que se refere à existência de repercussões metabólicas negativas para o organismo.
- Nas diferentes abordagens foi possível observar que o comprometimento do funcionamento hepático esteve relacionado com a desregulação do metabolismo energético, pela observação de modificações no metabolismo lipídico e resistência à insulina. No caso específico do estudo com modelo animal, foi possível observar, adicionalmente, que efeitos específicos podem ser gerados em decorrência da utilização de moléculas diferentes.

# Apêndices

**Apêndice A:**

Artigo - Hemostatic Dysfunction Is Increased in Patients with Hepatosplenic Schistosomiasis Mansoni and Advanced Periportal Fibrosis.

**Apêndice B:**

Artigo - Relationship between splenomegaly and hematologic findings in patients with hepatosplenic schistosomiasis.

**Apêndice C:**

Artigo - Lectin from *Crataeva tapia* Bark Improves Tissue Damages and Plasma Hyperglycemia in Alloxan-Induced Diabetic Mice.

**Apêndice D:**

Artigo – Splenectomy Improves Hemostatic and Liver Functions in Hepatosplenic Schistosomiasis Mansoni.

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# Hemostatic Dysfunction Is Increased in Patients with Hepatosplenic Schistosomiasis Mansoni and Advanced Periportal Fibrosis

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## Abstract

***Background:*** Schistosomiasis mansoni is an endemic parasitic disease and a public health problem in Northeast Brazil. In some patients, hepatic abnormalities lead to periportal fibrosis and result in the most severe clinical form, hepatosplenic schistosomiasis. This study aimed to evaluate whether abnormal blood coagulation and liver function tests in patients with hepatosplenic schistosomiasis ($n = 55$) correlate with the severity of their periportal fibrosis.

***Methodology/Principal Findings:*** Blood samples were used for liver function tests, hemogram and prothrombin time (International Normalized Ratio, INR). The blood coagulation factors (II, VII, VIII, IX and X), protein C and antithrombin IIa (ATIIa), plasminogen activator inhibitor 1 (PAI-1) and D-dimer were measured by photometry or enzyme linked immunosorbent assay. Hyperfibrinolysis was defined on the basis of PAI-1 levels and a D-dimer concentration greater than a standard cut-off of 483 ng/mL. Standard liver function tests were all abnormal in the patient group compared to healthy controls ($n = 29$), including raised serum transaminases ($p<0.001$) and lower levels of albumin ($p = 0.0156$). Platelet counts were 50% lower in patients, while for coagulation factors there was a 40% increase in the INR ($p<0.001$) and reduced levels of Factor VII and protein C in patients compared to the controls (both $p<0.001$). Additionally, patients with more advanced fibrosis ($n = 38$) had lower levels of protein C compared to those with only central fibrosis ($p = 0.0124$). The concentration of plasma PAI-1 in patients was one-third that of the control group ($p<0.001$), and D-dimer levels 2.2 times higher ($p<0.001$) with 13 of the 55 patients having levels above the cut-off.

***Conclusion/Significance:*** This study confirms that hemostatic abnormalities are associated with reduced liver function and increased liver fibrosis. Of note was the finding that a quarter of patients with hepatosplenic schistosomiasis and advanced periportal fibrosis have hyperfibrinolysis, as judged by excessive levels of D-dimer, which may predispose them to gastrointestinal bleeding.

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## Introduction

Schistosomiasis is a chronic parasitic liver disease that constitutes a major public health problem in several parts of the world. There are more than 200 million people affected by schistosomiasis worldwide and 600 million people are at risk of infection [1–3]. The disease caused by *Schistosoma mansoni* is the most prevalent liver disease in the Northeast region of Brazil [4]. Around 5–7% of patients infected by *S. mansoni* progress to the most severe form, hepatosplenic. Many patients exhibit high morbidity and mortality associated with periportal fibrosis, portal hypertension and splenomegaly, which lead to frequent episodes of upper gastrointestinal bleeding [5].

Periportal fibrosis constitutes the pathognomonic lesion of the liver in hepatosplenic schistosomiasis [6–8]. This process results from massive deposition of collagen products in the periportal spaces and leads in turn to progressive occlusion of the portal vein, portal hypertension, splenomegaly, collateral venous circulation and bleeding of the upper gastrointestinal tract. GI bleeding episodes are one of the causes of hepatic dysfunction in

## Author Summary

Schistosomiasis is a parasitic disease that affects the liver and in the severe hepatosplenic form results in periportal fibrosis. This disease is a major public health problem in Northeast Brazil. Our study aim was to evaluate whether abnormal blood coagulation and liver function tests in patients with hepatosplenic schistosomiasis depended on the severity of their fibrosis and could be used to inform diagnosis and treatment. We verified, by analyzing blood samples and by abdominal ultrasound of 55 patients, that blood clotting abnormalities are associated with reduced functioning of the liver and with increased liver fibrosis. Our results additionally suggested that reduced levels of protein C in plasma are a good marker of liver fibrosis progression. Also of note was our finding that a quarter of patients with advanced fibrosis have hyperfibrinolysis, a severe blood clotting disorder which may increase their risk of gastrointestinal bleeding. Therefore, we recommend, for patients eligible for surgical procedures, that certain blood tests (D-dimer, prothrombin time and platelet count) be measured during the pre-surgical evaluation to better assess risk of bleeding.


schistosomiasis; areas of hepatic necrosis can occur due to hypotension and loss of blood, which in liver regeneration can distort the hepatic parenchyma. When patients bleed more than once, hepatic dysfunction and compromised hemostasis ensue [6].

A more extensive pattern of fibrosis reflects the prognosis and severity of the chronic hepatosplenic condition [6], although it is generally reported that liver function remain preserved. On the other hand, some studies have found reduced levels of blood coagulation proteins, which are synthesized by liver cells [9–11]. Classically, it is believed that liver cell function is preserved in hepatosplenic schistosomiasis and that the compromised hemostasis is due to a consumptive coagulopathy related to the enlarged liver and spleen. In a previous study, it was suggested that early liver dysfunction in schistosomiasis may contribute to the problems with hemostasis [9]. However, it remains unclear whether an advanced pattern of fibrosis is linked to an adverse effect on hemostasis and liver function. Here, our aim was to determine whether abnormal blood coagulation and liver function tests in patients with hepatosplenic schistosomiasis correlate with the severity of their periportal fibrosis.

## Materials and Methods

### Ethical statement

The study was conducted according to the Helsinki Declaration and was approved by the Human Research Ethics Committee of the Federal University of Pernambuco (Number 028/11), in Brazil. All patients and healthy subjects received an explanation about the scope of the study, such as objectives, procedures and potential risks, and signed an informed consent statement before inclusion in the study.

### Patients

Fifty-five patients diagnosed with hepatosplenic schistosomiasis, and previously treated with praziquantel (50 mg/Kg) at least 6 months before the present study, were the overall of those attending as outpatients between 2010 and 2012 at the Gastroenterology Department, Clinical Hospital of the Federal University of Pernambuco, Recife, Brazil. When first seen at the clinic all patients had hepatosplenomegaly and portal hypertension, but without ascites, jaundice, encephalopathy and/or pulmonary hypertension, and a history of contact with river water within municipalities located in "Zona da Mata", an endemic area for schistosomiasis in Pernambuco State. Some had reported at least one episode of upper gastrointestinal bleeding.

The diagnosis of schistosomiasis was based on clinical history, physical examination and an abdominal ultrasonography which showed periportal fibrosis. Using the World Health Organization (Niamey Working Group, 2000) protocol [12] patients were classified as having peripheral fibrosis (Pattern C), central fibrosis (Pattern D), advanced fibrosis (Pattern E) or very advanced fibrosis (Pattern F) [12,13]. Patients who presented with advanced or very advanced fibrosis were grouped together for data analyses (Pattern E+F).

Patients were excluded if they reported alcohol abuse (>60 g ethanol/day for men and >40 g/day for women) or had a history of splenectomy, hepatic cirrhosis, systemic diseases such as diabetes mellitus, acute or chronic hepatitis B or C, collagenosis, heart and blood diseases. Use of hepatotoxic drugs, acetylsalicylic acid, anticoagulant drugs, or receiving a blood transfusion were also criteria for exclusion if less than 90 days prior to data collection. The control group consisted of twenty nine healthy individuals from the same age range (18 to 65 years) and socioeconomic background, as evaluated by a standardized questionnaire that enabled family budget, education level and lifestyle to be matched with those of the patients.

### Sample collection and processing

Three venous blood samples were collected under aseptic conditions without stasis using vacuum tubes (Vacutainer; *Becton Dickinson*, USA). The first tube contained 0.106M trisodium citrate at a 1:9 ratio to blood for coagulation tests. The second contained 0.362M EDTA-K3 and was used directly for platelet quantification, while the third blood collection tube was used for liver function tests. Tubes one and three were centrifuged for 10 min at 2000 *g* and the plasma and serum stored in 0.5 ml aliquots at −80°C until assayed.

### Parasitological diagnosis

Stool samples from control and patient groups were taken on two consecutive days and each tested twice by the Kato-Katz method. The mean egg counts are reported.

### Biochemical, hepatic and other tests

The routine liver function tests included aspartate and alanine aminotransferases (AST and ALT), alkaline phosphatase (ALP), γ-glutamyltransferase (γGT), bilirubin (Total, Direct and Indirect) and albumin and were measured by automated spectrophotometry (Cobas C501, Roche, Diamond Diagnostics, USA). Determinations of HBsAg, anti-HBc and anti-HCV were made by Chemiluminescence Microparticle Immuno Assay (CeMIA) using the ARCHITECT i2000 automatic light detector and test reagents (Abbott, North Chicago, USA) to exclude enrollment of patients with Hepatitis B or C. Abdominal ultrasound avoided inclusion of patients with hepatic cirrhosis and steatosis, and the anamnesis excluded patients with active use of alcohol.

### Platelet count and blood coagulation tests

Platelet counts (normal range $150–400 \times 10^9$/L) were measured by electrical impedance using the Pentra-120 (ABX Diagnostics, São Paulo, SP, Brazil). Coagulation tests were performed with an automated photooptical coagulometer, (Trinity Biotech, Acton, USA) and included measuring prothrombin time (PT, expressed as

the INR), partial thromboplastin time (PTT), thrombin test (TT) and fibrinogen.

Blood coagulation factors II, VII, VIII, IX, X were assayed using a Destiny Plus analyzer (Trinity Biotech, Acton, USA) and were based on correcting the long PT of factor deficient plasma by addition of test plasma diluted with clotting factor deficient plasma. Results were expressed as a percentage of activity for each factor. Protein C in test plasma was measured in the Destiny Plus analyzer with a specific snake venom protein C activator, thus inhibiting factor V and VIII in the added Protein C deficient plasma reagent and prolonging the subsequent PTT test, while antithrombin IIa was determined using saline buffer and specific reagents.

Antigenic assays to quantify tissue plasminogen activator (t-PA), plasminogen activator inhibitor −1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI) in plasma were measured by sandwich enzyme-linked immunosorbent assays ELISA (Asserachrom, Diagnostica Stago, France). Each sample was tested in duplicate and measured according to the supplier's instructions. D-dimer was assayed in an automated photo-optical coagulometer; values above 483 ng/mL were defined as hyperfibrinolysis [14].

### Statistical analysis

Unpaired Student's *t* test was used to compare differences between normally distributed variables of the hepatosplenic schistosomiasis patients (combined total) and control group, while the fibrosis pattern groups were compared by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test. Mann-Whitney and Kruskal-Wallis followed by Dunn's multiple comparison tests were used to compare differences among non-normally distributed variables. Variables were expressed as mean±Standard Error of the mean. P-values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using Statview SAS Inc. (1998, NC, USA).

## Results

All patients presented with some degree of periportal fibrosis. The Niamey classification [12] revealed a predominance of advanced periportal fibrosis, Pattern E (n = 30; 54.6%), followed by central fibrosis, Pattern D (n = 17; 30.9%), while only 8 of the patients (14.5%) showed Pattern F, very advanced fibrosis. No patient was classified as having Pattern C (peripheral fibrosis). For easiness of data analysis, the two patterns of advanced periportal fibrosis were combined into a single group (E+F; n = 38; 69.1%).

As a total group, the patients with hepatosplenic schistosomiasis showed abnormal liver function tests compared to the healthy controls with significantly ($p<0.05$) increased levels of serum AST, ALT, γ-GT, ALP and total bilirubin, and a lower concentration of albumin (Table 1). These differences were also seen when the two patients groups (Patterns D and E+F) were compared separately with the controls, except for the albumin level in the Pattern D group which was not significantly reduced. No differences were noted when patients with central fibrosis (Pattern D) were compared to those with advanced periportal fibrosis (Pattern E+F) although the level of γ-GT was 64% higher in the latter group ($p = 0.0501$; Table 1).

The combined total groups of patients all showed significant increases in the INR, PTT and TT values compared to the healthy controls, while the platelet count ($\times 10^9$/L) was 50% lower (128±13 vs. 261±10; $p<0.001$) (Table 2). All blood coagulation factors (II, VII, VIII, IX, X and antithrombin IIa) were lower in the total patient group, and these significant differences were also seen when Pattern D and Pattern E+F were compared with the healthy controls as separate groups (Table 2). However, only protein C was significantly different between the two patient groups (74.5±5.1% for Pattern D vs. 61.6±3.3% for Pattern E+F; $p = 0.0124$).

The levels of D-dimers were significantly higher in the total patient group compared to controls (210 ng/mL [61–3,224 ng/mL] vs. 96 ng/mL [47–190 ng/mL]; median [range]; $p<0.001$) and as shown in Figure 1 higher levels were a common feature in the patients with advanced periportal fibrosis (229 ng/mL [60.7–3,224 ng/mL]). Using the cut-off value of 483 ng/mL for D-dimer as a measure of hyperfibrinolysis [14], we found that 11 of 38 patients (29%) with advanced periportal fibrosis (Pattern E+F) and a history of upper digestive bleeding exhibited D-dimer levels above this value.

PAI-1 levels were decreased in the patients compared to the control group (65 ng/mL [5–162] vs. 202 ng/mL [17–448 ng/mL]; median [range]; $p<0.001$), but there were no significant differences ($p>0.05$) in plasma levels of t-PA and TAFI (data not shown).

All controls and patients were negative for elimination of Schistosoma mansoni eggs in the stool, and also for hepatitis B and C virus markers.

## Discussion

Periportal fibrosis is the main liver consequence of severe infection by *S. mansoni*. It plays a key role in the genesis of portal hypertension and in distorting hepatic parenchyma, which can cause hepatic dysfunction when the fibrosis is extensive. These effects may persist in some patients even after treatment and cure of the infection [7]. Moreover, bleeding episodes can allow progression to decompensated liver disease due to areas of hepatic necrosis caused by hypotension and loss of blood. In our study, we evaluated different patterns of advanced periportal fibrosis in patients with compensated hepatosplenic schistosomiasis, but without ascites, jaundice or hepatic encephalopathy. Pattern E [12] was the most prevalent, and 45% had a previous history of gastrointestinal bleeding. Consistent with our findings reported here, Correia et al [5] observed a high frequency (82%) of thrombocytopenia in patients with hepatosplenic schistosomiasis while other studies have demonstrated progressive deterioration of hepatic function in advanced stages of schistosomiasis disease [9–11].

Although we report that central fibrosis (Pattern D) is already associated with liver damage, our findings also suggest that γ-GT levels are the best marker of hepatic fibrosis progression. This supports the use of γ-GT as one of three biological markers proposed by Camacho-Lobato and Borges [9] to evaluate the progression of liver dysfunction in schistosomiasis, while Köpke-Aguiar et al. [15] also found γ-GT to be a sensitive indicator along with the platelet count and INR to differentiate patients with or without portal hypertension. Moreover, elevated γ-GT along with ALP was also a feature in hepatosplenic patients with anicteric cholangiopathy, who have alterations in biliary ducts (ductopenia intermediary and small caliber branches) reflecting advanced fibrosis [16].

