UNVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA
PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

TESE DE DOUTORADO

FATORES DE RISCO PARA DOENÇAS CRÔNICA DEGENERATIVAS NÃO TRANSMISSÍVEIS EM PACIENTES COM ESQUISTOSSOMOSE CRÔNICA

ORIENTANDO: ADENOR ALMEIDA PIMENTA FILHO

ORIENTADORA: PROFA. DRA. VERA LÚCIA DE MENEZES LIMA

RECIFE, 2013

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Tese apresentada ao Programa de Pós-graduação em Bioquímica e Fisiologia como requisito final para a obtenção do título de doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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ADENOR ALMEIDA PIMENTA FILHO

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

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“O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém”.

Dalai Lama

RESUMO

A esquistossomose é uma doença parasitária presente em regiões de climas tropicais e subtropicais. Aproximadamente 200 milhões de pessoas ao redor do mundo estão infectadas pela doença. A fase crônica é caracterizada por comprometimento de órgãos e tecidos principalmente fígado e baço. A forma hepatoesplênica (HS) é o estágio mais avançado, no qual se observam maiores danos no parênquima hepático, hipertensão portal e congestão hepática. Em muitos pacientes na forma HS se faz necessária cirurgia de esplenectomia (pacientes HSS). Na forma HS, ocorrem grandes alterações no metabolismo de lipídios. Alterações lipídicas são consideradas importantes fatores de risco para Doenças Crônica Degenerativas Não Transmissíveis (DCDNT) tais como *diabetes mellitus*, resistência insulínica (RI) e doenças cardiovasculares (DCV). Além disso, fatores genéticos também podem influenciar no padrão e grau de alteração lipídicas. O polimorfismo do gene da Apolipoproteína E (Apo E) tem sido reportado como um importante fator contributivo para alterações lipídicas. O objetivo deste estudo foi investigar em pacientes esquistossomóticos a ocorrência de fatores de risco para DCDNT tais como RI e dislipidemias bem como a influência do polimorfismo do gene da Apo E nas alterações lipídicas. Para tanto foram obtidas amostras de plasma sangüíneo de indivíduos HS e HSS bem como de indivíduos saudáveis. Foram determinadas as concentrações plasmáticas de insulina e glicose de jejum, triglicerídios, HDL-c, VLDL-c, LDL-c, colesterol total, LDL-oxidada, Albumina, transaminase glutâmico oxalacética (TGO), transaminase glutâmico pirúvica (TGP), fosfatase alcalina e gama glutaril transferase (GGT). Além de terem sido determinados os valores de HOMA-IR e da razão triglicerídios por HDL-c para acessar a resistência e os índices de Castelli para acessar o risco de desenvolver DCV, além de extrair DNA leucocitário para determinar o polimorfismo do gene da Apo E. Os indivíduos HS e HSS apresentaram níveis mais elevados de LDL-oxidada, das enzimas hepáticas, bem como da glicemia e insulina de jejum e HOMA-IR, além de níveis mais baixos de Colesterol, triglicerídios e albumina, quando comparado ao grupo controle. Não houve diferença significativa da razão triglicerídios/HDL-c, bem como dos índices de Castelli entre os grupos. O polimorfismo do gene da Apo E também influenciou na maneira pela qual a esquistossomose modula o metabolismo de lipídios. Desta maneira, os resultados demonstraram que os indivíduos portadores da esquistossomose crônica apresentaram alterações no metabolismo lipídico, elevadas concentrações de LDL-oxidada e um quadro de resistência insulínica. Estes, por sua vez, representam importantes fatores de risco para o desenvolvimento de DCDNTs.

Palavras-chaves: Esquistossomose, dislipidemias, resistência insulínica, Apolipoproteína E.

ABSTRACT

Schistosomiasis is a parasitic disease present in regions of tropical and subtropical climates. Approximately 200 million people around the world are infected with the disease. The chronic phase is characterized by impairment of organs and tissues especially liver and spleen. The hepatosplenic (HS) is the most advanced stage, in which we observe further damage in the liver parenchyma, portal hypertension and liver congestion. In many patients as HS surgery is required splenectomy (HSS patients). In HS form is observed changes occur in the lipid metabolism. The major changes occur in the lipid metabolism. Lipid disorders are considered important risk factors for Chronic Degenerative Diseases Non-communicable (CDDNC) such as diabetes mellitus, insulin resistance (IR) and cardiovascular disease (CVD). Furthermore, genetic factors may also influence the pattern and degree of lipid alteration. The polymorphism of the gene Apolipoprotein E (Apo E) has been reported as a major contributory factor for lipid disorders. The aim of this study was to investigate the occurrence of schistosomiasis patients risk factors for CDDNC such as IR and dyslipidemia as well as the influence of gene polymorphism of Apo E in lipid disorders. For this samples were obtained from blood plasma from individuals HS and HSS as well as healthy subjects. The concentrations of plasma insulin and fasting glucose, triglycerides, HDL-C, VLDL-C, LDL-C, total cholesterol, oxidized LDL, albumin, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (SGPT), alkaline alkaline and gamma glutaryl transferase (GGT). Besides having been determined values of HOMA-IR and triglycerides reason for HDL-c resistance and Castelli's index to access the risk of developing CVD, and to extract leukocyte DNA determining the polymorphism of Apo E. ndividuals HS and HSS had higher levels of oxidized LDL, liver enzymes and blood glucose and fasting insulin and HOMA-IR, and lower levels of cholesterol, triglycerides and albumin when compared to the control group. There was no significant difference in the ratio triglycerides/HDL-c, as well as Castelli's index between groups. The polymorphism of the Apo E gene also influence the way in which schistosomiasis modulates lipid metabolism. Thus, the results showed that individuals with chronic schistosomiasis showed alterations in lipid metabolism, high concentrations of oxidized LDL and a framework of insulin resistance. These, in turn, represent important risk factors for the development of CDDNC.

Keywords: Schistosomiasis, dyslipidemia, insulin resistance, Apolipoprotein E.

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LISTA DE ABREVIATURAS

ANOVA: Análise de Variância

Apo A-I: Apolipoproteína A-I

Apo B: Apolipoproteína B

Apo E: Apolipoproteína E

DCDNT: Doença Crônica Degenerativa Não Transmissível

DCV: Doença Cardiovascular

DM: *Diabetes mellitus*

ECLIA: electrochemiluminescence

ELISA: Enzyme-Linked Immunosorbent Assay

FI: Forma Intestinal

FHE: Forma Hepatoesplênica

FHI: Forma Hepatointestinal

HDL: *High Density lipoprotein*

HOMA-IR: Homeostasis Model Assessment Insulin Resistance

HS: Hepatosplenic

HSS: Hepatosplenic splenectomized

IDL: *Intermediate Density lipoprotein*

IL: Interleucina

INF-γ: Interferon Gama

IR: Insulin resistance

LCAT: Lecitina Acil-Transferase

LDL: *Low Density Lipoprotein*

LDL-ox: LDL-oxidized

S.E.M: Error Mean

TNF-α: Fator de Necrose Tumoral Alfa

VLDL: *Very Low Density Lipoprotein*

SUMÁRIO

1 INTRODUÇÃO

1.1 Esquistossomose

A esquistossomose ou bilharzíase é uma doença parasitária intravascular, que está presente em regiões de climas tropical e subtropical causada por helmintos trematódeos do gênero *Schistosoma*. Estima-se que ao redor do mundo existam, aproximadamente, 200 milhões de pessoas infectadas e que outros 600 milhões de pessoas vivam em condições de risco. Estudos epidemiológicos revelam que 76 países e territórios são endêmicos para esta parasitose (Figura 01). Entretanto, 85% dos casos concentram-se no continente africano (ENGELS *et al.*, 2002, RIDI *et al.*, 2004).

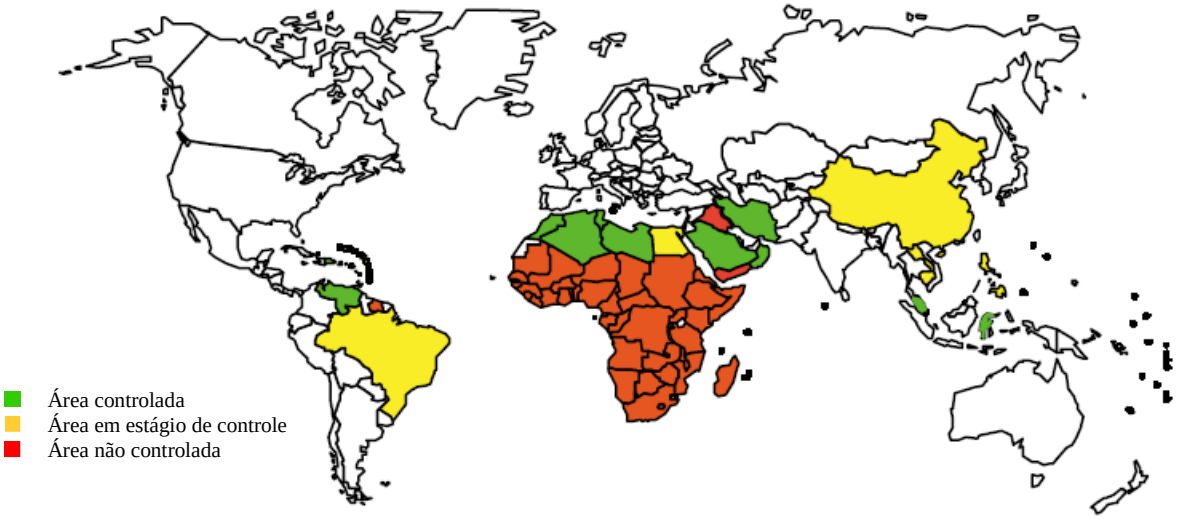


Figura 01: Distribuição global da esquistossomose e seu estado de controle (ENGELS *et al.*, 2002).

O primeiro caso de esquistossomose humana foi descrito pelo patologista alemão Theodore Maximilian Bilharz (1825-1862). Após fazer autópsias em indivíduos infectados no Egito, ele descreveu os vermes adultos, machos e fêmeas, ambos parasitando o sistema porta e a bexiga. Também descreveu a morfologia do ovo com sua peculiar espícula terminal. Os parasitos foram denominados *Distomum*

(Schistosoma) haematobium. Contemporaneamente ao patologista alemão, no Japão, outros pesquisadores relatavam à ocorrência de outro trematoda de morfologia similar que parasitava o sistema porta, o *Shistosoma japonicum*. Em 1907, na Inglaterra, Luigi Sambon (1865-1931) observou a ocorrência de ovos com espículas laterais nas fezes de indivíduos doentes, que mais tarde denominara ovos de *S. mansoni*. No Brasil, o primeiro caso da esquistossomose mansônica foi descrito em 1907 por Pirajá da Silva no estado da Bahia (COON, 2005; AMARAL *et al.*, 2006).

O gênero *Schistosoma* possui várias espécies que infectam animais e algumas que acometem o homem. Contudo, existem três espécies de maior interesse médico: *S. haematobium*, *S. japonicum* e *S. mansoni*. Os vermes adultos são brancos ou cinzas, medem 7-20 mm de comprimento, possuem um tegumento complexo por onde absorvem os nutrientes oriundos do hospedeiro e excretam metabólitos. Diferentemente de outros trematódeos, estes parasitas apresentam dimorfismo sexual, sendo os machos achatados dorso-ventralmente e com uma fenda longitudinal, denominado canal ginecóforo, onde abriga as fêmeas que são cilíndricas e afiladas (Figura 02) (GRYSEELS *et al.*, 2006).

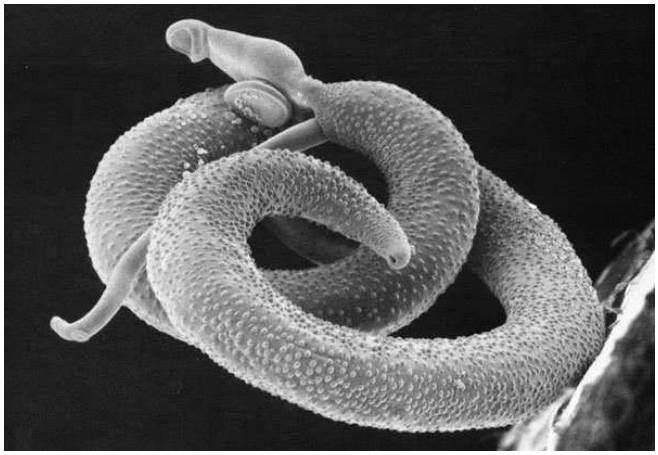


Figura 02 Morfologia do *S. mansoni* (Disponível em: <http://vineetgupta.files.wordpress.com/2008/06/schistosoma_mansoni2.jpg>. Acesso em 22de janeiro de 2013).