The liver play a major role in the control of hemostasis, and disturbed liver parenchymal cell function affects the hemostastic system. Such studies report a frequent thrombocytopenia associated with the splenomegaly and portal hypertension [17]. The thrombocytopenia in hepatosplenic schistosomiasis is compensated, at least in part, by increased levels of von Willebrand factor

**Table 1.** Liver function tests in hepatosplenic schistosomiasis patients with different patterns of periportal fibrosis.

| Characteristics | Controls (C) | Hepatosplenic Schistosomiasis Patients | | | p-value | | | |
|---|---|---|---|---|---|---|---|---|
| | | Overall | D fibrosis pattern | E+F fibrosis pattern | Overall vs. C | D vs. C | E+F vs. C | D vs E+F |
| Subjects (n) | 29 | 55 | 17 | 38 | - | - | - | |
| AST (U/L) | 21.9±1.2 | 51.6±4.7 | 54.4±9.3 | 50.4±5.4 | <0.0001 | 0.0003 | 0.0001 | 0.6276 |
| ALT (U/L) | 18.6±0.9 | 49.2±5.8 | 57.5±10.9 | 45.4±6.9 | 0.0003 | 0.0005 | 0.0027 | 0.2399 |
| ALP (U/L) | 65.2±3.4 | 170.7±23.5 | 162.3±21.4 | 174.4±32.8 | 0.0017 | 0.0287 | 0.0026 | 0.7716 |
| γ-GT (U/L) | 27.2±2.6 | 145.0±19.2 | 103.4±24.1 | 169.4±25.1 | <0.0001 | 0.0313 | <0.0001 | 0.0501 |
| Albumin (g/dL) | 4.30±0.07 | 3.98±0.08 | 4.03±0.12 | 3.96±0.11 | 0.0156 | 0.1189 | 0.0170 | 0.6834 |
| Total bilirubin (g/dL) | 0.65±0.05 | 1.23±0.12 | 1.18±0.16 | 1.26±0.16 | 0.0011 | 0.0239 | 0.0017 | 0.7345 |
| Direct bilirubin (g/dL) | 0.31±0.03 | 0.52±0.08 | 0.43±0.06 | 0.57±0.12 | 0.1050 | 0.5333 | 0.0667 | 0.3624 |
| Indirect bilirubin (g/dL) | 0.31±0.04 | 0.71±0.07 | 0.75±0.15 | 0.69±0.08 | 0.0002 | 0.0017 | 0.0008 | 0.6343 |

Values are expressed as mean±Standard Error (SE). Unpaired Student's *t* test or one-way ANOVA followed by Fisher's PLSD post test.
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that enable platelets to adhere and aggregate at sites of vascular injury [5,18,19]. Köpke-Aguiar et al. [20] also reported that levels of thrombopoetin and reticulated platelets are normal in schistosomiasis patients with portal hypertension and that the bone marrow produces normal amounts of platelets. Thrombocytopenia in schistosomiasis patients may occur because of splenic retention due to poor portal blood drainage, or because platelets are trapped in the sinusoidal spaces of the fibrotic liver [20]. Our study confirms that thrombocytopenia is common in patients with hepatosplenic schistosomiasis, and that this tends to be higher in the advanced stages of periportal fibrosis.

A number of studies have demonstrated reduced vitamin K-dependent coagulation factors in patients with hepatosplenic schistosomiasis [9–11] and our findings agree with these reports. Several mechanisms may explain the substantial reductions in coagulation factors, including reduced hepatic synthesis and increased consumption. Impaired carboxylation of precursor molecules is also proposed for factors II, VII, IX and X [11], due to premature release of the protein from damaged hepatocytes, or because of vitamin K-dependent carboxylase deficiency and production of abnormal proteins [10,11]. Moreover, Tripodi et al. [21] recently highlighted the occurrence of concomitant decreases of both procoagulant and anticoagulant factors in chronic liver disease, mainly in cirrhotic patients. These features escaped attention for many years [11,21].

Levels of protein C and antithrombin were significantly lower in our patients compared to the healthy controls, presumably reflecting hepatic dysfunction caused by portal hypertension and advanced periportal fibrosis [10,20–22]. Although bleeding events in hepatosplenic schistosomiasis are associated with portal hypertension [20], the deficient production of coagulation factors does not seem to aggravate the situation due to a balance between the reductions in pro- and anti-coagulation proteins. Our results also show that in almost all cases the changes in blood coagulation

**Table 2.** Coagulation parameters from hepatosplenic schistosomiasis patients with different patterns of periportal fibrosis.

| Characteristics | Controls (C) | Hepatosplenic Schistosomiasis Patients | | | p-value | | | |
|---|---|---|---|---|---|---|---|---|
| | | Overall | D fibrosis pattern | E+F fibrosis pattern | Overall vs. C | D vs. C | E+F vs. C | D vs. E+F |
| Subjects (n) | 29 | 55 | 17 | 38 | - | - | - | - |
| Platelets Count ($\times 10^9$/L) | 261.1±9.8 | 128.4±12.7 | 146.6±23.1 | 120.3±14.0 | <0.0001 | <0.0001 | <0.0001 | 0.1112 |
| INR | 1.01±0.02 | 1.44±0.06 | 1.38±0.09 | 1.47±0.07 | <0.0001 | 0.0028 | <0.0001 | 0.4828 |
| TT (Seconds) | 11.9±0.2 | 13.8±0.2 | 13.56±0.4 | 13.85±0.3 | <0.0001 | 0.0001 | <0.0001 | 0.4770 |
| PTT (Seconds) | 31.1±0.6 | 37.9±1.5 | 36.4±1.9 | 38.6±2.0 | 0.0012 | 0.0510 | 0.0010 | 0.3969 |
| Fibrinogen (mg/dL) | 342.9±21.0 | 267.5±9.9 | 263.4±15.6 | 269.4±12.7 | 0.0002 | 0.0045 | 0.0007 | 0.9770 |
| Factor II (%) | 92.7±3.3 | 66.6±2.3 | 67.8±4.2 | 66.0±2.8 | <0.0001 | <0.0001 | <0.0001 | 0.6377 |
| Factor VII (%) | 84.8±4.1 | 49.9±2.4 | 56.6±4.2 | 46.9±2.7 | <0.0001 | <0.0001 | <0.0001 | 0.0839 |
| Factor VIII (%) | 120.2±6.5 | 90.5±4.2 | 96.3±8.2 | 87.9±4.8 | 0.0001 | 0.0164 | <0.0001 | 0.3238 |
| Factor IX (%) | 100.1±3.3 | 59.9±2.5 | 65.3±4.5 | 57.5±2.9 | <0.0001 | <0.0001 | <0.0001 | 0.1375 |
| Factor X (%) | 88.8±3.7 | 63.8±3.7 | 59.1±7.4 | 65.91±4.3 | <0.0001 | 0.0001 | 0.0001 | 0.4842 |
| Protein C (%) | 100.1±2.2 | 65.6±2.9 | 74.5±5.1 | 61.6±3.3 | <0.0001 | <0.0001 | <0.0001 | 0.0124 |
| Antithrombin IIa (%) | 112.0±3.4 | 92.8±3.5 | 96.0±8.8 | 91.3±3.3 | <0.0001 | 0.0319 | 0.0007 | 0.4560 |

Values are expressed as mean±Standard Error (SE). Unpaired Student's *t* test or one-way ANOVA followed by Fisher's PLSD post test.
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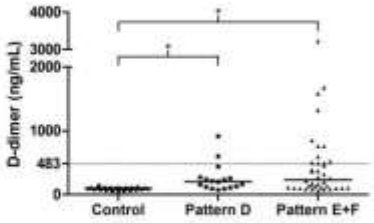


**Figure 1. Plasma D-dimer levels in healthy control subjects (Control) and hepatosplenic schistosomiasis patients with either central fibrosis (Pattern D) or advanced periportal cirrhosis (Pattern E+F).** The solid horizontal lines show D-dimer median values (96, 199 and 229 ng/mL), while the dashed line indicates the cut-off value for hyperfibrinolysis [14]. *p<0.001; Kruskal-Wallis test.
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proteins were greater in patients with advanced periportal fibrosis (Pattern E+F) than those with central fibrosis (Pattern D), although statistical significance for the difference was only reached for protein C. Nevertheless, our data suggest that platelet counts, Factor VII and protein C are good predictors of advanced fibrosis.

Fibrinolysis was studied in patients with decompensated hepatosplenic schistosomiasis by El-Bassiouni et al. [23] who reported high concentrations of t-PA and low levels of PAI-1. Although PAI-1 was also decreased in our patients, the level of t-PA (and TAFI) did not differ between our patients and controls. In addition, we found increased levels of D-dimer, most notably in those patients with advanced periportal fibrosis (Pattern E+F). Plasma D-dimer concentration reflects the degree of thrombin turnover and consequently increased levels are a good marker of recent coagulation and fibrinolysis. Indeed, Primignani et al. [14] suggested D-dimer as a predictor of death in patients with liver cirrhosis. Using the cut-off value proposed by Primignani et al. [14], one-third of our patients with Pattern E+F were considered to have hyperfibrinolysis, which may increase their risk of a bleeding event. Therefore, we recommend for patients eligible for surgical procedures that D-dimer be measured during the pre-surgical evaluation, in addition to PT and platelet count, to better assess the risk of bleeding.

One limitation of our study is that it was conducted at a single hospital, the Hospital das Clínicas, UFPE. This is the reference hospital for schistosomiasis in Pernambuco State and receives the most severe cases of schistosomiasis, usually patients with a history of one or more episodes of gastrointestinal bleeding and hence a high proportion with abnormal liver function tests. Thus, the findings from our study may not extrapolate to all patients from endemic areas who present with the hepatosplenic form of the disease.

In conclusion, our study verified that coagulation abnormalities in hepatosplenic schistosomiasis are due to liver fibrosis and portal hypertension, and additionally demonstrated that these abnormalities increase in advanced periportal fibrosis and that reduced levels of protein C may be a good marker of hepatic fibrosis progression.

## Supporting Information

**Checklist S1** STROBE checklist.
(DOC)

## Author Contributions

Conceived and designed the experiments: LACI, VLdML, ALCD EPL. Performed the experiments: LACI, AAPF SMLM. Analyzed the data: LACI, CSMdF BSdS RdCdSF EPL ALCD VLdML. Contributed reagents/materials/analysis tools: VLdML. Wrote the paper: LACI, CSMdF BSdS EPL ALCD VLdML JSO.

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Original Article

# Relationship between splenomegaly and hematologic findings in patients with hepatosplenic schistosomiasis

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***Background:*** *Schistosomiasis is a tropical disease. Patients who develop hepatosplenic schistosomiasis have clinical findings including periportal fibrosis, portal hypertension, cytopenia, splenomegaly and gastrointestinal hemorrhage.*

***Objective:*** *The aim of this study was to analyze the hemostatic and hematologic findings of patients with schistosomiasis and correlate these to the size of the spleen.*

***Methods:*** *Fifty-five adults with hepatosplenic schistosomiasis and 30 healthy subjects were selected through a history of contact with contaminated water, physical examination and ultrasound characteristics such as periportal fibrosis and splenomegaly in the Gastroenterology Service of the Universidade Federal de Pernambuco. Blood samples were collected to determine liver function, blood counts, prothrombin (international normalized ratio), partial thromboplastin time and fibrinogen and D-Dimer levels using the Pentra 120 hematological analyzer (HORIBA/ABX), Density Plus (test photo-optical Trinity Biotech, Ireland) and COBAS analyzer 6000 (Roche). Furthermore, the longitudinal size of the spleen was measured by ultrasound (Acuson X analyzer 150, Siemens). The Student t-test, the Fisher test and Pearson's correlation were used to analyze the results with statistical significance being set for a p-value < 0.05.*

***Results:*** *The mean age was higher for the Study Group than for the Control Group (54 ± 13.9 vs. 38 ± 12.7 years). The average longitudinal diameter of the spleen was 16.9 cm (Range: 12.5-26.3 cm). Anemia is a common finding in patients with schistosomiasis (36.3%). The mean platelet and leukocyte counts of patients were lower than for the Control Group (p-value < 0.001). Moreover, the international normalized ratio (1.42 vs. 1.04), partial thromboplastin time (37.9 vs. 30.5 seconds) and D-Dimer concentration (393 vs. 86.5 ng/mL) were higher for the Study Group compared to the Control Group.*

***Conclusion:*** *This study suggests that hematological and hemostatic abnormalities are associated with splenomegaly, hypersplenism and portal hypertension.*

***Keywords:*** *Schistosomiasis mansoni; Schistosomiasis; Splenomegaly; Hypersplenism; Thrombocytopenia*

## Introduction

Schistosomiasis mansoni is a chronic parasitic disease and the most prevalent tropical liver disease in the northeastern region of Brazil. There are 200 million people affected by *Schistosoma mansoni* worldwide with 600 million people being exposed. Around 5-7% of the patients infected by *S. mansoni* progress to the most severe form, hepatosplenic schistosomiasis (HS). Many of these patients exhibit high morbidity associated with periportal fibrosis, portal hypertension, splenomegaly, upper digestive tract bleeding and cytopenia[1-3].

Hypersplenism is a consequence of massive splenomegaly and is a common finding in chronic liver diseases. In schistosomiasis, this results from hyperplasia of the reticuloendothelial system and consequently venous congestion caused by portal hypertension. Studies have reported a correlation between the increase of spleen size and drops in blood cell counts, mainly the platelet count. These findings depend on the severity of portal hypertension as some studies have shown that thrombocytopenia is more common in HS patients, especially after episodes of digestive tract bleeding[4-8].

Some studies have reported that compensated HS patients exhibit normal hepatic function even with some abnormalities in blood clotting. Other reports have called attention to hemostatic abnormalities in decompensated HS patients who develop ascites and upper digestive tract bleeding including increases in the prothrombin time/international normalization ratio (PT/INR), partial thromboplastin time (PTT), thrombin time (TT) and abnormalities in K-dependent factors. The liver plays an important role in the control of blood coagulation and disorders of parenchymal liver cell function affect the hemostatic system. Liver disorders may be associated with the reduction of coagulation protein synthesis, in particular in cirrhosis patients. These findings are still unclear and represent a contradiction for medical research, especially in studies involving liver diseases such as cirrhosis and schistosomiasis and other liver disorders that lead to progressive hepatic damage[9-11].

The purpose of this study was to evaluate the hematological and hemostatic abnormalities in patients with severe forms of schistosomiasis, and possible associations with splenomegaly and portal hypertension.

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## Methods

### Diagnosis

A prospective study was undertaken involving 55 compensated HS patients previously treated using praziquantel (50 mg/kg) and 30 healthy subjects in the Outpatient Gastrointestinal Service of *Hospital das Clínicas* of the *Universidade Federal de Pernambuco* (UFPE), Recife, Brazil from 2010 to 2012. Patients and controls had comparable socioeconomic conditions including similar salaries, educations and lifestyles. The diagnosis and consecutive selection of HS patients was based on clinical history, physical examination and an abdominal ultrasonography that showed periportal fibrosis and splenomegaly. The abdominal ultrasound was performed using an ultrasound analyzer (Acuson X 150, 3.5 MHz, Siemens). For this, we used the Cairo and Niamey protocols to measure the longitudinal diameter of the spleen and classify the pattern of fibrosis as central fibrosis (Pattern D), advanced fibrosis (Pattern E) or very advanced fibrosis (Pattern F)[12-14].