Durante o ciclo de vida do parasita (Figura 3), as fêmeas podem produzir centenas de ovos por dia (A), os quais são eliminados pelas fezes (*S. japonicum* e *S. mansoni*) ou pela urina (*S. haematobium*) de indivíduos infectados. Cada um desses ovos possui uma larva ciliada denominada miracídio (B). Quando os ovos entram em contato com a água, sob condições ideais de luz e temperatura, eclodem liberando os miracídios, que nadam até encontrar o caramujo (C), o hospedeiro intermediário. Após a penetração no caramujo, os miracídios multiplicam-se assexuadamente em esporocistos que darão origem as cercárias. Cerca de 4-6 semanas depois da penetração, as cercarias (D) saem do caramujo e permanecem nadando no meio aquático por até 72h. Quando o indivíduo entra em contato com coleções de águas contaminadas, as cercárias penetram ativamente através da pele, transformam-se em esquistossômulos e migram pela corrente sanguínea passando pelos pulmões, local de alongamento, até chegar ao sistema porta-hepático, onde alcançam a maturidade sexual (E) e finalmente seguem para as veias mesentéricas para ovoposição (BLANCHARD, 2004).

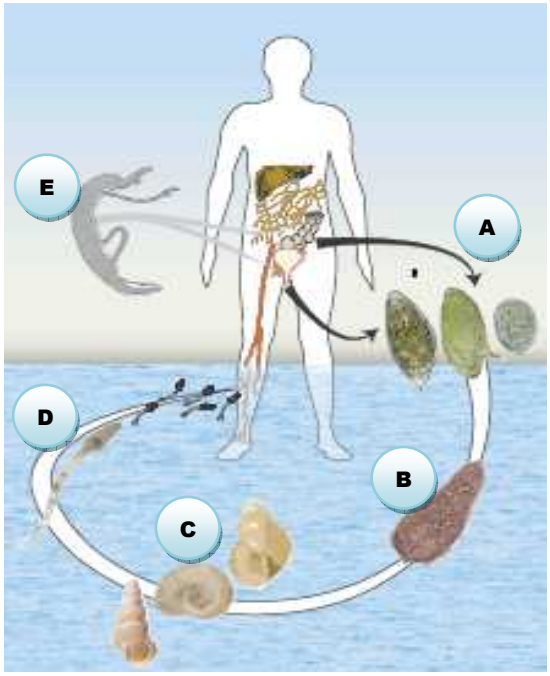


Figura 03 Ciclo de vida do *S. mansoni* (GRYSEELS *et al.*, 2006).

Dentre as espécies do gênero *Schistosoma*, o *S. mansoni* é o agente etiológico da esquistossomose no Brasil. O verme tem origem africana e teria sido introduzido no país durante o período colonial através do tráfico de escravos oriundos daquele continente. O sucesso da perpetuação do parasito na região foi atribuído a dois fatores principais: condições climáticas semelhantes ao *habitat* original e a existência de um hospedeiro intermediário compatível com o seu ciclo de vida, os moluscos do gênero *Biomphalaria.* No Brasil, existem três espécies deste gênero: *B. glabrata, B. straminea, B. tenagophila* (PARAENSE, 1983; GRYSEELS *et al.*, 2006). Destas três espécies o *B. glabrata* é o que apresenta maior susceptibilidade a infecção pelo *S. mansoni* devido ao seu maior tamanho, melhor adaptação ao parasito e um maior número de larvas infectantes liberadas (MALAGUEÑO *et al.*, 1994).

Estima-se que no Brasil existam cerca de 2,5 milhões de casos da esquistossomose mansônica, sendo bastante endêmico na região nordeste. O estado de Pernambuco – Nordeste – Brasil possui uma das prevalências médias mais elevadas de pessoas infectadas pelo *S. mansoni*, com uma concentração dos casos predominantemente nas zonas da mata e litorânea (AMARAL & PORTO, 1994; BARBOSA *et al.*, 1996; BARBOSA *et al.*, 2001; BARBOSA *et al.*, 2006).

A patologia da esquistossomose mansônica é dividida em duas fases: fase aguda e fase crônica (GRYSEELS, 2006). A fase aguda é caracterizada por uma reação imune decorrente da penetração das cercárias através da pele do hospedeiro, é autolimitante e resolvida espontaneamente. Os principais sintomas da fase aguda são: diarréia, febre, perda de peso, tosse, mialgia, atralgia, eosinofilia, leucocitose e urticária (dermatite cercariana). Geralmente não é observada nas populações de áreas endêmicas, sendo

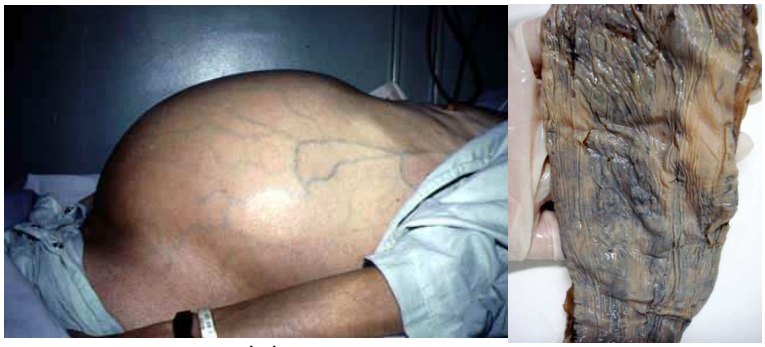
mais comum em indivíduos oriundos de áreas não-endêmicas que entram em contato com antígenos do parasito pela primeira vez (CALDAS *et al.*, 2008).

A fase crônica é caracterizada pelo comprometimento de órgãos e tecidos ocasionados por reações granulomatosas, que são desencadeadas pela deposição dos ovos do parasito nos mesmos. De acordo com os órgãos envolvidos e seu grau de comprometimento, a fase crônica é subdividida em três subfases: fase intestinal (FI), fase hepatointestinal (FHI) e fase hepatoesplênica (FHE) (BLANCHARD, 2004; GRYSEELS, 2006). Tradicionalmente, a ultra-sonografia tem sido o método mais utilizado para diagnóstico e acompanhamento de pacientes esquistossomóticos crônicos possibilitando, inclusive, a caracterização das subfases (BEZERRA *et al.*, 2004).

A FI é caracterizada por lesões nas alças intestinais promovidas pela postura dos ovos, pela fêmea do parasito, nos vasos sanguíneos do plexo mesentérico causando granulomas, pseudopólipos e microulcerações na mucosa intestinal. Enquanto que a FHI constitui uma fase intermediária entre a FI e a FHE, na qual podem ser observadas as primeiras lesões granulomatosas no fígado, decorrente da deposição dos ovos (GRYSEELS, 2006).