Upper digestive tract endoscopy was used either to confirm or exclude the presence of esophageal varices and viral marker and liver function tests were performed to exclude viral hepatitis B and C. Patients suffering from alcoholism (> 60 g/day for men and > 40 g/day for women)[15], systemic diseases such as diabetes mellitus, collagenosis, blood diseases (lymphoproliferative disease and lymphomas), those that were taking hepatotoxic, antiaggregant or anticoagulant drugs and every patient with a history of splenectomy or blood transfusions (within the previous 3 months) were carefully excluded. Stool samples were examined to detect intestinal parasites by the Lutz-Hoffman followed by Kato-Katz method. Routine liver tests included analysis of aspartate transaminase (AST) alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (γGT) and albumin. The Control Group had the same exclusion criteria as described above and were checked for possible contact with water contaminated by the cercariae of *S. mansoni* and positive parasitological tests. The compensated hepatosplenic form corresponds to the severe form of disease and includes patients with hepatosplenomegaly and portal hypertension, but without ascites, jaundice or encephalopathy[16].

### Sample collection

About 15 mL of venous blood was drawn under aseptic conditions without stasis and placed in vacuum tubes (Vacutainer, Becton Dickinson, UK). The first blood sample was placed in a polypropylene tube containing 0.106 M trisodium citrate at a blood-anticoagulant ratio of 9:1. These tubes were used for the coagulation tests (PT, PTT, fibrinogen and D-dimer). The blood samples were centrifuged at 2000 g for 15 minutes at 4°C. Subsequently, the platelet free plasma was quickly distributed in 0.5 mL aliquots in plastic-capped tubes and stored at -80°C for six months until processing. A second sample, placed in a tube containing ethylenediaminetetraacetic acid (EDTA), was used for the complete blood count (CBC). A blood smear was prepared and stained for conventional microscopic analysis. The CBC was carried out by electrical impedance or light dispersion using a Pentra 120 analyzer (ABX, São Paulo, SP, Brazil). A third sample was collected for routine liver tests (AST, ALT, γGT, ALP, albumin) using biochemistry tubes (Becton Dickinson, UK) and the assay was performed using the COBAS 6000 analyzer (Roche). The aliquots of plasma serum samples were also stored for the other tests. The hemostatic tests (PT/INR, PTT, fibrinogen and D-dimer) were carried out in an automated photo-optical coagulometer, (Trinity Biotech, Acton, Ireland) following the manufacturer's instructions. The study was conducted according to the norms of the Declaration of Helsinki. The protocols for the collection and use of human samples were submitted and approved by the Research Ethics Committee of the Health Sciences Center of the UFPE (N° 028/11). All subjects received an explanation about the study and signed informed consent forms.

### Statistical analysis

Statistical analyzes were performed using the unpaired Student t-test and Fisher's test for contingency analysis. These analyzes were performed using the Statistical Package for Social Sciences software (SPSS 17.0, Chicago, IL, USA). Continuous variables were expressed as means ± standard deviation (SD), median and range. Furthermore, the Pearson correlation test was employed to examine the relationship between the longitudinal diameter of the spleen and platelet counts. P-values of less than 0.05 were considered statistically significant.

## Results

The results are shown in Tables 1 & 2 and in Figures 1 to 4. The patients had a mean age of 54 ± 13.9 years and the 30 control subjects had a mean age of 38.3 ± 13.7 years (p-value < 0.001) but no difference was found in respect to the gender. The Niamey

Table 1 - Liver function tests in hepatosplenic schistosomiasis patients and the Control Group

| Variable | Control Group | Hepatosplenic Group | p-value |
|---|---|---|---|
| Participants | 30 | 55 | |
| AST (units/L) median (range) | 20 (15-29) | 39 (15-201) | < 0.0001 |
| ALT (IU/L) median (range) | 17.5 (9-27) | 36 (12-257) | < 0.0001 |
| ALP (IU/L) median (range) | 67.0(28-105) | 132 (58-1321) | < 0.0001 |
| γGT (IU/L) median (range) | 24.5 (9-75) | 93 (16-756) | < 0.0001 |
| Albumin (g/L) (mean ± SD) | 43 ± 5 | 38 ± 6 | < 0.0001 |

AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; γGT: gamma-glutamyl transferase.

Table 2 - Distribution of the hepatosplenic schistosomiasis patients according to type of cytopenia

| Cytopenias | n | % |
|---|---|---|
| No cytopenia | 12 | 21.8 |
| Leukopenia in isolation | 4 | 7.27 |
| Thrombocytopenia in isolation | 12 | 21.8 |
| Bicytopenia | 17 | 30.9 |
| Pancytopenia | 10 | 18.1 |
| Total | 55 | 100 |

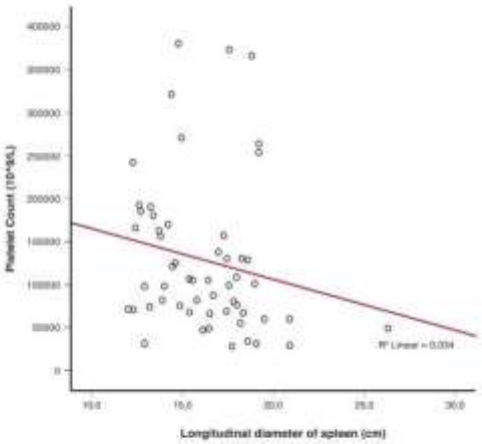


Figure 1 – Relationship between longitudinal diameter of the spleen and platelet counts in hepatosplenic patients

A direct positive correlation was observed between splenomegaly and platelet counts in schistosomiasis patients ($r^2$ = 0.034, p-value = 0.02)

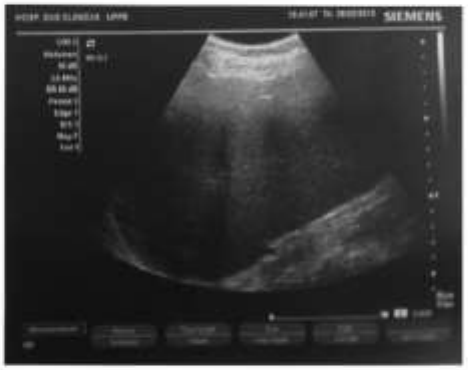


Figure 2 – Image showing a massive splenomegaly (18.7 cm) in a hepatosplenic schistosomiasis patient

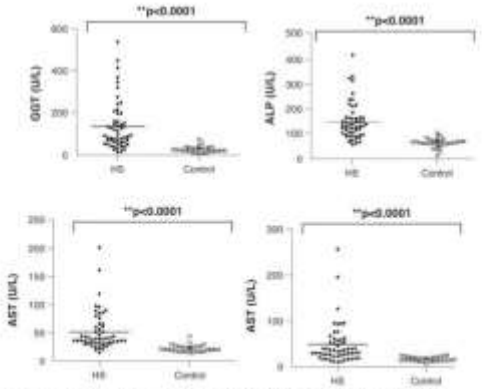


Figure 3 – Routine liver tests in the Control and Schistosomiasis Groups

Data are expressed as means. Controls vs. hepatosplenic schistosomiasis (HS) patients
AST: Aspartate transaminase; ALT: Alanine transaminase; γGT: gamma glutamyl transferase

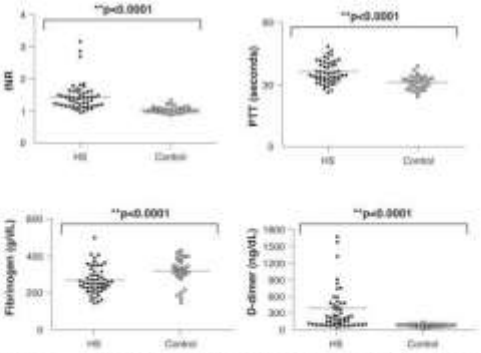


Figure 4 – Coagulation and fibrinolytic parameters in Control and Schistosomiasis Groups

Data are expressed as means
Controls vs. hepatosplenic schistosomiasis (HS) patients
INR: international normalized ratio; PTT: partial thromboplastin time
The D-dimer concentration was used for measure the fibrinolytic status and risk of bleeding

classification of fibrosis showed predominance of advanced (pattern E - 30 patients; 54.5%), followed by pattern D (17 patients; 30.9%) with only eight patients (14.5%) having very advanced fibrosis (pattern F). The routine liver tests showed significant differences in relation to parameters such as albumin, AST, ALT, ALP and γGT between the groups (p-value < 0.001; Figure 1).

Upper gastrointestinal bleeding was found in 34 (61.8%) patients. The mean longitudinal diameter of the spleen of the 55 patients was 16.9 cm (Range: 12.3-26.3 cm) and the median platelet count was 101.0 x $10^9$/L. A total of 38 (69.1%) patients had thrombocytopenia (platelet counts below 150 x $10^9$/L). Furthermore, 14.5% of patients had platelet counts below 50 x $10^9$/L (Table 1). There was an inverse correlation between the longitudinal diameter of the spleen and the platelet count (Figure 2). It was also found that 36.3% of patients had anemia and 47.3% presented leukopenia (cytopenias were different in each

patient). Table 2 shows the distribution of HS patients according to the type of cytopenia. Figure 3 demonstrates the massive splenomegaly of one patient with schistosomiasis.

Figure 4 demonstrates that HS patients presented with increased INR, PTT, and fibrinogen and D-dimer concentrations. There were significant differences in respect to these parameters on comparing the Control Group with patients (p-value < 0.001).

## Discussion

Portal hypertension is one of the most important consequences of *S. mansoni* infections. Due to the fibrotic process and venous congestion, HS patients develop hemodynamic changes associated with splenomegaly and high morbidity rates[16]. In a recently study in the same region, Dias et al.[17] showed that 61% of HS patients had advanced or very advanced periportal fibrosis. In the current study, all HS patients presented with a severe form of HS and portal hypertension, 38 (69%) had advanced and very advanced liver fibrosis (pattern E+F) and 34 (61.8%) patients had gastrointestinal bleeding. This proves that these patients have advanced disease and that changes in liver function tests may be related to the high frequency of advanced fibrosis.

Camacho-Lobato et al. and Tanabe reported that a large number of HS patients present conserved liver function even with periportal fibrosis[9,11]. However, this study demonstrates elevated liver function tests and remarkable changes in coagulation tests. Some studies have reported varied frequencies of thrombocytopenia (30 to 75%) depending on the stage of portal hypertension[9,16]. Lower values of platelet counts are seen especially in patients with HS after gastrointestinal bleeding. This was also seen in the current work with the possible cause being the increase of spleen and hypersplenism leading to frequent thrombocytopenia, leukopenia and anemia.

Thrombocytopenia is a common feature in chronic liver disease. Correia et al.[9] showed that thrombocytopenia can be compensated by increased levels of von Willebrand factor in HS patients. Other studies have shown that nearly 50% of HS patients exhibit thrombocytopenia, 16% bicytopenia and 7% anemia or leukopenia[18]. Anemia in patients with liver disease is often multifactorial and may be associated with iron and folic acid deficiency or even an inflammatory process. Red blood cell survival is often shortened in chronic liver disease with the increased spleen being a major site of red blood cell destruction[17]. In this study, high frequencies of thrombocytopenia (74.1%), leukopenia (47.3%), anemia (36.3%), bicytopenia (30.9%) and pancytopenia (18.2%) were found. The high frequencies of cytopenias found may be associated with the increased spleen size and the consequent hypersplenism leading to an increase in scavenging and retention of blood cells in the spleen, mainly platelets and white blood cells. Furthermore, anemia may be accentuated because of the low socioeconomic situation of the patients and due to blood loss from upper digestive tract bleeding.

Berzigotti et al.[19] used the diameter of the spleen combined with platelet count as a noninvasive marker of portal hypertension; these tools can also be used to predict esophageal varices and upper gastrointestinal bleeding episodes, especially in asymptomatic patients with cirrhosis. In this study, an inverse relationship was found between platelet count and longitudinal diameter of the spleen. Concomitant enlargement of the spleen from 15 to 20 cm together with a platelet count below 100 x $10^9$/L is an excellent predictor of portal hypertension, esophageal varices and upper digestive tract bleeding, even in asymptomatic patients with schistosomiasis. These two parameters combined with an increased INR could be a useful noninvasive tool to predict a different behavior of portal hypertension in patients with HS. Nevertheless, further studies are needed to identify other noninvasive markers and thus validate a score to classify the different stages of schistosomiasis and other liver diseases.

A recent study showed that the levels of thrombopoietin and reticulated platelets were normal in schistosomiasis patients with portal hypertension and that the bone marrow produces platelets normally[20]. The cytopenias in schistosomiasis patients may occur because the cells are retained in the spleen due to difficulty in draining portal blood. Additionally, platelets may be retained in the sinusoidal spaces of the fibrotic liver. Some studies have shown that the degree of liver fibrosis is associated with the degree of esophageal varices and that the risk of bleeding is associated with the degree of liver fibrosis[21]. Studies by Köpke-Aguiar et al.[20] report that there are no changes in the production of platelets in the bone marrow in schistosomiasis patients.

## Conclusion

The present study suggests that the hematological abnormalities seen in HS patients are associated with splenomegaly, hypersplenism and portal hypertension. However, further studies are needed to verify whether the platelet count might be a non-invasive tool to assess portal hypertension.

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*Research Article*

# Lectin from *Crataeva tapia* Bark Improves Tissue Damages and Plasma Hyperglycemia in Alloxan-Induced Diabetic Mice

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*Crataeva tapia* is a plant popularly used for diabetes treatment, in Brazil. Progressive decline in renal and hepatic functions has been described in patients with diabetes mellitus, and mortality rate is increased in patients with chronic liver and renal disease. This study aimed to evaluate whether *Crataeva tapia* bark lectin (CrataBL) improves hyperglycemia and renal and hepatic damage in diabetic mice. CrataBL was purified by ion exchange chromatography on CM-cellulose, and intraperitoneal administration of CrataBL to alloxan-induced diabetic mice at dose of 10 mg/Kg/day and 20 mg/Kg/day for 10 days significantly reduced serum glucose levels by 14.9% and 55.9%, respectively. Serum urea, creatinine, aspartate aminotransferase, and alanine aminotransferase were also significantly reduced after treatment with both doses of CrataBL. Furthermore, histological analysis of liver, kidney, and pancreas revealed an improvement in the tissue morphology upon treatment with CrataBL. The results suggest that CrataBL has a beneficial hypoglycemic activity and improves the renal and hepatic complications of diabetes. Therefore, this lectin may be a promising agent for the treatment of diabetes, and this might be the basis for its use in the folk medicine as an alternative treatment to manage diabetes-related complications such as hyperglycemia and tissue damage.

## 1. Introduction

*Crataeva tapia* (also known as *Crateva tapia*), a plant of Capparidaceae family, is commonly found in Pluvial Tropical Atlantic Forest and Pantanal Tropical Forest in Brazil [1]. *C. tapia* is known by Northeast Brazilian people as "pauadalho" or "tapiá" and its bark is largely used in the folk medicine for the treatment of diabetes. Recently, a lectin with a molecular weight of 40 kDa (CrataBL) was purified from the aqueous extract of *Crataeva tapia* bark [2]. Lectins are carbohydrate binding proteins, of nonimmunogenic origin, that bind specifically and reversibly to different types of carbohydrates or glycoproteins and can be obtained from several sources, mainly from vegetal [3]. Several plant lectins have been demonstrated to possess a variety of biological activities including antitumor [4–6], anti-inflammatory [7, 8], antimicrobial [9–11], analgesic [4], antioxidant [3] insecticidal [2, 12–14], anticoagulant [15], and hypoglycemic [16, 17].

Diabetes mellitus is a chronic disease considered to be one of the five leading causes of death in the world, and it is a complex metabolic disease with great development of pathological changes in many tissues [18]. The

disease is characterized by alteration in the carbohydrate metabolism resulting in an increase of the glucose levels [19]. Approximately 360 million of adult people have diabetes, corresponding to 8.3% of the world with diabetes, and this is projected to rise to 552 million by 2030, corresponding to 9.9% of the world population [20]. The hyperglycemia in diabetes produces superoxide anions, which generate hydroxyl radicals, promoting cell membrane damages as a result of lipid peroxidation and protein glycation of membrane [18]. In diabetic individuals the major alterations occur in renal and hepatic tissue and have been associated with functional and morphological damage in these organs [21, 22]. Among the common complications of diabetes the nephropathy is a chronic disease that affects 40% of individuals. Diabetic nephropathy is responsible for 50% of chronic renal failure cases [23]. Furthermore, hepatic dysfunction promoted by diabetes can result in nonalcoholic steatosis, hepatomegaly amongst others [24].

Studies have reported that the doubts about the efficacy and safety of some of the oral hypoglycemic agents have prompted a search for safer and more effective drugs in the treatment of diabetes [25]. Thus, the aim of the present study was to investigate whether CrataBL from *C. tapia* bark is a metabolite with potential antihyperglycemic activity.

## 2. Material and Methods

*2.1. Chemicals.* Alloxan monohydrated and CM-cellulose was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Insulin (Humolin N) was purchased from Lilly, Brazil. All the other chemicals used were in an analytical grade.

*2.2. Plant Material.* *C. tapia* barks were collected from the Recife City, PE, Northeast Brazil. The plant was identified by *Instituto Agronômico de Pernambuco* (IPA) and a voucher specimen was deposited (n° 61.415).

*2.3. Purification of Crataeva Tapia Bark Lectin.* *C. tapia* bark lectin was obtained through a sequential purification protocol as previously reported by Araújo et al. [2]. Powdered bark (10 g) was suspended in 0.15 M NaCl (100 mL). After homogenization in a magnetic stirrer (16 h at 4°C), followed by filtration through gauze and centrifugation (4,000 ×g, 15 min), the supernatant (crude extract) was taken as starting material. Soluble proteins in crude extract were fractionated with ammonium sulphate and the 30–60% precipitate fraction (30–60 F) was submitted to dialysis (3,500 Da cut-off membrane, 4°C) against distilled water (2 h) followed by 10 mM citrate-phosphate buffer pH 5.5 (2 h). The 30–60 F was applied (11 mg of protein, hemagglutinating activity of 1024) into a CM-cellulose chromatography column (5.2 cm × 1.6 cm) equilibrated with 10 mM citrate-phosphate buffer pH 5.5 at flow rate of 20 $mL h^{-1}$. The unabsorbed proteins were eluted with the buffer solution until the absorbance at 280 nm was lower than 0.05; CrataBL was eluted with 0.5 M NaCl. Protein concentration was determined according to Lowry et al. [26] using bovine serum albumin as standard.

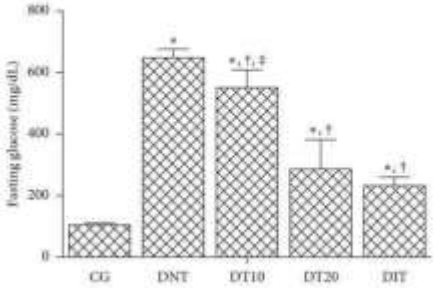


FIGURE 1: Fasting serum glucose levels in diabetic mice after treatment with CrataBL. CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT; $^{\ddagger}P < 0.05$ versus DIT.

*2.4. Animals.* Female albino Swiss mice (*Mus musculus*), six weeks of age, weighing 30 ± 5 g, bred in the Biotherium of *Departamento de Antibióticos*, UFPE, Brazil, were housed in colony cages (six mice per cage) at room temperature of 22 ± 2°C, relative humidity 40–60%, and 12 h light and 12 h dark cycle. The mice were fed standard rodent diet (Labina, Purina Brazil Ltd., Brazil) and water *ad libitum*. The experimental protocol was approved by the Animal Care and Use Committee at the Federal University of Pernambuco, Brazil (CEEA-UFPE-Oficio n° 40/06). All experimental procedures were conducted in accordance with the ethical guidelines for Care and Use of Laboratory Animals.

*2.5. Induction of Diabetes in Mice.* Experimental diabetes was induced in overnight-fasted mice by a single intraperitoneal injection of freshly prepared alloxan monohydrated (80 mg/kg in 0.9% NaCl solution). After alloxan administration, all animals were relocated to their cages and given free access to food and water. Diabetes was confirmed by measuring the fasting blood glucose levels 72 h after alloxan injection. The mice with serum glucose of >250 mg/dL were considered diabetic and were included in the study.

*2.6. Experimental Design.* The mice were split into four groups ($n = 6$, for group) as follows:

Group (I)—normoglicemic mice receiving saline solution (0.9%), as control group;

Group (II)—diabetic control mice, named diabetic nontreated;

Group (III)—diabetic mice treated with CrataBL (10 mg/kg/day, intraperitoneally) in saline solution (0.9%) for 10 days, named diabetic treated 10;

Group (IV)—diabetic mice treated with CrataBL (20 mg/kg/day, intraperitoneally) in saline solution (0.9%) for 10 days, named diabetic treated 20;

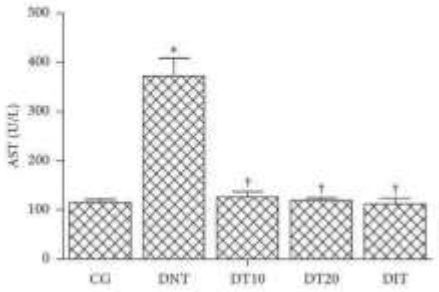


Figure 2: Serum aspartate aminotransferase levels in diabetic mice after treatment with CrataBL. CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT.

Group (V)—diabetic mice treated with insulin (10 mg/kg/day, intraperitoneally) for 10 days, named diabetic insulin treated.

Before and at the end of the experimental period, overnight fasting mice were anaesthetized with 2% xylazine hydrochloride (10 mg/kg) and 10% ketamine hydrochloride (115 mg/kg); blood samples were withdrawn with a capillary from mice-cavernous sinus for biochemical parameters determination [27]. The mice were sacrificed by cervical dislocation. Thereafter, pancreas, liver, and kidneys were excised and immediately fixed in 10% neutral buffered formalin for histological analysis.

*2.7. Effect of CrataBL on Biochemical Data.* Mice blood samples were centrifuged at 2,500 g for 15 min at 4°C (Sorvall RC6, NC, US). Sera were obtained and the levels of the glucose, urea, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by enzymatic colorimetric methods (Labtest Diagnostica, Brazil/SA) in a chemistry autoanalyzer (COBAS 6000, Roche Diagnostics, England).

*2.8. Histological Analysis of Pancreas, Kidneys, and Liver.* Pancreas, kidney, and liver from all groups were subjected to standard routine tissue processing technique: dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections of 5 $\mu$m thickness were cut from each block and stained with haematoxylin-eosin for histological examination. Prepared slides were studied by light microscopy and all sections were evaluated for the tissue injury.

*2.9. Statistical Analysis.* Values were expressed as the mean ± SD. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. For all analysis the 0.05 level of probability was used as the criterion of significance. The analyses were carried out using software PRISMA (GraphPad Software, Inc., San Diego, CA, version 5.01).

Table 1: Serum urea and creatinine levels in diabetic mice after treatment with CrataBL.

| Groups | Urea | Creatinine |
|---|---|---|
| CG | 34.3 ± 6.8 | 0.30 ± 0.01 |
| DNT | 58.9 ± 5.8* | 0.39 ± 0.04* |
| DT10 | 46.7 ± 6.6*,† | 0.33 ± 0.05† |
| DT20 | 44.0 ± 2.9*,† | 0.32 ± 0.04† |
| DIT | 43.1 ± 2.6† | 0.32 ± 0.02† |

CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT.

## 3. Results and Discussion

*3.1. Effect of CrataBL on Fasting Glucose.* Diabetes is a complex metabolic disorder with a characteristic modulation of glucose metabolism. Chronic hyperglycemia promotes tissue damage which can be found in many organs and systems, with consequent often serious disease [28]. Alloxan, a prominent diabetogenic chemical with ability to generate reactive oxygen species formation that induce death of $\beta$ cell of the pancreas by necrosis [29], is considered a good model for reproducible induction of the metabolic state of this disease in experimental animals [30–33]. Thus, in this study the mice subjected to alloxan injection showed symptoms of severe diabetes such as hyperglycemia. Insulin treatment, as a positive control, validates our model by showing the improvement in diabetes.

In a previous study, the acute toxicity of CrataBL was determined in mice; at the doses from 300 mg/kg to 2,000 mg/kg, mice did not present weight loss or death, and $LD_{50}$ of CrataBL was 2,500 mg/kg [4]. Therefore, CrataBL concentrations used in the present study are considered safe, without problem of toxicity, and indicate that the lectin is a potential pharmaceutical substance.

As demonstrated in Figure 1, CrataBL proved to be an effective hypoglycemic agent after 10 days of treatment and showed significant antihyperglycemic activity in a dose-dependent manner, in alloxan-induced diabetic mice, and at the dose of 20 mg/kg/day it exhibited better glucose reduction (56%) than 10 mg/kg/day (15%), and it was similar to that found by the treatment with the standard drug, insulin (64%), without no significant difference ($P > 0.05$). Studies with soya bean lectin reported a decrease of 17.3% in blood glucose, and it was suggested that this effect is due to an increase in pancreatic growth stimulated by the lectin [34]. Wang et al. [35] demonstrated that *Agaricus bisporus* lectin administration could partially reverse the impaired $\beta$-cell growth potential by regulating cell cycle proteins (cyclin D1, cyclin D2, and Cdk4). So, induction of pancreatic $\beta$-cell proliferation by lectins suggests the therapeutic potential in decreasing blood glucose and treating experimental diabetes mellitus [34, 35].

Medicinal plants are gaining wide acceptably worldwide because they are the potential sources of bioactive agents in use as pharmaceutics. In a fast changing world, a number of procedures to evaluate hypoglycemia as well as the kidney

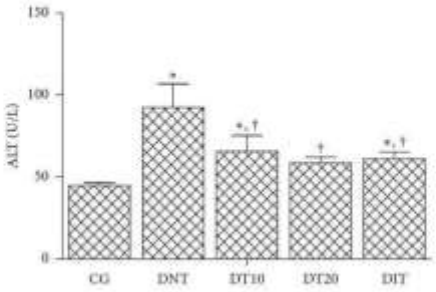


FIGURE 3: Serum alanine aminotransferase levels in diabetic mice after treatment with CrataBL. CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT.

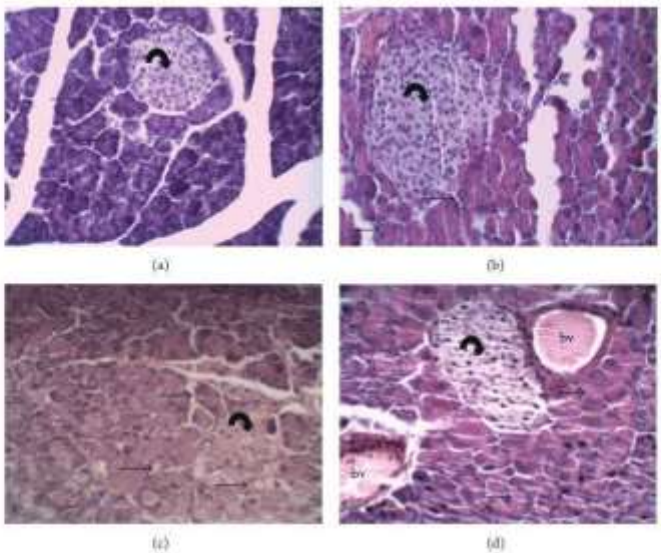


FIGURE 4: Histopathological changes in pancreatic tissue. (a) Control group—preserved pancreatic islet of Langerhans (curved arrow) and preserved pancreatic acinar cells (straight arrows); (b) diabetic nontreated—atrophic pancreatic islet of Langerhans with few cells (curved arrow) and the presence of some vacuoles in the pancreatic acinar cells (straight arrows); (c) diabetic treated with CrataBL (10 mg/kg)—pancreatic islet of Langerhans (curved arrow) and preserved pancreatic acinar cells (straight arrows); (d) diabetic treated with CrataBL (20 mg/kg)—preserved pancreatic islet of Langerhans (curved arrow) and preserved pancreatic acinar cells (straight arrows) and blood vessel (bd). Haematoxylin-eosin: 400x.

and liver damage have been used to investigate the effectivity of new natural agents which are explored by experts and clinicians [36–39].

*3.2. Effects of CrataBL on Markers of Kidney Damage.* As shown in Table 1, levels of urea and creatinine known as kidney function markers were significantly increased in sera of alloxan-induced diabetic mice, in comparison with normal mice. After 10 days of treatment with CrataBL, the levels of urea and creatinine significantly decreased. The diabetic mice treated with CrataBL at doses of 10 and 20 mg/kg reduced serum levels of urea by 20.7% and 25.3%, respectively, and the same doses decreased creatinine concentration by 15.4% and 17.9%, respectively. Insulin, the positive control for treatment,

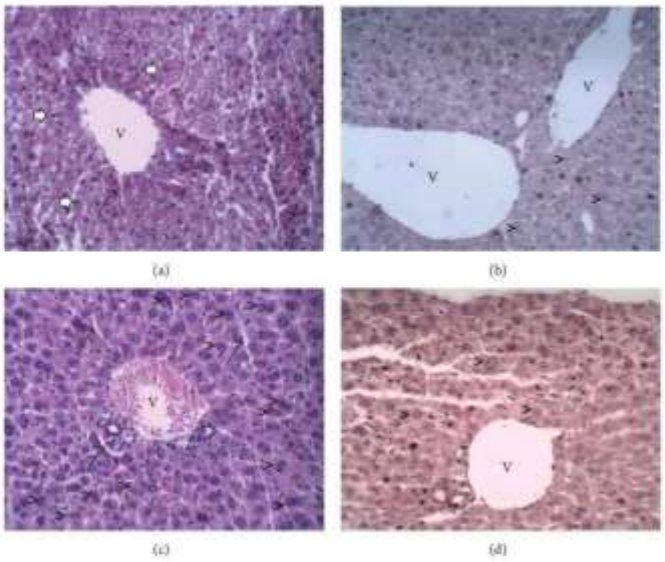


FIGURE 5: Histopathological changes in hepatic tissue. (a) Control group—centrilobular vein (V), preserved hepatocytes (white arrows), and sinusoidal capillaries (thin arrows); (b) diabetic nontreated—centrilobular vein with many red blood cells (V), intense mitotic activity in hepatocytes (arrowheads), and the presence of sinusoidal capillaries (thin arrows); (c) diabetic treated with CrataBL (10 mg/kg)—centrilobular vein (V) and considerable mitotic activity in hepatocytes (arrowheads); (d) diabetic treated with CrataBL (20 mg/kg)—centrilobular vein (V) and preserved hepatocytes (arrowheads). Haematoxylin-eosin: 400x.

decreased these markers of renal damage by 26.8% and 17.9%. Our results are in agreement with recent reports by Kumar et al. [39], Omara et al. [40], and Yankuzo et al. [41] who demonstrated that renal damage can be ameliorated when the levels of serum urea and creatinine are decreased by treatment with extracts of medicinal plants.

Kidney damage is usually associated with diabetes. In the initial course of disease the presence of hypertrophy of the glomeruli and tubular cells, matrix expansion, and enhanced renal blood flow is common, and these alterations have been postulated to cause loss of renal function [40, 41]. High levels of urea and creatinine are usually reported as one of the most sensitive markers of kidney damage, and it is reported that renal hypertrophy in diabetic animals is caused by an increased formation of advanced glycation end products and accumulation of glycogen granules in distal tubules [42, 43].

Thus, our results clearly indicate that CrataBL possesses an effective potential to improve kidney damage induced by alloxan-diabetes.