A FHE é a estágio mais avançado da doença e geralmente se estabelece após a primeira década da infecção, sendo encontrada principalmente em áreas hiperendêmicas onde ocorrem reinfecções sucessivas. No decorrer desta fase observa-se maior grau de lesões hepáticas, alterações fisiopatológicas e manifestações clínicas (ABDALLAHI *et al.*, 1999). A entidade anátomo-patológico característica da FHE é representada pela lesão periportal, descrita por Symmers em 1904, conhecida como fibrose de Symmers, que é resultante de uma intensa neoformação conjuntiva nos sinusóides hepáticos, desencadeada por antígenos solúveis secretados pelos ovos viáveis. Durante esta fase

também pode ser observado o aparecimento de varizes esofagianas, que podem se romper e ocasionar hemorragias digestivas; circulação colateral e ascite causada pela combinação da hipoalbuminemia e hipertensão portal (Figura, 04); aumento no tamanho do baço (esplenomegalia) resultante da congestão da circulação porta-hepática e proliferação celular acentuada do sistema fagocítico-mononuclear (hiperesplenismo) que, por sua vez, leva ao aparecimento de anemia, leucopenia e trombocitopenia (ANDRADE *et al.,* 1962; STAVITSKY, 2004; GRYSEELS *et al.*, 2006).



(A) (B)

Figura 04 (A) Paciente apresentando ascite e circulação colateral. (B) Esôfago apresentando varizes esofagianas. Disponível em <www.hepatocentro.com.br>. Acesso em 21de janeiro de 2013.

De acordo com as fases da doença, o hospedeiro expressa diferentes padrões de resposta imune, que são mediadas por células T auxiliares CD4+. Durante a fase aguda o padrão citocínico Th1 (IL-2. TNF-α e IFN-γ) é predominantemente expresso, estando relacionado com funções citotóxicas e inflamatórias. Enquanto que na fase crônica predomina o padrão Th2 (IL-4, IL-5, IL-6, IL-10 e IL-13) que tem um papel estimulante para produção de anticorpos, principalmente IgE, proliferação e ativação de eosinófilos. O balanço entre as respostas Th1/Th2 está relacionado com a regulação da intensidade da reação granulomatosa bem como do grau de fibrose hepática observado. O equilíbrio

estabelecido entre estes padrões inflamatórios é fator determinante para a sobrevivência mútua na relação parasito hospedeiro (BRUNET *et al.*, 1998; WYNN *et al.*, 2004; ABATH *et al.,* 2006; CALDAS *et al.,* 2008).

O tratamento clínico padrão da esquistossomose é baseado na utilização de drogas esquistossomicidas, principalmente a Oximiniquine e o Praziquantel. Atualmente, o Praziquantel, um derivado pirazino-isoquinolona, tem sido a droga de escolha para o tratamento da esquistossomose devido ao seu menor custo/tratamento, alta eficácia e baixa toxicidade em relação aos demais quimioterápicos (CIOLI & PICA-MATTOCIA, 2003; DAYAN, 2003; McFADYEN, 2006; KATZ & COLEHO, 2008). Entretanto, alguns pacientes portadores da FHE mais avançada necessitam de uma intervenção cirúrgica, que consiste na retirada do baço, a esplenectomia, no intuito de diminuir a hipertensão portal e a congestão hepática, o que resulta numa melhora do quadro hemodinâmico do paciente, o que minimiza os riscos de hemorragia digestiva aguda, uma das principais causas de mortalidade, além de promover uma melhora na função metabólica hepática (BRANDT *et al.*, 2005).

1.2 Alterações no metabolismo lipídico em estados infecciosos e/ou inflamatórios.

Alguns estudos reportam uma associação entre estados infecciosos e/ou inflamatórios com alterações no metabolismo de lipídios, lipoproteínas e apolipoproteínas, particularmente com relação à apolipoproteína A-I, o principal componente protéico da HDL (*High Density Lipoprotein*), e a apolipoproteína B, o principal constituinte protéico da VLDL (*Very Low Density Lipoprotein*), IDL (*Intermediate Density Lipoprotein)* e LDL (*Low Density Lipoprotein*). Estas alterações

estariam relacionadas com a ação direta do parasito sobre o hospedeiro, como também ao padrão de resposta imune expresso durante as fases agudas e crônicas das infecções (KHOVIDHUNKIT *et al.*, 2000).

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 (FEINGOLD *et al.*,1989). 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 lecitina colesterol acil-transferase (LCAT) estando relacionada com alterações no metabolismo do colesterol, interferindo no seu transporte reverso (LIMA *et al.*,1998). Outros estudos também relataram alterações no metabolismo de triglicerídeos e lipoproteína de muito baixa densidade (VLDL) tendo sido observados níveis elevados em camundongos, que desenvolveram a infecção por *S. mansoni* (FEINGOLD *et al.*,1989; DOENHOFF *et al.*, 2002, RAMOS *et al.*, 2004 e 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 triglicerídios (SILVA *et al.*, 2001; FACUNDO *et al.*, 2004). Desta forma pode-se evidenciar que as alterações metabólicas observados 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.

1.3 Alterações no metabolismo lipídico em doenças crônicas degenerativas não-transmissíveis.

Doenças crônicas degenerativas não-transmissíveis (DCDNT) correspondem a um grupo de doenças não contagiosas incapacitantes, cuja patogênese está geralmente associada a alterações metabólicas que são influenciadas pelo ambiente, fatores genéticos e estilo de vida do indivíduo (sedentarismo, tabagismo, alcoolismo dentre outras). São classificados como DCDNT o *diabetes mellitus*, resistência insulínica, doenças cardiovasculares (DCVs) e câncer, entre outros (MALTA *et al*, 2006).

Este grupo de patologias é responsável por cerca de 60% dos 56,5 milhões de óbitos anuais que ocorrem no mundo. Parte destes óbitos, aproximadamente 17 milhões, é causada por doenças cardiovasculares, que segundo o "Relatório sobre a saúde do mundo (2002)" da OMS, tem como principais fatores de risco a obesidade, hipertensão e dislipidemias (ORGANIZAÇÃO PANAMERICANA DE SAÚDE/OMS, 2003). O Sistema Único de Saúde (SUS) gasta cerca de 12 milhões de reais/ano em internações decorrentes das DCDNTs e suas complicações. Esse valor corresponde a aproximadamente 80% dos gastos com assistência médica-hospitalar oferecida a população (LAURENTI *et al*, 2000).

Dados epidemiológicos reportam uma estreita relação entre alterações lipídicas com a patogênese de DCDNT tais como resistência insulínica, *diabetes mellitus* e DCVs. Modificações quantitativas nos componentes lipídicos plasmáticos tais como a

diminuição nas concentrações plasmáticas de HDL-c, Apo A-I e elevações nas concentrações plasmáticas de colesterol, LDL-c, triglicerídios e VLDL-c, além de alterações qualitativas, como a oxidação de lipoproteínas, são considerados importantes fatores de risco para desenvolvimento das DCDNT (GOTTLIEB *et al*, 2011).