*3.3. Effects of CrataBL on Markers of Liver Damage.* As compared to the control groups, the activities of the markers of liver damage serum AST and ALT were significantly ($P < 0.05$) reduced in alloxan-induced diabetic mice after treatment with 10 or 20 mg/kg of CrataBL; the activity of AST was reduced by 66.2% and 67.9%, respectively (Figure 2) and ALT activity was decreased by 28.9% and 36.6%, respectively (Figure 3). These percentages of reduction were similar to those observed with insulin treatment. Therefore, administration of CrataBL for 10 days reversed the elevated levels of liver marker enzymes, which reflects the capability to conserve the membrane integrity of cellular and mitochondrial membranes of hepatocyte in alloxan-diabetic mice treated with this lectin.

Our results are in agreement with those of Mansour et al. [25] who reported that hepatic damage can be improved by decreasing the levels of serum AST and ALT in alloxan-induced diabetic rats subjected to treatment with herbal bioactive agents. It is well known that liver is the focal organ of oxidative and detoxifying processes [22]. Liver diseases are a high problem of health worldwide and the release of intracellular localized marker enzymes such as AST and ALT into the blood when cell and mitochondria are subjected to injury indicates hepatocytes damage [44, 45]. Furthermore, the elevated serum levels of AST and ALT in nontreated diabetic mice (Figures 2 and 3) indicate that alloxan caused liver damage and loss of the functional integrity of the hepatocyte membranes, as also evidenced in a study reported by Rajesh and Latha [45] about hepatotoxicity of polyherbal formulation.

As indicated by serum levels of AST and ALT CrataBL is able to improve liver damage.

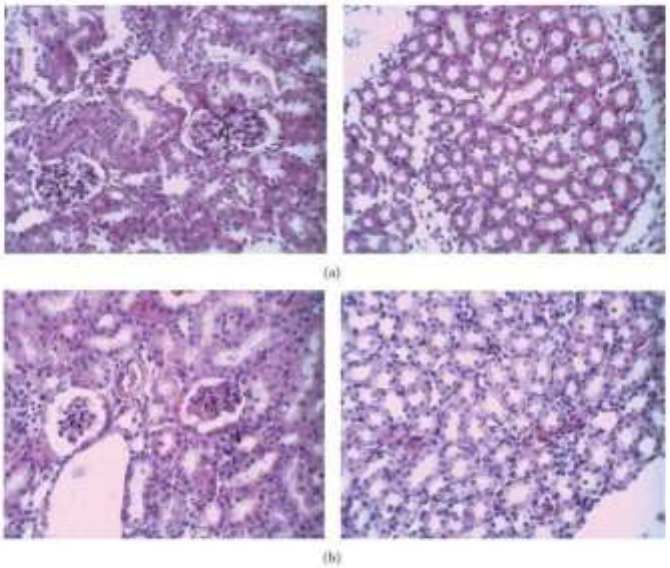

FIGURE 6: Histopathological changes in renal tissue of the normal and diabetic mice. (a) Control group—renal glomeruli (G) with preserved subcapsular spaces (left) and collecting tubules (stars) without changes in the medullary region (right); (b) diabetic nontreated—retracted glomerular tufts (G) with increased subcapsular space and evident thickening of parietal layer of Bowman's capsule due to have been entirely replaced by the cuboidal cells (left) and preserved collecting tubules (stars) (right). Haematoxylin-eosin: 400x.

*3.4. Effects of CrataBL on the Histopathological Changes of the Pancreas, Liver, and Kidneys.* The structure of the pancreas of the control and diabetic mice are shown in Figure 4. Pancreas of control group showed normal pancreatic islet of Langerhans and acinar cells (Figure 4(a)). By contrast, in alloxan-induced diabetic mice the acinar cells were altered with presence of vacuoles; furthermore deterioration of pancreatic islets was also observed (Figure 4(b)). CrataBL (10 mg/kg) treatment increased the number of pancreatic islets as compared to that of diabetic nontreated animals (Figure 4(c)). Interestingly, pancreatic section of diabetic mice treated with CrataBL (20 mg/kg) showed pancreatic islet similar to that of the control group (Figure 4(d)).

The histopathological analysis of pancreas isolated from mice administrated with alloxan alone revealed tissue damage with deterioration of pancreatic islets. In this connection, it may be observed that several authors reported such changes in pancreas tissues of mice exposed to prominent diabetogenic alloxan for its ability to induce reactive oxygen species (ROS) formation, resulting in the selective necrosis of beta cells in pancreatic islets [29, 39, 46, 47]. However, the diabetic animals treated with lectin from *C. tapia* bark showed normal architecture of pancreatic tissue, suggesting the regeneration of pancreatic islet by CrataBL administrations. The ability of lectins to stimulate pancreatic growth has been reported [48]. The regenerative action of CrataBL corroborates with *Agaricus bisporus* lectins (ABL). The ABL administration could partially reverse the impaired $\beta$-cell growth potential by induction of pancreatic islet proliferation [35]. Thus, the antidiabetic effect observed by CrataBL administration suggests the therapeutic potential in preventing and/or treating diabetes.

Figure 5 shows the photomicrographs of hepatic tissues of control group and diabetic experimental groups. The section of liver tissue of control mice demonstrates preserved hepatocytes, centrilobular vein, and sinusoidal capillaries (Figure 5(a)). In the alloxan-induced diabetic mice the histopathological analysis of hepatic tissue shows intense mitotic activity in hepatocytes (Figure 5(b)). CrataBL (10 mg/kg) treatment exhibited considerable mitotic activity in hepatocytes (Figure 5(c)). Similar to the control group, diabetic mice treated with CrataBL (20 mg/kg) also revealed an equivalent architecture of hepatic tissue (Figure 5(d)).

The photomicrographs of renal tissues are represented in Figures 6 and 7. Figures 6(a) and 6(b) represent the renal tissues of control group and diabetic nontreated group, respectively. Kidneys of control group show normal architecture of tissue with preserved subcapsular spaces in glomeruli and collecting tubules without change in the medullary region. Differently, the renal tissue of alloxan-induced diabetic mice shows retracted glomerular tufts with increased subcapsular space and evident thickening of Bowman's membrane due

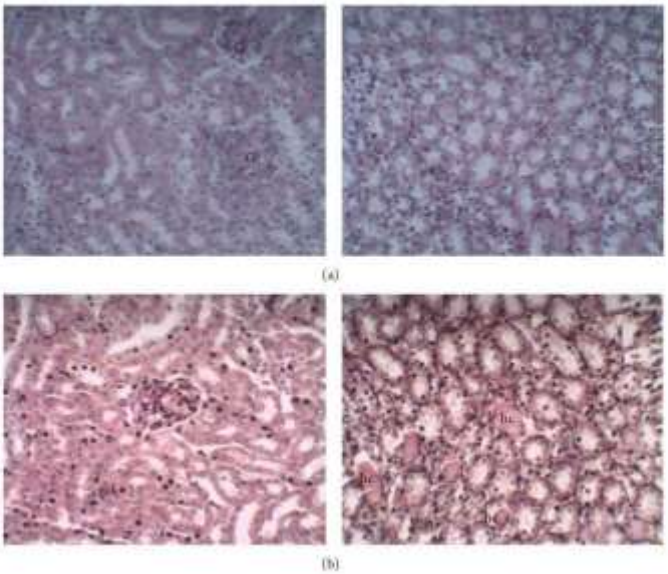

Figure 7: Histopathological changes in renal tissue of the diabetic mice treated with CrataBL. (a) Diabetic treated with CrataBL (10 mg/kg)—renal glomeruli (G) with irregular subcapsular spaces and some distinctly collapsed (left) and collecting tubules with slight swelling of the tubular epithelium (stars) (right); (b) diabetic treated with CrataBL (20 mg/kg)—preserved renal glomeruli (G) (left) and presence in the medullary region of collecting tubules (stars) with evident swelling of the tubular epithelium and hyaline casts (hc) (right). Haematoxylin-eosin: 400x.

to the cuboid appearance of epithelial cells. In kidneys of alloxan-induced mice treated with CrataBL (10 mg/kg) renal glomeruli were evident with irregular subcapsular spaces and some distinctly collapsed (Figure 7(a)). However, renal sections of diabetic mice treated with CrataBL (20 mg/kg) show preserved renal glomeruli and presence in the medullary region of collecting tubules with evident swelling of the tubular epithelium and hyaline casts presence (Figure 7(b)).

The elevated levels of glucose contribute to the generation of ROS in the diabetes, which promotes to the increase of oxidative stress in various organs and tissues [49, 50]. In addition, the hyperglycemia provokes hepatic and renal damage and consequently has been associated with histological and functional alterations and liver and kidneys [51, 52]. In fact, these organs are the focal of important organic functions and damage promoted by diabetes can result in severe complications with nephropathy and nonalcoholic steatosis [23, 24]. The current study demonstrated that CrataBL treatment improves the hepatic and renal histologic damage induced by diabetes. These findings correlated with improved biochemical markers of liver and renal functions by CrataBL. Taken together, these results may contribute to a better understanding of the regenerative effect of CrataBL in pancreas and protective in liver and kidneys, emphasizing the utilization of this lectin in the treatment of complications associated with diabetes mellitus.

## 4. Conclusion

Our results indicate that CrataBL is a good agent in controlling diabetes induced by alloxan and improves the damage on kidneys and liver tissues. The findings of this study also indicate that the active principle present in *C. tapia* is CrataBL, which is a lectin responsible for the antihyperglycemic activity found in this study and that could explain the basis for its use in the folk medicine as an alternative treatment for diabetes. Therefore, we conclude that CrataBL serves as an excellent candidate for an alternative therapy in the treatment of diabetes mellitus since it revealed an antidiabetic activity and other beneficial effects that ameliorate diabetes and associated complications.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Acknowledgments

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RESEARCH ARTICLE

# Splenectomy Improves Hemostatic and Liver Functions in Hepatosplenic Schistosomiasis Mansoni

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## Abstract

### Background

Schistosomiasis mansoni is a chronic liver disease, in which some patients (5–10%) progress to the most severe form, hepatosplenic schistosomiasis. This form is associated with portal hypertension and splenomegaly, and often episodes of gastrointestinal bleeding, even with liver function preserved. Splenectomy is a validated procedure to reduce portal hypertension following digestive bleeding. Here, we evaluate beneficial effects of splenectomy on blood coagulation factors and liver function tests in hepatosplenic schistosomiasis mansoni compared to non-operated patients.

### Methodology/Principal Findings

Forty-five patients who had undergone splenectomy surgery were assessed by laboratory analyses and ultrasound examination and compared to a non-operated group (n = 55). Blood samples were obtained for liver function tests, platelet count and prothrombin time. Coagulation factors (II, VII, VIII, IX and X), protein C and antithrombin IIa, plasminogen activator inhibitor-1 were measured by routine photometric, chromogenic or enzyme-linked immunosorbent assays, while hyperfibrinolysis was defined by plasminogen activator inhibitor-1 levels. Both groups had similar age, gender and pattern of periportal fibrosis. Splenectomized patients showed significant reductions in portal vein diameter, alkaline phosphatase and bilirubin levels compared to non-operated patients, while for coagulation factors there were significant improvement in prothrombin, partial thromboplastin times and higher levels of factor VII, VIII, IX, X, protein C and plasminogen activator inhibitor-1.

**Competing Interests:** The authors have declared that no competing interests exist.

## Conclusion/Significance

This study shows that the decrease of flow pressure in portal circulation after splenectomy restores the capacity of hepatocyte synthesis, especially on the factor VII and protein C levels, and these findings suggest that portal hypertension in patients with hepatosplenic schistosomiasis influences liver functioning and the blood coagulation status.

## Introduction

Schistosomiasis causes one of the most prevalent liver diseases, affecting more than 200 million people in over 74 different countries and is a major public health problem in the Northeast region of Brazil [1–2]. Nearly 10% of patients infected by *Schistosoma mansoni* progress to the most severe form, hepatosplenic (HS) schistosomiasis, which is characterized by periportal fibrosis (PPF), obstruction by eggs of intrahepatic veins, presinusoidal portal hypertension, splenomegaly, hemodynamic and lipid abnormalities, frequently resulting in upper digestive bleeding [3–5].

Upon blocking the terminal branches of the portal vein, the deposition of numerous *S. mansoni* eggs provokes granulomatous reactions with subsequent fibrosis, intrahepatic portal vein obstruction and increased resistance of blood flow to the sinusoids [6]. Splenomegaly results from the congestion caused by egg obstruction and fibrosis and also from hyperplasia of cells of the reticuloendothelial system, induced by immunological stimulation due to antigens released by the worms and eggs [7–9]. In addition, it has been reported that splenomegaly leads to thrombocytopenia, associated with hypersplenism, in more than 60% of patients with HS schistosomiasis, especially in the advanced stages of the disease; however, relatively few patients present symptoms due to hypersplenism and need surgery [10].

The increased resistance to portal inflow and the hyperflux in the spleno-portal territory due to massive splenomegaly both trigger presinusoidal portal hypertension [11–13]. In schistosomiasis mansoni, PPF forms around the portal branches, whilst maintaining the architecture of the hepatic parenchyma and a normal synthetic capacity of the hepatocytes [6,14]. Nevertheless, PPF may induce slight increases in the liver enzymes, alkaline phosphatase (ALP) and gamma glutamyl transferase (γGT) [14]. On the other hand, we previously reported that in HS schistosomiasis the common markers of liver injury, alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP, γGT and bilirubin, are all significantly higher than in a control group of uninfected individuals [15].

Patients with advanced HS schistosomiasis often have abnormalities in hemostasis and fibrinolysis. These include prolongation of prothrombin time (PT), partial thromboplastin time (PPT), thrombin time (TT) as well as thrombocytopenia, hypofibrinogenemia and decreases of vitamin-K-dependent factors, which relate to the low degree of disseminated intravascular coagulation [14–17]. Moreover, some patients with HS schistosomiasis may present with hyperfibrinolysis and a consequent tendency for bleeding; levels of D-dimer and tissue plasminogen activator are increased, while plasminogen activator inhibitor-1 (PAI-1) is reduced [18].

Splenectomy with ligation of the left gastric vein and esophagogastric disconnection has become a good therapeutic option to reduce portal hypertension after episodes of gastrointestinal bleeding [8,13]. Nevertheless, 13 to 53% of patients with HS schistosomiasis develop portal vein thrombosis following this procedure [13,19]. To better understand this complication, the present study has compared serum levels of liver enzymes and the hemostatic profiles in

patients with HS schistosomiasis, one group having undergone surgical splenectomy and the other being non-operated.

## Materials and Methods

### Ethical Statement

Each patient received an explanation of the study and signed a free and informed consent form. The study was approved by the Ethics Committee for Research on Humans at the Federal University of Pernambuco, Brazil (Number 028/11), in accordance with the Helsinki Declaration of 1975.

### Patients

One hundred patients with HS schistosomiasis, 45 that had been splenectomized and 55 non-operated, were consecutively selected during attendance at the outpatient clinic of the Gastroenterology Division, Hospital das Clínicas of the Federal University of Pernambuco, Recife, Brazil, between April 2011 and December 2012. All patients had been previously treated with praziquantel (50 mg/kg) at least 6 months before enrolment in the study.

The diagnosis of schistosomiasis was based on clinical history, earlier contact with water bodies in the endemic zone, history of positive parasitology for *S. mansoni*, specific treatment and ultrasound examination revealing PPF. Abdominal ultrasound was performed by a single researcher (ALCD) through the Acuson X 150 device, with a 3.5 mHz convex transducer (Siemens), for diagnosis, to classify the different patterns of PPF and to exclude other liver diseases such as steatosis and cirrhosis. The Niamey classification of PPF was used: pattern D (central or moderate fibrosis), pattern E (advanced fibrosis) and pattern F (very advanced fibrosis) [20–21].