Recentemente, um estudo realizado em indivíduos cirróticos revelou que 96% destes indivíduos apresentaram um quadro de glicemia de jejum alterada e destes, 30% foram posteriormente diagnosticados com *diabetes mellitus* tipo 2. Além de evidenciarem esta relação, ainda observaram que aqueles indivíduos portadores de algum distúrbio no metabolismo insulínico desenvolviam um quadro de esteatose, mesmo quando não apresentavam alterações significativas no perfil lipídico, sobretudo nos níveis de triglicerídios e VLDL-c, que, por sua vez, aumentava o grau e a velocidade de progressão da fibrose hepática, além de promover o estresse oxidativo nos hepatócitos (GARCIA-COMPEAN *et al*, 2009).

1.4 Resistência insulínica

A insulina tem grande importância no metabolismo orgânico, pois a maioria dos tecidos, com exceção do cerebral, renal e sangüíneo, não consegue utilizar a glicose diretamente, sendo então necessária a interação da insulina com receptores de membrana para ativação de uma proteína transportadora da glicose (RASMUSSEN *et al*, 1990).

Os receptores de insulina são glicoproteínas semelhantes estruturalmente às imunoglobulinas, localizados nas membranas dos tecidos-alvos, compostos por duas subunidades α (125KDa a 153KDa) e duas subunidades β (90KDa), dispostas de forma

simétrica e unidas por duas pontes dissulfeto. A subunidade α é completamente extracelular e contém o sítio de ligação com a insulina, enquanto a subunidade β apresenta um segmento extracelular, um domínio transmembranar hidrofóbico e um segmento intracelular (SHULMAN, 2000).

O número de receptores para insulina, existentes na membrana celular, é regulado, em parte, pela própria insulina, ou seja, quando há um aumento na insulinemia ocorre uma diminuição no número de receptores (*down regulation*), com o inverso ocorrendo na diminuição dos níveis de insulina plasmático (*up regulation*) (RASMUSSEN *et al*, 1990; SHULMAN, 2000).

A ação da insulina inicia-se com a ligação deste hormônio com o seu receptor de membrana. Em seguida, ocorre a ativação da enzima tirosina-cinase que é responsável pela autofosforilação da subunidade β do receptor e pela fosforilação nos resíduos de tirosina de uma proteína citoplasmática, de peso molecular de 131 KDa, denominada IRS-1 (substrato 1 do receptor de insulina). A IRS-1 fosforilada interage com outras proteínas específicas intracelulares, como, por exemplo, a Grb2 (associated binding protein 2), fosfatidilinositol-3-cinase, fosfotirosinas e fosfatases. Estas, por sua vez, desencadeiam de forma ainda desconhecida, múltiplas cascatas de fosforilações e desfosforilações resultando nos efeitos biológicos da insulina (CARVALHEIRA *et al*, 2002) (Figura 05).

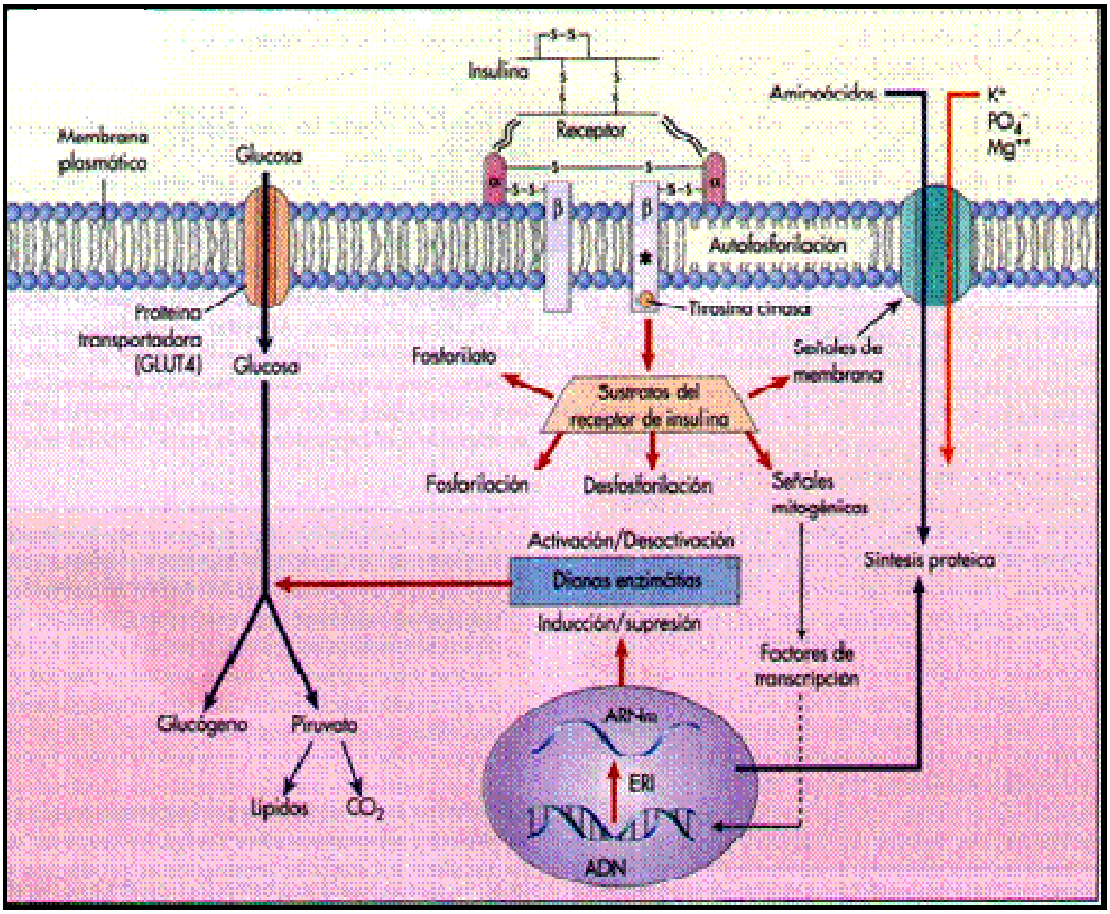


Figura 05 Mecanismos de ação da insulina nas células. <http://www.afh.bio.br/img/insulina%20e%20>. Acesso em 22 de janeiro de 2013.

Após ocorrer a ligação, os complexos hormônio-receptor, são agrupados para serem internalizados em depressões especiais da membrana plasmática, denominadas *coated pits*, que são revestidas por um peptídeo (clatrina). No citoplasma, os complexos agrupados fundem-se com os lisossomos, a insulina é degradada pelas enzimas lisossomais e os receptores retornam para a membrana (VIRKAMÄKI *et al*, 1999).

A insulina tem efeitos diretos em muitos órgãos e tecidos como o fígado, tecido muscular, tecido adiposo e efeitos sobre substratos energéticos circulantes. Além disso, acredita-se que a insulina atue como fator de crescimento celular. No tecido hepático, a

insulina estimula a captação de glicose e o seu armazenamento como glicogênio, ativando a enzima glicocinase e a glicogênio sintetase; aumenta o fluxo na via glicolítica e na via das pentoses; inibe a gliconeogênese e glicogenólise (OSEI, 1999).

A captação da glicose pelas células do tecido muscular é mediada pela insulina, que aumenta a síntese e a capacidade dos receptores de glicose (GLUT 4). Parte dessa glicose captada é oxidada, mas a maior parte é transformada em glicogênio pela glicogênio-sintetase muscular. Ainda no tecido muscular, a insulina tem efeito anabólico no metabolismo das proteínas estimulando o transporte de aminoácidos, aumentando, em nível ribossomal, a eficiência do processo de tradução e inibindo a degradação protéica (SHULMAN, 2000).

No tecido adiposo, a insulina estimula a lipogênese e inibe a mobilização dos ácidos graxos. Da mesma forma que no tecido muscular, o hormônio atua nos receptores GLUT 4 aumentando a síntese e a capacidade dos transportadores. Quando a glicose combina-se com os receptores, é fosforilada, formando o α-fosfoglicerato, que será utilizado como substrato na esterificação dos ácidos graxos na síntese de triglicéridios (SALTIEL & KAHN, 2001).

Além disso, a insulina acelera a captação de ácidos graxos, que se encontram na circulação em moléculas de lipoproteínas, como os quilomícrons e a VLDL, através da enzima lipase lipoprotéica. Esta enzima é estimulada pela insulina e catalisa a hidrólise das lipoproteínas liberando os ácidos graxos que serão captados pelos adipócitos e combinados com α-fosfoglicerato, diminuindo, com isso, a formação dos corpos cetônicos (*op cit*).

A resistência insulínica é um distúrbio metabólico, caracterizado pela diminuição da capacidade dos receptores dos tecidos em interagir com a insulina, e conseqüente diminuição na captação da glicose plasmática. Com isso, há um aumento na concentração de glicose no sangue e, conseqüente, secreção de insulina compensatória, levando à hiperinsulinemia (SIMONE *et al*, 2003).

Durante o estado de resistência insulínica, podem ocorrer vários distúrbios metabólicos no organismo, 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 hiperternsão arterial sistêmica. Os principais órgãos e tecidos afetados são o tecido adiposo, o tecido muscular, o fígado e os rins (*op cit*).

No tecido adiposo, a resistência insulínica está relacionada com a diminuição dos níveis de HDL-c, na formação de uma LDL aterogênica que é menor e mais densa, aumento nos níveis plasmáticos de triglicéridios 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 triglicéridios, o que provoca, conseqüentemente, desordens metabólicas, como, por exemplo, a esteatose hepática, esteatoepatite, cirrose e carcinoma hepatocelular (HOLZL *et al*, 1998; OLIVEIRA *et al*, 2004; CUSSONS *et al*, 2005).

As LDLs são afetadas em sua composição, pois são produzidas a partir de VLDLs anormais, incorporando em suas moléculas grandes quantidades de triglicerídios, 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 triglicéridios para a HDL; a troca entre colesterol que foi esterificado pela atuação da enzima lecitina colesterol aciltransferase (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 *et al* 2005).

1.5 Apolipoproteína E *versus* metabolismo de lipídios.

Além de fatores ambientais, diferentes fatores intrínsecos influenciam no metabolismo lipídico. Os fatores genéticos são determinantes para ditar o ritmo metabólico de cada indivíduo ou grupos específicos de indivíduos. Dentre os fatores genéticos, o gene polimórfico da Apolipoproteína E (Apo E) tem sido muito estudado nos últimos anos (MERKEL *et al*, 2002).

A Apo E é uma glicoproteína anfipática de aproximadamente 34 KDa, formada por 299 resíduos de aminoácidos. No plasma, a Apo E está presente em diversas lipoproteínas, nas quais possui várias funções, dentre as quais manter a estrutura e regular o metabolismo (MAHLEY & RALL, 2000). Suas funções no metabolismo lipídico estão relacionadas à mediação do reconhecimento e internalização hepática de lipoproteínas ricas em triglicerídeos (TRLs), quilomícrons e VLDLs, e seus remanescentes através de sua ação como ligante de LDLR, LRP e HSPG (MAHLEY & HUANG, 1999; HARRIS, EVANS & OWEN, 2006) e também a tecidos periféricos (HAGBERG, WILUND & FERREL, 2000).

Além disso, a Apo E também tem papel fundamental no metabolismo das lipoproteínas ricas em colesterol, como LDL, em que atua na distribuição de colesterol para os tecidos periféricos, nos quais este lipídeo é utilizado principalmente na síntese de membranas e como precursor de hormônios esteróides, ou ainda como constituinte de algumas frações de HDL contribuindo com o transporte reverso de colesterol através da participação desta apolipoproteína na internalização hepática destas lipoproteínas (MAHLEY, 1982; MAHLEY & INNERARITY, 1983).

Além de atuar como ligante de receptores, a Apo E 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 LCAT (GREENOW, PEARCE & RAMJI, 2005)

O gene da Apo E, nos seres humanos, está localizado no braço longo do cromossomo 19 (19q13.2) (MASEMOLA, ALBERTS & URDAL, 2007), bastante próximo e com sobreposição parcial aos genes da Apo C-II e LDLR (JACKSON,

BRUNS & BRESLOW, 1984). O gene tem 3,6 kilobases com 3 íntrons (4 éxons) e forma uma proteína precursora de 317 aminoácidos (FRANCKE, BROWN & GOLDSTEIN, 1984).

O polimorfismo existente na Apo E foi primeiramente descrito nos estudos do pesquisador alemão Uttermann e seus colaboradores em 1977 e 1980 utilizando focalização isoelétrica e por Zannis e Breslow em 1981, utilizando eletroforese bi-dimensional (UTERMANN *et al*, 1980; ZANNIS & BRESLOW, 1981). As três principais isoformas da Apo E, chamadas E2, E3 e E4, são produtos dos três alelos ε2 (lê-se épsilon), ε3, ε4. Três fenótipos homozigotos (APOE2/2, E3/3 e E4/4) e três heterozigotos (Apo E2/3, E2/4, E3/4) provêm da expressão de dois destes três alelos (MAHLEY, 1988).