Patients were not included in the study groups if they reported alcohol abuse (>60 g/day of ethanol for men and >40 g/day for women), pregnancy, diabetes mellitus, hepatitis B or C, fatty liver diseases, cirrhosis, collagenosis, chronic lymphoproliferative diseases, or any use of hepatotoxic, antiplatelet or anticoagulant drugs. A transfusion of blood within 90 days of data collection also constituted an exclusion factor. All patients were tested for markers of hepatitis B virus (HBsAg and anti-HBc), hepatitis C (anti-HCV) and HIV (anti-HIV).

### Collection and Processing of Samples

Venous blood samples were collected aseptically with minimal stasis using vacuum tubes (Vacutainer, Becton Dickinson, USA) into three tubes. The first, containing 0.106 M trisodium citrate (1:9 to blood), was for blood coagulation tests, the second without anticoagulant was for liver function tests, including AST, ALT, ALP, γGT, bilirubins and albumin, while the third tube with 0.562M ethylenediaminetetraacetic acid (EDTA-K3) was used for platelet quantification. The first two blood samples were centrifuged for 10 min at 2000 g and the plasma and serum divided into 0.5 mL aliquots and stored at -80°C until assayed.

### Biochemical and Coagulation Analysis

The serum concentration of each enzyme was divided by the upper normal value according to gender (for women and men, respectively, AST 31 and 35 U/L; ALT 31 and 41 U/L; γGT 38 and 55 U/L; and ALP 128 and 141 U/L) and expressed as the resulting ratio. Bilirubin (total, direct and indirect) and albumin were measured as μmol/L and g/L, respectively. All liver function tests were measured by automated spectrophotometry (6000 analyzer series Cobas, Roche, USA). HBsAg, anti-HBc, anti-HCV and anti-HIV markers were detected by

Chemiluminescence Microparticle Immuno Assay (CeMIA) using the ARCHITECT i2000 automatic light detector and test reagents (Abbott, North Chicago, USA) to exclude enrolment of patients with hepatitis B or C, and immunodeficiency.

The platelet counts were measured by electrical impedance (Pentra DF 120, HORIBA ABX SAS Diagnostics, Brazil). Coagulation tests were performed by the chromogenic method using a Destiny Plus automatic analyzer (Trinity Biotech, Ireland) and included the determination of PT, PTT, TT and fibrinogen; coagulation factors (II, VII, VIII, IX, X), protein C and antithrombin IIa were also measured with the Destiny Plus automatic analyzer (Trinity Biotech, Ireland), while quantification of PAI-1, a measure of fibrinolysis, was by ELISA (Asserachrom Diagnostica, Stago, France).

## Statistical Analysis

Differences between continuous variables in splenectomized and non-operated HS schistosomiasis patients were compared by unpaired Student's t test, while the Mann-Whitney test was used for comparisons of non-normally distributed variables. Continuous variables were expressed as mean ± standard error of the mean, or as median and range, while qualitative variables were expressed as absolute frequencies (percentage). The Pearson chi-square test was used to compare the different patterns of PPF. All statistical analyses were performed using StatView SAS Inc. (1998, NC, USA); $P < 0.05$ was considered statistically significant.

## Results

The two groups of patients with HS schistosomiasis did not differ in relation to age or gender, and had similar PPF patterns. However the mean portal vein diameter in the 55 non-operated patients was 30% greater ($P<0.0001$) than in the 45 patients who had undergone splenectomy (Table 1). The average time post-splenectomy was 11.5 ± 8.6 years, ranging from 2 to 33 years, with a median of 9 years.

Serum levels of AST, ALT, γGT and albumin were not significantly different between the two patient groups. However, non-operated HS patients showed significantly increased levels of ALP and of total, direct and indirect bilirubin (Table 2).

**Table 1. Demographic characteristics and ultrasound parameters of patients with hepatosplenic schistosomiasis who have undergone surgical splenectomy or are non-operated.**

| Characteristics | Hepatosplenic schistosomiasis patients | |
|---|---|---|
| | Non-operated | Splenectomized |
| Number of patients | 55 | 45 |
| Age (years) | 50.20 ± 1.86 | 50.19 ± 1.41 |
| Gender | | |
| Male | 50.9% | 37.8% |
| Female | 49.1% | 62.3% |
| Diameter portal vein (cm) | 1.28 ± 0.04 | 0.96 ± 0.26* |
| Fibrosis pattern | | |
| D | 30.9% | 15.6% |
| E | 54.6% | 73.3% |
| F | 14.5% | 11.1% |

Values are expressed as mean ± standard error and compared by unpaired Student's t-test; D, moderate; E, advanced; F, very advanced fibrosis
*P<0.0001.

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**Table 2. Liver function tests in patients with hepatosplenic schistosomiasis who have undergone surgical splenectomy or are non-operated.**

| Liver Parameters | Hepatosplenic schistosomiasis patients | |
|---|---|---|
| | Non-operated | Splenectomized |
| Number of patients | 55 | 45 |
| AST/ULN | 1.58 ± 0.14 | 1.45 ± 0.09 |
| ALT/ULN | 1.40 ± 0.16 | 1.29 ± 0.12 |
| ALP/ULN | 1.10 ± 0.08 | 0.88 ± 0.06* |
| γ-GT/ULN | 3.11 ± 0.37 | 3.35 ± 0.42 |
| Albumin (g/L) | 39.8 ± 0.79 | 40.4 ± 0.10 |
| Total bilirubin (μmol/L) | 21.03 ± 2.05 | 14.17± 0.66* |
| Direct bilirubin (μmol/L) | 8.89 ± 1.36 | 5.71 ± 0.51* |
| Indirect bilirubin (μmol/L) | 12.14 ± 1.19 | 8.48 ± 0.56* |

Values are expressed as mean ± standard error, and compared by unpaired Student's t-test; ULN, Upper Limit of Normal

*P<0.05.

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Splenectomized HS schistosomiasis patients had twice the platelet count of the non-operated patients (P<0.0001); they also exhibited less prolonged PT and PTT than those of the non-operated HS group. In addition, the levels of coagulation factors VII, VIII, IX, X, and protein C were significantly higher in the splenectomized group than in the HS schistosomiasis patient group, whereas no differences were noted for TT and the levels of factor II, antithrombin IIa and fibrinogen (Table 3).

The median plasma level of PAI-1 in splenectomized patients was 3-fold greater (221.5 vs. 65.2 ng/mL; P = 0.0003) than in the non-operated patients (Fig 1).

**Table 3. Coagulation and fibrinolytic parameters in patients with hepatosplenic schistosomiasis who have undergone surgical splenectomy or are non-operated.**

| Coagulation tests | Hepatosplenic schistosomiasis patients | |
|---|---|---|
| | Non-operated | Splenectomized |
| Number of patients | 55 | 45 |
| Platelets Count ($x10^3/mm^3$) | 128.4 ± 12.0 | 254.1 ± 10*** |
| PT (Sec) | 19.3 ± 0.63 | 13.8 ± 0.63*** |
| TT (Sec) | 13.7 ± 0.21 | 13.9 ± 0.26 |
| PTT (Sec) | 37.9 ± 1.47 | 26.9 ± 0.16*** |
| Fibrinogen (g/L) | 2.62 ± 0.10 | 2.82 ± 1.2.1 |
| Factor II (%) | 66.5 ± 2.3 | 71.6 ± 1.6 |
| Factor VII (%) | 49.9 ± 2.5 | 65.0 ± 2.4*** |
| Factor VIII (%) | 90.5 ± 4.1 | 106 ± 6.3 * |
| Factor IX (%) | 59.9± 2.4 | 82.7 ± 4.3*** |
| Factor X (%) | 63.7 ± 3.6 | 77.7 ± 3.7** |
| Protein C (%) | 65.6 ± 2.8 | 77.6 ± 4.1** |
| Antithrombin IIa (%) | 92.7 ± 3.5 | 96.2 ± 3.6 |

Values are expressed as mean ± standard error (SE) and compared by unpaired Student's t- test

*P<0.05

**P<0.01

***P<0.0001.

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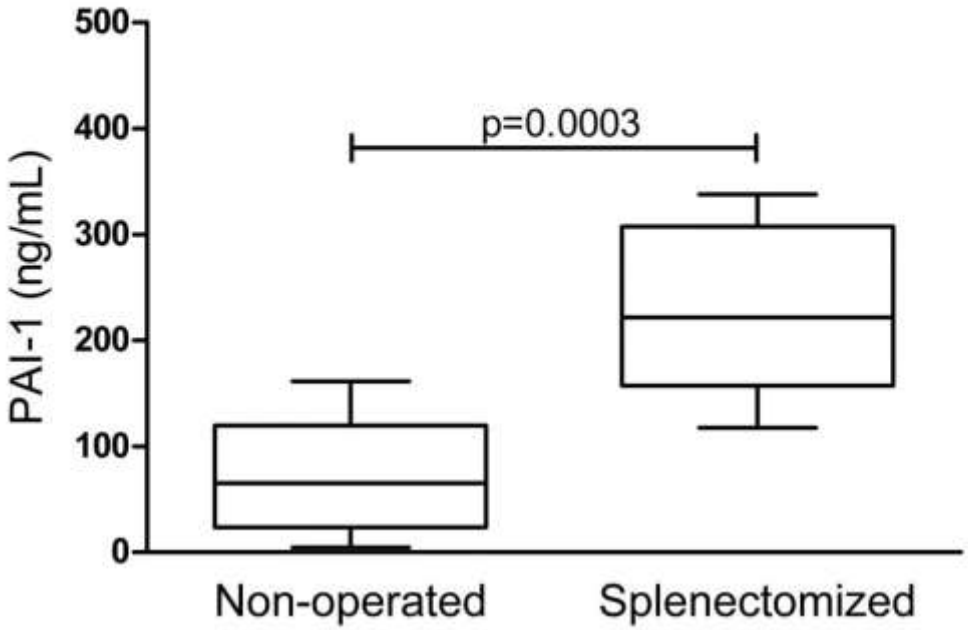


**Fig 1. Plasma levels of PAI-1 in patients with hepatosplenic schistosomiasis who have undergone surgical splenectomy, or are non-operated.** The box shows the 25th to 75th percentile of the PAI-1 distribution, while the horizontal bar inside the box shows the median values (65.2 vs 221.5 ng/mL). The upper and lower bars indicate the maximum and minimum values, respectively. Mann Whitney test was used to analyze the difference between the two groups.

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## Discussion

Portal hypertension associated with *S. mansoni* infection has a major impact on morbidity and mortality, due to the possibility of bleeding from esophageal or gastric varices [22]. Splenectomy is an established therapeutic procedure to treat and help prevent new episodes of gastrointestinal bleeding; the marked reduction of pre-sinusoidal portal hypertension improves hemodynamic abnormalities [12]. In this study we have measured markers of liver function and blood coagulation parameters in HS schistosomiasis patients, comparing those who have undergone splenectomy with a non-operated group.

Recently, we reported that in HS schistosomiasis the common indicators of liver injury ALT, AST, ALP and bilirubin are twice the levels of those in healthy individuals, while γGT is five-fold higher [15]. Mechanisms proposed to explain elevated ALP and γGT in HS patients, include the compression of small intrahepatic bile ducts by schistosomal granulomas [23], though this mechanism was not confirmed by Amaral et al. [24] who found no changes in intra or extra-hepatic biliary tracts by ultrasound examination. Our previous study also demonstrated that levels of γGT increase with progression of PPF, suggesting that this enzyme is a useful marker for stratifying the different patterns of PPF [15]. The severity of PPF reflects host immunogenic response and degree of infection, which amplify splenic volume and

consequently increase hyperflux in the spleno-portal region. These factors in combination raise portal hypertension [11–13].

In the present study, although both groups of HS schistosomiasis patients had similar age, gender and pattern of PPF, we still found a significant reduction in the portal vein diameter of the splenectomized patients, reflecting decreased portal hypertension following surgery. There were also significant differences in some liver function tests between our two patient groups; serum levels of bilirubin and ALP were decreased in splenectomized patients, whereas γGT levels were similar. Previous studies have demonstrated that the greater the portal blood flow the higher the levels of ALP and γGT, findings linked to possible anatomical changes in the biliary tree caused by fibrosis in the portal region [25]. Additionally, serum ALP is higher in schistosomiasis patients with portal hypertension than those without, although no differences are noted in γGT levels [26]. Hence, we conclude that our finding of high serum ALP is related to portal hypertension insofar as splenectomy reduces the portal flow, which in turn decreases ALP levels. In contrast, the elevation of γGT in schistosomiasis is probably associated not only with portal hypertension but also with advanced PPF.

The second indicator of improved liver function in our splenectomized patients was their lower serum levels of bilirubin (total, direct and indirect) compared to non-operated HS schistosomiasis patients. The low indirect bilirubin fraction in splenectomized patients could result from reversal of the accelerated hemolysis that occurs in the splenic parenchyma due to hypersplenism. Furthermore, the reduction in direct bilirubin could reflect a better synthetic capacity of hepatocytes following the decrease of portal pressure in our splenectomized patients. Indeed, Toledo et al. [26] also observed lower total and direct serum bilirubin in schistosomiasis patients without portal hypertension compared to those with.

Serum albumin levels in our two patient groups were within the normal range, and no increased tendency was evident in splenectomized patients. As the diseased liver in schistosomiasis largely preserves hepatocyte architecture and synthetic capacity, it is perhaps not surprising that albumin levels were unchanged. However, another liver-secreted protein, lecithin-cholesterol acyltransferase (LCAT) [27], is considered a more sensitive serum test of hepatocyte synthetic capacity [28,29] and in a previous study we showed that splenectomy significantly improved low plasma LCAT activity by around 50%, compared to non-operated HS schistosomiasis patients [30]. This finding supported an earlier proposal that LCAT assay might be a useful test in schistosomiasis mansoni for assessing disease severity [31].

Although hepatocyte synthetic capacity is largely conserved in schistosomiasis, some patients in an advanced stage of the disease have hemostatic abnormalities and altered mechanisms of fibrinolysis [14–16]. In the present study, we observed prolongation of the PT and PTT in the non-operated patients with HS schistosomiasis. However, the splenectomized group had no increases in PT and PTT, suggesting that when prolongation occurs it reflects not only hepatocyte synthetic capacity, but also the degree of portal hypertension. Our earlier study found that the changes in PT, PTT and TT in HS schistosomiasis were more pronounced with disease progression [15]. Here, we also noted slightly higher fibrinogen levels in the splenectomized patients compared to the non-operated group, although this difference was not significant. The prolongation of PT, PTT and TT as well as hypofibrinogenemia is a well-established finding in patients with schistosomiasis [16].

Reduced levels of factors VII, IX, X, and protein C have also been reported in human schistosomiasis [16]. Indeed, we found lower levels of factors VII, VIII, IX, X, protein C in the non-operated patients with HS schistosomiasis compared with the splenectomized group (Table 3). It is assumed that the reduction of vitamin K in HS schistosomiasis patients is caused by impaired hepatic synthesis or increased consumption of these coagulation factors, [15,16]. Our

present findings suggest that the lower portal pressure after splenectomy will improve hepatic synthetic capacity and reduce consumption of the factors.

An additional finding, potentially significant, from our study relates to low levels of PAI-1 in HS schistosomiasis, highlighted by El-Bassiouni et al. [18] and ourselves [15]. There is evidence that PAI-1 is synthesized by endothelial cells [32], as well as hepatocytes [33–34]. Thus, the higher level of serum PAI-1 in our splenectomized patients suggests that the reduction of portal pressure may not only improve hepatic synthetic function, but also the generation of PAI-1 by endothelial cells. Therefore, this possibility and other functional properties of endothelial cells merit further investigation.

One limitation of our study is that it was conducted at a single hospital, the Hospital das Clínicas, UFPE. This is the reference hospital for schistosomiasis in Pernambuco State and receives the most severe cases of schistosomiasis, usually patients with a history of one or more episodes of gastrointestinal bleeding and hence a high proportion with abnormal liver function tests. Furthermore, we had no information on liver function and blood coagulation parameters in HS schistosomiasis patients before they had undergone splenectomy, due to the wide time frame around 11.5 years post-splenectomy; hence, they were compared with a non-operated group of patients with HS schistosomiasis. Therefore, the findings from the present study may not extrapolate to all patients from endemic areas who present with the HS form of schistosomiasis mansoni.

In summary, we conclude that the abnormal changes observed in liver function tests and components of hemostasis in non-operated HS schistosomiasis are less severe, or not present, in splenectomized patients. This implies that portal hypertension is an important factor in the pathogenesis of the liver fibrosis and hemostatic dysfunction observed in human HS schistosomiasis mansoni. Moreover, a key point arising from this study is that splenectomy ameliorates liver function tests in patients with the most severe form of schistosomiasis; this may eventually reduce clinical symptoms and perhaps prolong life, a significant possibility as, in general, cure of HS schistosomiasis mansoni is not possible.

## Author Contributions

Conceived and designed the experiments: LACL VLML ALCD. Performed the experiments: LACL ALCD SMLM AAPF RCSF CSMF. Analyzed the data: VLML JSO ALCD EPAL. Contributed reagents/materials/analysis tools: VLML ALCD EPAL. Wrote the paper: LACL ALCD EPAL BSS VLML JSO.

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# Anexos

**Anexo A:**

Guia de Revista: PLOS ONE

**Anexo B:**

Guia de Revista: The Journal of Lipid Research

Style and Format
Manuscript Organization
**Parts of a Submission**
**Title**
Author list
Cover letter
Title page
Abstract
Introduction
Materials and Methods
Results, Discussion, Conclusions
Acknowledgments
References
Supporting Information
Figures and tables
Data reporting
Accession numbers
Striking image
Additional Information Requested at Submission
Guidelines for Specific Study Types

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| | |
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| | |
|---|---|
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|---|---|---|---|
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| | | | |
|---|---|---|---|
| **Short title** | 50 characters | State the topic of the study | Cigarette Smoke Exposure and Innate Immunity<br>SODIS and Childhood Diarrhoea |

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Acknowledgments

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| Source | Format |
|---|---|
| **Published articles** | Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1586.<br><br>Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.006<br><br>*Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.* |
| **Accepted, unpublished articles** | Same as published articles, but substitute "In press" for page numbers or DOI. |
| **Web sites or online articles** | Huynen MMTE, Martens P, Hilderink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14. |
| **Books** | Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992. |
| **Book chapters** | Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28. |
| **Deposited articles (preprints, e-prints, or arXiv)** | Kristi T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity.; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014. |
| **Published media (print or online newspapers and magazine articles)** | Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Accessed 17 March 2014. |
| **New media (blogs, web sites, or other written works)** | Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/. |
| **Masters' theses or doctoral dissertations** | Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available: http://cumincad.scix.net/cgi-bin/works/Show?2e09. |
| **Databases and repositories (Figshare, arXiv)** | Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214. |
| **Multimedia (videos, movies, or TV shows)** | Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM. |

**Supporting Information**

Authors can submit essential supporting files and multimedia files along with their manuscripts. All Supporting Information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a Supporting Information file as long as it contains an "S" and number. For example, "S1 Appendix" and "S2 Appendix," "S1 Table" and "S2 Table," and so forth.

Supporting files should be publication-ready, as they are not copyedited.

Supporting information captions

List Supporting Information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

**Example caption**

**S1 Text. Title is strongly recommended.** Legend is optional.

In-text citations

We recommend that you cite Supporting Information in the manuscript text, but this is not a requirement. If you cite Supporting Information in the text, citations do not need to be in numerical order.

Read the Supporting Information guidelines for more details about submitting Supporting Information and multimedia files.

**Figures and tables**

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order upon first appearance in the manuscript file.

Read the guidelines for figures.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

**Data reporting**

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Read our policy on data availability

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

**Accession numbers**

All appropriate datasets, images, and information should be deposited in public resources. Please provide the relevant accession numbers (and version numbers, if appropriate). Accession numbers should be provided in parentheses after the entity on first use.

Suggested databases include, but are not limited to:

- ArrayExpress
- BioModels Database
- Database of Interacting Proteins
- DNA Data Bank of Japan [DDBJ]
- DRYAD
- EMBL Nucleotide Sequence Database
- GenBank
- Gene Expression Omnibus [GEO]
- Protein Data Bank
- UniProtKB/Swiss-Prot
- ClinicalTrials.gov

In addition, as much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Providing accession numbers allows linking to and from established databases and integrates your article with a broader collection of scientific information.

**Striking Image**

You can choose to upload a "Striking Image" that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

The PLOS content license also applies to striking images. Read more about the content license.

**Additional Information Requested at Submission**

**Funding statement**

This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission.

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role.

Read our policy on disclosure of funding sources.

**Competing interests**

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

Read our policy on competing interests.

**Manuscripts disputing published work**

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite input from the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

**Related manuscripts**

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into "parts." Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

Guidelines for Specific Study Types

**Human subjects research**

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s)**. If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed.
- **Whether informed consent was written or oral**. If informed consent was oral, it must be stated in the manuscript.
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

**The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.**

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

**Clinical trials**

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

*A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.*

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

*PLOS ONE* supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as Supporting Information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as Supporting Information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's "Fig 1"

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

**Animal research**

We work in consultation with the *PLOS ONE* Animal Research Advisory Group to develop policies. Animal Research Advisory Group members may also be consulted on individual submissions.

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

If we note differences between an IACUC-approved protocol and the methods reported in a submitted manuscript, we may report these discrepancies to the relevant institution or committee.

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s). Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why.
- Relevant details for efforts taken to ameliorate animal suffering.

**Example ethics statement**

*This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.*

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

Where unregulated animals are used or ethics approval is not required, authors should make this clear in submitted articles and explain why ethical approval was not required. Relevant regulations that grant exemptions should be cited in full. It is the authors' responsibility to understand and comply with all relevant regulations.

We reserve the right to reject work that the editors believe has not been conducted to a high ethical standard, even if authors have obtained formal approval or approval is not required under local regulations.

We encourage authors to follow the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information. Please note that inclusion of a completed ARRIVE Checklist may be a formal requirement for publication at a later date.

Non-human primates

Manuscripts describing research involving non-human primates must include details of animal welfare, including information about housing, feeding, and environmental enrichment, and steps taken to minimize suffering, including use of anesthesia and method of sacrifice if appropriate, in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF).

Humane endpoints

Manuscripts describing studies that use death as an endpoint will be subject to additional ethical considerations, and may be rejected if they lack appropriate justification for the study or consideration of humane endpoints.

**Observational and field studies**

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

**Paleontology and archaeology research**

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

*All necessary permits were obtained for the described study, which complied with all relevant regulations.*

If no permits were required, please include the following statement:

*No permits were required for the described study, which complied with all relevant regulations.*

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

**Systematic reviews and meta-analyses**

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as Supporting Information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as Supporting Information

**Meta-analysis of genetic association studies**

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al*.

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

**Cell lines**

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

**For *de novo* (new) cell lines**, including those given to the researchers a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

**For established cell lines**, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

**Blots and gels**

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

**Antibodies**

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species
- The commercial supplier or source laboratory
- The catalogue or clone number and, if known, the batch number
- The antigen(s) used to raise the antibody
- For established antibodies, a stable public identifier from the Antibody Registry

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

**Methods, software, databases, and tools**

*PLOS ONE* will consider submissions that present new methods, software, or databases as the primary focus of the manuscript if they meet the following criteria:

**Utility**
The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

**Validation**
Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

**Availability**
Software should be open source, deposited in an appropriate archive, and conform to the Open Source Definition. Databases must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. Authors should provide a direct link to the deposited software or the database hosting site from within the paper.

Software submissions

Manuscripts describing software should provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

**New taxon names**

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

***Anochetus bolton*** Fisher **sp. nov.** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub:XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

**Solanum aspersum** S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11401 (holotype, COL; isotypes, F [F-1336119], US [US-1799731])

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase.

In the **Methods** section, include a sub-section called "Nomenclature" using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

***Hymenogaster huthii***, Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called "Nomenclature" using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

> The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
>
> In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

**Qualitative research**

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

# Instructions for authors

Click on headings in the Table of Contents to skip directly to that section:


Overview of submission and review process

Before preparing the manuscript
- Scope
- Prior publication
- Charges to authors
- Human subjects and patient-oriented research
- Ethical use of animals in research
- Article types

Preparing text and tables
- Text and table formatting requirements
- Order of sections
- Title page
- Abstract
- Supplementary keywords
- Tables
- Abbreviations and text conventions
- References
- Editing and proofreading services
- Monoclonal antibodies
- Lipid classification, nomenclature and structural representation
- Depositing novel lipid structures in the LIPID MAPS database
- Genomic and proteomic studies

Preparing figures
- Color figures
- Figure format and resolution
- Figure dimensions
- Text and lines in figures

Preparing PDF for submission

Preparing supplemental data

Cover letter

Resubmissions

Contact us



**More Author Tools**

- SUBMIT MANUSCRIPT
- Online submission help
- Editorial policy
- Editors and Editorial Board
- Copyright permissions

General Help

Feedback


## Overview of submission and review process

Follow all instructions on his site exactly to avoid delay of the manuscript review process. The following is an overview:

1. Prepare manuscript text and tables in Microsoft Word. See Preparing text and tables.
2. Prepare publication-quality figures. See Preparing figures.
3. Combine text, tables and figures into a single PDF file (maximum size 5 MB). See Preparing PDF for submission.
4. Prepare any allowable supplemental data files. See Preparing supplemental data.
5. Prepare a cover letter. See Cover letter.
6. Submit the manuscript PDF, supplemental data files and cover letter at the submission site. See Submission help.
7. Reviewers will recommend whether the manuscript should be accepted, revised or declined. See JLR's editorial policy.
8. If you are asked to submit a revised manuscript for review, prepare and submit a revised manuscript. See Resubmissions.
9. If your manuscript is accepted for publication, it will be published as a Paper in Press within 24 hours of acceptance. You should upload the manuscript source files (but not the supplemental data) to the redactory office according to the instructions that will be given to you following acceptance.

Click here to return to the top of the page.

## Before preparing the manuscript

### Scope

The Journal of Lipid Research (JLR) publishes original articles and reviews in the broadly defined area of biological lipids. We encourage the submission of manuscripts relating to lipids, including those addressing problems in biochemistry, molecular biology, structural biology, cell biology, genetics, molecular medicine, clinical medicine and metabolism. Major criteria for acceptance of articles are new insights into mechanisms of lipid function and metabolism and/or genes regulating lipid metabolism along with sound primary experimental data. Interpretation of the data is the authors' responsibility, and speculation should be labeled as such. Manuscripts that provide new ways of purifying, identifying and quantifying lipids are invited for the Methods section of the Journal. JLR encourages contributions from investigators in all countries, but articles must be submitted in clear and concise English.

### Prior publication

Except for review articles, the JLR will not publish articles in which a significant portion of the data, in the form of text, figures and tables, has been published or submitted for publication elsewhere. In accordance with this policy, the JLR requires that the authors state in their cover letter that no significant amount of data reported in their manuscript has been published elsewhere or is under consideration for publication elsewhere. If some of the data presented in the manuscript have previously been published, or such is contemplated, a reprint or a copy of the manuscript must accompany the article. If, in the Editor's judgment, the prior publication renders the submitted article non-original, the manuscript will be returned to the authors immediately, without review.

### Charges to authors

Authors are charged for all published manuscripts:

- $85 per page for pages 1-10
- $170 per page for page 11 and each page thereafter
- $50 per color figure

Corresponding authors who are ASBMB Regular Members are eligible for a $10 per page discount and free color figures. Join ASBMB NOW!

Please note: Any figure containing ANY color will be reviewed and processed with the understanding that the figure will be published in color.
All page and color fees must be paid by the authors. ASBMB will consider requests for waiver of publication page charges if research funds are not available that can cover publication costs. Waiver requests must be made at the same time that you submit the paper for review. Requests must be emailed to the editorial office and uploaded as a supplemental file under the category: "Other (will not appear online)". The request should also be mentioned in the cover letter. The request must be co-signed by an institutional official certifying that the authors do not have research funds that can be used for publication costs. Waiver requests cannot be made after the paper has been accepted for publication.

All articles published in JLR are automatically deposited in PubMed Central and regular scientific content will be made available to non-subscribers 12 months after publication.

Author Choice option: In addition to the standard publication charges, for an additional publication fee — $1,500 for ASBMB members and $2,000 for nonmembers — the final version of the manuscript will be released immediately on both the JLR web site and PubMed Central and will be covered under the Creative Commons Attribution license (CC BY). Join ASBMB NOW!

### Human subjects and patient-oriented research

The research described in papers submitted to JLR that involve the use of human beings, including patient-oriented research articles, must adhere to the principles of the Declaration of Helsinki, as well as to Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, revised Jan. 15, 2009, effective July 14, 2009. These studies must be approved by an appropriate Institutional Review Board or Committee, and informed consent must be obtained from the human subjects or their representatives. If registration of clinical studies is required in accord with the International Committee of Medical Journal Editors (ICMJE) (see http://clinicaltrials.gov/ct2/manage-recs/resources#InternationalCommittee and http://www.icmje.org), then such registration must be indicated in a footnote in the manuscript.

The Methods section of the paper must include:

1. A statement of protocol approval from an Institutional Review Board or Committee;
2. A statement that informed consent was obtained from the human subjects or their representatives.

### Ethical use of animals in research

All investigations involving animals that are reported in manuscripts submitted to JLR must be conducted in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research (ILAR) Guide for Care

and Use of Laboratory Animals. All such investigations must be approved by an appropriate Institutional Review Board or Committee.

All papers submitted to JLR that include investigations involving animals must include in the Methods section:

1. A statement indicating that the research was conducted in conformity with this PHS policy;
2. A statement that the studies were approved by an appropriate Institutional Review Board or Committee.

### Article Types

1. Regular research articles
   These present original research and address a clearly stated specific hypothesis or question. Papers should provide novel approaches and new insights into the problem addressed. Manuscripts dealing with mechanisms are especially encouraged.

   Review process. Manuscripts are assigned to Associate Editors who with the aid of Editorial Board Members determine whether the methods are adequate and if data support the conclusions drawn and provide new and exciting information that is of the high quality expected by JLR. Papers will generally be assigned to at least two editorial board members and/or reviewers in addition to the Associate Editor, and the final determination of publication will be made by the Associate Editor in consultation with the Editorial Board.

2. Methods papers
   The editors will consider significant new/novel contributions in the field of lipid methodology; the paper must provide sufficient details so that the method can be readily reproduced. These papers should be short and concise. At the time of submission the authors should indicate that the manuscript is a Methods manuscript.

   Review process. Similar to that of a regular research article.

3. Review articles
   The Journal welcomes critical reviews on current, timely topics. Reviews should be concise and should provide a balanced analysis and summary of the topic. The information should be understandable to scientists in other related fields. A good and critical review will develop new insights into the field and propose potential new research opportunities.

   Review process. Reviews need to stand up to the scrutiny of experts in the field; the manuscripts will be reviewed by an Associate Editor and at least one member of the Editorial Board.

4. Patient-oriented and epidemiological research articles
   These include studies in which human subjects play an important role and where at least one of the authors has had direct contact with the subjects, as opposed to using samples from a blood or a tissue bank. To be considered Patient-Oriented Research, the study must make connections between the results and the human sources of the samples (individuals or groups), but it does not matter whether the samples are subsequently used in an in vivo, ex vivo or in vitro analysis (for example, the incubation of lipoproteins with cells). Patient-Oriented Research could, for example, include genetic studies where participants were seen by one of the authors and where the study obtained either baseline or pre- and post-perturbation biochemical, biophysical and/or demographic data. See published JLR patient-oriented articles for more examples.