As modificações que dão origem a estes três alelos acontecem em dois nucleotídeos presentes no éxon 3 e formarão proteínas que diferem nos aminoácidos 112 e/ou 158. A Apo E3 possui um resíduo de cisteína na posição 112 e um resíduo de arginina na posição 158, enquanto a Apo E2 contem duas cisteínas (Arg_{158}→ Cys) e a Apo E4 contem duas argininas em ambos os sítios (Cys_{112}→ Arg) (MAHLEY & RALL, 2000). Foi relatado que o polimorfismo da Apo E pode provocar até 20% da variação dos níveis de Apo E plasmáticos (KAPRIO *et al*, 1991).

Desde a descoberta da existência da Apo E tem-se estudado sua participação no metabolismo lipídico (UTERMANN *et al*, 1977). No decurso destes estudos tem sido observada uma grande importância do polimorfismo do gene da Apo E na gênese de distúrbios metabólicos de grande interesse para a saúde pública do mundo (BRESLOW *et al*, 1982; ALVIM *et al*, 2010).

Doenças como as DCVs e o Diabetes Mellitus tipo 2 possuem as maiores taxas de morbi-mortalidade entre as DCDNTs e estão fortemente associadas a distúrbios no metabolismo lipídico/lipoprotéico, no qual a Apo E está intimamente relacionada (FERREIRA *et al*, 2011; TAO *et al*, 2011).

O elegante trabalho de Lahoz e cols. (2001) apresentado a partir do *Framingham Heart Study* demonstrou um aumento do risco de desenvolvimento de doença arterial coronariana (DAC) em homens portadores do alelo ε4, mesmo quando eliminada a influência da idade e de outros fatores de risco, como diabetes, tabagismo, pressão arterial sistólica, índice de massa corpórea e hipertrofia ventricular esquerda (LAHOZ *et al*, 2001). Outros estudos também têm encontrado associações significativas entre Apo E4 e Doença Cerebrovascular Isquêmica (MCCARRON, DELONG & ALBERTS, 1999). Além disso, enquanto portadores do alelo ε4 apresentaram risco um pouco maior de desenvolver DAC do que indivíduos ε3/ε3, portadores de Apo E2 apresentaram risco 20% menor (BENNET *et al*, 2007).

Além dos distúrbios cardiovasculares, as dislipidemias também apresentam relação bem estabelecida com o diabetes mellitus tipo 2. Indivíduos diabéticos frequentemente apresentam níveis elevados de triglicerídeos e diminuídos de HDL-c (TOCCI *et al*, 2011). De maneira que o interesse para o estudo da influência do polimorfismo da Apo E nestes indivíduos tem produzido diversos estudos ao longo das últimas décadas (WEN *et al*, 2011). Recentemente, uma elegante meta-análise – utilizando 30 estudos e mais de 13000 indivíduos – apresentou dados bastante relevantes para este entendimento. Foi observado que a presença de Apo E2 é um fator

de risco independente para o desenvolvimento de diabetes mellitus tipo 2 (ANTHOPOULOS, HAMODRAKAS & BAGOS, 2010).

Outros estudos têm encontrado associação de Apo E4 com a Síndrome Metabólica X em idosos (TAO *et al*, 2011), mas não em pacientes com obesidade mórbida (FERREIRA *et al*, 2011). Também tem sido relatado que pacientes diabéticos portadores de ε4 apresentam duas vezes mais risco de desenvolvimento de Doença de Alzheimer (DA) (PEILA *et al*, 2002) e lesões mais graves entre os indivíduos com DA (MESSIER, 2003; MARTINS *et al* 2006).

2 JUSTIFICATIVA

Apesar de alguns estudos reportarem alterações metabólicas ocorridas em processos patológicos que envolvem danos hepáticos, sobretudo na esquistossomose crônica, ainda são divergentes as opiniões quanto ao papel dessas desordens metabólicas na patogênese das DCDNT, tais como o *diabetes mellitus*, resistência insulínica e doenças cardiovasculares (DCVs), bem como a forma pela qual o polimorfismo do gene da Apo E interage com estas alterações.

Desta maneira, o estabelecimento de dados que possam ser utilizados com fins de auxiliar no diagnostico e/ou prognóstico, bem como na avaliação de predisposição e na investigação de fatores de risco para essas patologias, que possam acometer pacientes com esquistossomose, mesmo após o tratamento antiparasitário, são considerados de grande importância para melhoria das condições de vida e longevidade deste grupo de indivíduos.

3 OBJETIVOS

3.1 Geral

Investigar a presença de fatores de risco para DCDNTs em indivíduos portadores da esquistossomose mansônica crônica.

3.2 Específicos

- Obter um grupo de indivíduos portadores de esquistossomose crônica e outro grupo de indivíduos saudáveis para compor o grupo controle.
- Determinar o perfil lipídico (colesterol total, triglicerídeos, HDL-colesterol, LDL-colesterol e VLDL-colesterol) em todos os indivíduos participantes da pesquisa.
- Quantificar as concentrações plasmáticas das Apoliproteínas A-I e B das amostras obtidas e determinar a relação Apo B/Apo A-I de os todos indivíduos participantes da pesquisa.
- Acessar o risco para desenvolver DCVs nos indivíduos com esquistossomose a partir dos índices CT/HDL-c, LDL-c/HDL-c e Apo B/Apo A-I.
- Quantificar as concentrações plasmáticas de LDL-oxidada.
- Determinar os níveis plasmáticos de glicose e insulina de todos os indivíduos participantes da pesquisa.
- Avaliar a presença de resistência insulínica através do HOMA-IR (*Homeostasis Model Assessment Insulin Resistance)* e da relação triglicerídios/HDL-colesterol.
- Obter DNA genômico e determinar os genótipos de todos indivíduos participantes.
- Determinar as freqüências dos alelos e genótipos do gene da Apo E nos indivíduos participantes.

- Investigar a associação dos alelos e genótipos da Apo E com as alterações lipídicas observadas nos indivíduos.

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5 Resultados

5.1 Capítulo I: Artigo a ser submetido CCA.

Chronic schistosomiasis causes oxidation of low density lipoprotein predisposing infected individuals to development of cardiovascular diseases.

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Abstract

Worldwide, estimated 200 million people are affected by schistosomiasis. During the HS from several metabolic changes are observed mainly in the lipid metabolism. Dislipidemics are directly relationship with cardiovascular diseases (CVD). Sit still controversial role of lipid changes caused by schistosomiasis in the pathogenesis of CVDs. Thus, the present study aimed to evaluate the potential risk of developing CVD in patients HS and HSS across lipid profile, Castelli's index, Apo A-I, Apo B, Apo B/Apo A-I ratio and LDL-ox. The infected individuals presented lower levels of TC, LDL-c, HDL-c, VLDL-c, Triglycerides, Apo A-I, Apo B and high levels of LDL-oxidized when compared to control group. No had significant difference of Castelli's index and Apo B/Apo A-I ratio between groups. Despite decreased lipids levels, the infected individuals presented high levels de LDL-oxidized an independent risk factor to develop CVDs. Still, the alterations observed in the lipid profile and Apo A-I and Apo B plasma levels decreased the sensitivity of the index to characterize predisposition to CVDs in these individuals. Thus this study opens a new perspective for investigating the development of CVDs in individual with chronic diseases suggesting the existence of a mechanism independent of elevation of plasma lipids.