   These manuscripts must include a statement of institutional approval of the study and informed consent of the participants.

   Review process. Similar to that of a regular research article.

Click here to return to the top of the page.

## Preparing the text and tables

Please note that, if accepted, JLR will automatically publish the accepted version of the manuscript online as a JLR Paper in Press (in PDF format), without copyediting or typesetting. Therefore, it is critical that the manuscript should be prepared with great care before submitting online. It is particularly important that the title and authors be correct, since this information will become part of the paper's permanent record in PubMed.

JLR does not set a specific word limit for submitted manuscripts but encourage authors to be concise. Authors pay publication charges on a per-page basis.

### Text and table formatting requirements

The text and tables must be:

- Prepared using Microsoft Word version 6.0 (Microsoft 2001 for Mac) or a later version;
- double-spaced;
- 11-point Times New Roman font;
- 8.5-by-11-inch paper size (U.S. letter);
- have all pages numbered, including those with figures. Manuscripts without page numbers will be returned to authors for correction.

**Order of sections**

Each section must begin on a new page and all pages must be numbered.

1. Title page
2. Abstract and keywords
3. Introduction
4. Materials and Methods
5. Results
6. Discussion
7. Acknowledgments/grant support
8. References
9. Footnotes to text (if any)
10. Figure legends
11. Tables
12. Figures

**Title page**

The title page should include the following information. Footnotes should be kept to a minimum, numbered consecutively and printed on a separate page.

Title: Limited to two printed lines, about 110 characters including spaces
Author(s): Full name, mailing addresses, and institutions — indicate which author will accept correspondence and proofs
Contact information for corresponding author: Mailing address, telephone and fax numbers and E-mail address.
Running title: Abbreviated title of maximum 60 characters including spaces.
Abbreviations: Any abbreviations not found on the JLR abbreviation list.
Authors whose names are normally represented by non-Latin characters can provide these characters in parenthesis after the transliterated version (see example below).
Zi-Zhen Wu (吳子祯)[1], De-Pei Li (李德培)[2], Shao-Rui Chen (陈少瑞)[3], and Hui-Lin Pan (潘惠麟)[3,4]

Only characters that can be encoded in Unicode such as Chinese, Japanese, Korean, Cyrillic and Arabic are acceptable. Non-Latin characters can only be used for author names, not author affiliations or titles.

**Abstract**

- No longer than 200 words
- States objectives and new findings
- Ends with a short conclusion of two or three sentences
- Contains no reference numbers
- Must be included in manuscript PDF and also copy and pasted into submission web site in unformatted text

**Supplementary keywords**

- At least five keywords from this list
- Up to five free-form keywords
- Maximum of ten keywords in total
- Should not appear in title or running title
- Listed on the abstract page

**Tables**

- Prepared using the Table Feature of Microsoft Word
- One table per page
- Titles should be descriptive
- Double-spaced and numbered with Arabic figures
- Intelligible without reference to the text
- Footnotes indicated by superscripted, lowercase italic letters

**Abbreviations and text conventions**

- Abbreviations on the JLR abbreviation list may be used without definition in JLR.
- All other abbreviations should be explained in an unnumbered footnote on the title page.
- Abbreviated terms should also be defined on first occurrence in the abstract and text.
- Single letter abbreviations are discouraged.

For common abbreviations and other points of style, JLR follows the conventions of: Scientific Style and Format, The CBE Manual for Authors, Editors, and Publishers, 1994, 6th edition, prepared by the Council of Biology Editors, Inc., and published for the Council of Biology Editors by the Press Syndicate of the University of Cambridge, New York; Nomenclature of Lipids (Recommendations 1976), IUPAC-IUB Commission of Biochemical Nomenclature, J. Lipid Res. 1978. 19: 114-128; for chemical nomenclature, Chemical Abstracts; and for enzyme terminology, Enzyme Nomenclature, Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press, Inc., New York.

**References**

- Responsibility for the accuracy of the references lies solely with the author(s).
- References should be cited in text using only numbers in parentheses (Vancouver citation style).
- Citations should be numbered consecutively in order of appearance in the text.
- Reference list should be double-spaced.

- All authors, titles and inclusive pages are to be listed ("et al." is not acceptable and will result in a delay in copyediting).
- Abbreviations of journals should conform to those used in Index Medicus, National Library of Medicine.

In-text citation example:
"Lipoproteins were isolated from serum by precipitation (1). A Student's t-test was performed (2)."

Reference list formatting examples:

1. Burstein, M., H. R. Scholnick, and R. Morfin. 1970. Rapid method for isolation of lipoproteins from human serum by precipitation with polyanions. J. Lipid Res.11: 583–595.
2. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. fifth ed. Iowa State University Press, Ames, IA. 342–343.
3. Sweeley, C. C., and G. Dawson. 1969. Lipids of the erythrocyte. In Red Cell Membrane. G. A. Jamieson and T. W. Greenwalt, editors. J.B. Lippincott, Philadelphia, PA. 172–228.

Unpublished observations, personal communications, submitted papers:

- Should not appear in reference list
- Cited in parentheses in text, including names and initials of all authors.
- Written approval by the person(s) cited in personal communications must accompany the manuscript.
- Other papers accepted for publication but that have not been published yet may appear in the reference list with the words "In press" in place of the volume number and page range and also include the DOI number. Submit a PDF file of "In press" articles with the manuscript.
- References to abstracts are permitted only when the abstract is the sole source of the information.

### Editing and proofreading services

Authors who are not native English speakers may appreciate assistance with grammar, vocabulary and style when submitting papers to the JLR. This can help maximize the accuracy and impact of the journal submission as well as aid in communicating ideas to fellow scientists and the JLR editors and reviewers. Several companies provide revising, editing and proofreading services for scientific and medical research documents. These include:

http://www.bioedit.co.uk
http://www.bioscienceeditingsolutions.com
http://www.biosciencewriters.com
http://www.bostonbioedit.com
http://www.sciencedocs.com
http://www.charlesworthauthorservices.com
http://www.editage.com/?ref=_referral
https://ebmenglishservices.squarespace.com
http://www.biologicalediting.com
http://www.peakmedicalediting.com

Please note that JLR has not used these services and thus cannot attest to the quality of their work.

### Monoclonal antibodies

Papers in which monoclonal antibodies constitute an essential reagent must contain a footnote indicating how a qualified investigator can obtain the antibody or a sample of the parent hybridoma cells.

### Lipid classification, nomenclature and structural representation

JLR recommends the use of the classification, nomenclature and structural representation of lipids used by the LIPID MAPS initiative (see Fahy et al. J. Lipid Res. 2005 46: 839–862 and Fahy et al. J. Lipid Res. 2009 50: S9–S14).

You can download lipid structures directly from the "Lipid Classification" section of the LIPID MAPS Web site (http://www.lipidmaps.org) or draw structures de novo from the "Tools" section of the website, and then insert them into your documents.

### Depositing novel lipid structures in LIPID MAPS database

JLR recommends that authors deposit all novel lipid molecules for registration in the LIPID MAPS structure database prior to publication.

Structures can be registered at http://www.lipidmaps.org/new/reg/index.html. Structures will be validated for uniqueness and checked for correct nomenclature before being placed in the public database. Questions regarding the submission of structures should be directed to webmaster@lipidmaps.org.

Benefits of using LIPID MAPS:

- Maintaining and expanding a comprehensive lipid database covering a wide variety of organisms;
- accurate classification of new lipid structures;
- application of consistent nomenclature standards with regard to systematic names and

abbreviations.
- consistent and unambiguous structural representation.

### Genomic and proteomic studies

Genomic, proteomic or other high-throughput data must be submitted to NCBI gene expression and hybridization array data depository (GEO, http://www.ncbi.nlm.nih.gov/geo/). GEO has a Web-based submission route, suitable for a small number of samples, or a batch submission tool (called SOFT).

The GEO accession number must be provided before the paper can be accepted. Information in the database must be available at the time of acceptance, because the paper will be published immediately as a Paper in Press.

Authors are encouraged to follow the MIAME checklist for submitting data. Proteomics papers must comply with the guidelines used by Molecular & Cellular Proteomics.

Click here to return to the top of the page.

## Preparing figures

Authors should keep the number of illustrations to a minimum. Figures should be intelligible without reference to the text.

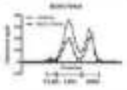
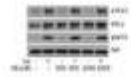
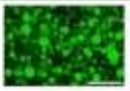
When you submit your manuscript for review, you should combine the figure files with the manuscript text in a single PDF file, with the figures at the end. The figure number must be clearly displayed on each figure in the PDF. See this example of a properly formatted manuscript.

If the paper is accepted, you will be asked to submit individual high-resolution figure files to the redactory office that satisfy all the requirements listed below.

### Color figures

All color figures must be in RGB format. When saving, embed any ICC profile you have been working with. JLR charges authors $50 per color figure (see page charges).

### Figure format and resolution requirements for accepted manuscripts

| Image type | Monochrome | Combination halftones | Halftones |
|---|---|---|---|
| |  |  |  |
| Description | Line art, graphs or diagrams in black-and-white (no gray). | Grayscale or RGB color images with text or lines. | Grayscale or RGB color images containing pictures only, with no text or thin lines. |
| Minimum resolution (raster images) | 1,000 dpi | 600 dpi | 300 dpi |
| Preferred format | Vector EPS | TIFF | TIFF |
| Acceptable formats (if processed correctly) | Raster EPS<br>TIFF<br>Microsoft Word/ Powerpoint/ Excel | JPEG<br>PDF<br>Microsoft Word/ Powerpoint/ Excel | JPEG<br>PDF<br>Microsoft Word/ Powerpoint/ Excel |

Additional requirements for JPEG, PDF and Microsoft Office files:
- Embed fonts in all files.
- JPEG files should be saved at maximum quality.
- PDF files should be saved with ZIP compression only and not with downsampled graphics.
- Word/Powerpoint/Excel files should not contain pattern or textured fills.
- Images placed into Word/Powerpoint/Excel files must be at least 1000 dpi for line art, 600 dpi for combination halftones or 300 dpi for halftones.
- When inserting images into Word/Powerpoint/Excel files, select "insert", not "insert link."

### Figure dimensions

- During typesetting, all figures will be reduced to fit either a single-column width (3.375 inches), 1.5 column widths (4.125 inches) or two column widths (7.1 inches).
- Maximum height for each figure (including legend) is 10 inches.

**Text and lines in figures**

- Use Helvetica typeface when possible.
- Lines must be 0.25 pt thickness or greater.
- Embed fonts in vector files, such as those created with Adobe Illustrator.

Click here to return to the top of the page.

## Preparing PDF for submission

Once you have finished preparing the manuscript, convert the text and figure files into a single PDF with a file size of less than 5 MB. This PDF will be used for peer review purposes. If your paper is accepted for publication, this PDF will be published as the "Paper in Press" version. Make sure that:

- The figures are at the end of the PDF file, not inserted within the flow of the main text;
- All figures are numbered with Arabic numerals;
- All pages are numbered, including those with figures;
- Text is double-spaced;
- File name is one word with no spaces and a .PDF file extension (e.g., manuscript.pdf);
- There is no password protection on the file;
- There are no restrictions on the extraction of content or modification of the PDF (as this will disable reference extraction).

Any paper that does not comply with the above will be returned to the author for correction.

You can create the PDF using Adobe Acrobat, which is available for purchase (http://adobe.com/products/main.html). Microsoft Word also has a "Save As" feature that will allow you to save a file in PDF format. (PC versions 2007 and higher, Macintosh 2004 and higher.) Be aware that figure resolution may be reduced if this option is used. To review your image resolution settings, please refer to the help section of Microsoft Word under "Compress Pictures."

Consult the Adobe Acrobat manual for detailed instructions on creating PDF files. We recommend the following settings:

- Text and Graphics LZW Compression ON
- Thumbnail Generation OFF
- Generating ASCII format Files OFF
- Subset Embedded Fonts ON
- Color Image Downsampling ON
- Color Image Downsampling Resolution 150
- Color Image Compression ON
- Color Image Compression JPEG Medium
- Grayscale Image Downsampling ON
- Grayscale Image Downsampling Resolution 150
- Grayscale Image Compression ON
- Grayscale Image Compression JPEG Medium
- Monochrome Image Downsampling ON
- Monochrome Image Downsampling Resolution 300
- Monochrome Image Compression ON
- Monochrome Image Compression CCITT Group 4

We cannot process your submission if your PDF is larger than 5 MB without special arrangements. See here for suggestions on reducing PDF file size. If you need more help, please contact us via the feedback form.

You are responsible for ensuring the accuracy and quality of the PDF. You should print the PDF file and carefully review the text and figures to make sure that there were no compression or font errors introduced by the conversion process. You should also check to make sure the PDF conversion process has not inserted blank pages into the middle of the document.

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## Preparing supplemental data

**Allowable supplemental data**

Manuscripts must NOT be dependent on supplemental data. The manuscript is a complete, stand-alone record of research. However, data that is impossible or impractical to include in the manuscript may be provided as an online supplement. Examples include videos, long lists of primers, 3-D structures/images, large sequence alignments and datasets obtained from microarray hybridization or mass spectrometry. Please see our instructions on how to create 3-D files for submission as supplemental files. Data that would be important and useful to specialists, such as chemical structures, spectra and kinetic plots that are not critical to the manuscript are also permitted.

Links to author or third-party sites for such information are not allowed because they lack permanence, but links to "official" databases like GenBank are encouraged.

Supplemental data should NOT contain preliminary data that simply extends the scope of the study, unnecessary "control" data, or data that are thought to be not rigorous enough for the main text. Some novel methodology may be presented in detail in the Supplemental Data, but it should not be viewed as a "depository" for most methods; the main text should contain sufficient methodology for an experienced investigator to replicate the experiments.

### Supplemental data review process

Supplemental data will be reviewed as a part of the normal manuscript review process and will be judged by the same rigorous criteria to be important but not essential to the stand-alone manuscript. Only data that are deemed appropriate for the online journal and substantially contribute to the manuscript will be accepted. Supplemental data submitted during review will require that the paper be reviewed again thus extending the review process. Supplemental data submitted after the paper has been accepted will not be published.

Authors should carefully review the Supplementary Data for factual, grammatical and typographical issues since this material will not be professionally copyedited but permanently posted "as is".

### Supplemental data file formats

Supplemental data, figures and tables should NOT be included in the main manuscript PDF file. Compatible data files MUST BE combined into a single PDF (click here to see an example). Movies and large Excel files should be submitted in their native formats. Supplemental tables and figures (one per page) should each have a descriptive title and should be prepared double-spaced and numbered with Arabic figures.

Source files for supplemental data are NOT needed at the time of acceptance. We will use the files included in your submission. Supplemental files cannot be added after acceptance.

Click here to return to the top of the page.

## Cover Letter

A cover letter must accompany the manuscript. The cover letter must be prepared in plain unformatted text, ready to be copied and pasted or typed directly into the web site. The cover letter must not be included in the body of the manuscript. The cover letter should include:

- Statement regarding prior publication (see Prior publication above)
- Statement disclosing any conflicts of interest
- If any listed authors have not seen and approved the manuscript as submitted, an explanation as to why, e.g., deceased author, author unreachable because out of country, etc.
- Indication of whether supplemental material is being submitted with the manuscript, and whether the supplemental material is for publication online or is intended for the reviewers only

## Resubmissions

When an author has been invited by an editor to resubmit a revised manuscript, the resubmission must be accompanied by a cover letter that includes a detailed point-by-point listing as to how each of the reviewers' comments has been addressed and describes any other changes made to the manuscript. During the online submission process, authors must include the original JLR manuscript number in the Previous Manuscript ID blank on the submission form. This ensures all correspondence with your original manuscript will be linked to your new resubmission. Please see the online submission help for details.

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