Key words: Chronic Schistosomiasis, Lipids, LDL-oxidized.

1. Introduction

Worldwide, approximately 200 million people are affected by schistosomiasis. A parasitic disease that can last for more than 10 years, whose form hepatosplenic (HS) is the most advanced stage. In this stage are observed more damage liver and spleen. Sometimes being required splenectomy in hepatosplenic individuals (HSS) for improving the hemodynamic status of the patient [01, 02].

During the HS from several metabolic changes are observed mainly in the metabolism of lipids and lipoproteins, such as reduction in plasma levels of total cholesterol (TC), triglycerides, cholesterol of very low density lipoprotein (VLDL-c), cholesterol of low density lipoprotein (LDL-c) and cholesterol of high lipoprotein density lipoprotein (HDL-c), beyond peroxidation of erythrocytes membrane lipids and decreased activity of enzyme lecithin cholesterol acil-tranferase (LCAT) [03, 04].

Besides the report of reduced plasma TC and LDL-c levels caused by schistosomiasis [03, 05, 06], it is also reported that such reduction of lipids levels has no effect on atherosclerosis development [07]. The role of lipid changes caused by schistosomiasis in the pathogenesis of cardiovascular diseases (CVDs) remains controversial. One possible factor may be some differences observed between human and rodent lipid metabolism [08]. Furthermore, studies have reported that decreased plasma concentration of TC and LDL-c in lipid profile does not exclude the possibility of developing CVD requiring the evaluation of other laboratorial parameters such as Apo A-I, Apo B, Apo B/Apo A-I ratio and LDL-oxidized (LDL-ox) plasma concentrations [09, 10].

Apo A-I is a major protein component of HDL, an anti-atherogenic lipoprotein. However Apo B is the main component of atherogenic lipoproteins. Epidemiological studies have reported a correlation between high values of the Apo B/Apo A ratio [09] beyond Castelli's I index (TC/HDL-c), Castelli's II index (LDL-c/HDL-c) [10] and increased of risk to develop CVD. Furthermore, LDL-ox is considered an independent factor to CVDs since their chemical and structural modifications stimulates phagocytosis by macrophages yielding the foam cells that migrate into layer intimal of the arteries trigger the atherosclerotic process [11, 12, 13].

Thus, the present study aimed to evaluate the potential risk of developing CVD in patients HS and HSS across lipid profile, Castelli's index, Apo A-I, Apo B, Apo B/Apo A-I ratio and LDL-ox.

2. Methods

2.1. Ethical Statement

The whole study was planned and executed following the Ethical Guidelines of the Helsinki Declaration. A written informed consent was obtained from all participants after a full 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. Ethical approval for all procedures was obtained from the committees on the ethics of human research of Center for Health Sciences of *Universidade Federal de Pernambuco* (Protocol No. 359/09).

2.2. Study subjects

A hundred eighten individuals with chronic schistosomiasis guided by ultrasound service of the gastroenterology outpatient at the *Hospital das Clínicas* of *Universidade Federal de Pernambuco* (HC-UFPE) enrolled this study. All patients were previously characterized on the basis of schistosomiasis forms. Forty eight healthy individuals with the same socioeconomic background, without epidemiological history compatible with schistosomiasis and three negative stool examinations. We considered subjects excluded from the study if they presented the following clinical conditions: Hepatitis B or C virus infection, diabetes mellitus diagnosed, chronic kidney disease, thyroid dysfunction or cancer. Individuals who were taking lipid-lowering drugs anytime within the past year were also excluded.

2.3. Processing of samples and measurement of biochemistry parameters

Blood samples were collected into tubes with EDTA (1mg/ml) and the plasma was obtained after centrifugation (Sorvall, USA) at 1500 x*g* for 15 minutes**.** Plasma concentrations of total cholesterol, triglycerides and HDL-cholesterol were determined by enzymatic-colorimetric methods (Roche-USA), while plasma levels Apolipoprotein A-I and Apolipoprotein B were determined by immuneturbidimetric assay (Roche-

USA), by using the Cobas c501 (Roche-USA). Plasma concentration of LDL-cholesterol and VLDL-cholesterol was calculated by the Friedewald's equation (LDL-c = TC – HDL-c – (TG/5). The Castelli's index was accessed by Castelli & Framinghan (1983): TC/HDL-c (Castelli's index I) and LDL-c/HDL-c (Castelli's index II) [10]. Levels of LDL-ox were obtained by Enzyme-Linked Immunosorbent Assay – ELISA – Mercodia (Sweden) [13].

In order to access liver function, aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) and albumin plasma levels were quantified by colorimetric assays.

2.4 Statistical analysis

Results were expressed as mean ± Standard Error Mean (S.E.M.). The differences among groups were obtained by analysis of variance (ANOVA). Statistical significance for all comparisons was assigned at $P<0.05$, and all tests were performed using Statview for Windows (EUA, 1998).

3. Results

From the total of 118 infected individuals, 62 (33 men/29 women) presented HS form and 56 (30 men/26 women) were characterized HSS with age mean 48±8 years. The control group presented 48 healthy individuals (26 men/22 women) with age mean 44±6 years. No significant differences by gender or age among groups were observed.

HS and HSS patients presented increased hepatic enzymes values and decreased levels of albumin, when compared control group (Table 01). Plasma levels of TC, LDL-c, HDL-c, TG, VLDL-c (Figure 01), Apo A-I and Apo B (Figure 02) were significantly lower in the HS and HSS when compared to control group. Otherwise, infected individuals showed significant high levels of LDL-ox (Figure 03). There were no significant differences in the values of the Apo B/Apo AI ratio (Figure 02) or Castelli index among groups (Figure 04).

4. Discussion

Infection states, mainly when cause hepatic damage, are relationship with metabolic disorders, in special to lipids metabolism [14]. Ours patients presented several alterations in the lipid profile and in hepatic parameters used for liver function monitoring.

The high levels of the hepatic enzymes and lower levels serum albumin presented by ours infected patients showed existence of hepatic damage and impairment of synthesis liver function respectively. These findings were also observed in other study with schistosomiasis patients and attributed this fact to aggression liver due to granulomatous reaction and subsequent liver fibrosis [15].

In the infected individuals were observed lower levels of TC and LDL-c similar to those observed in other studies [16, 17]. The decreased on the plasma concentrations of TC and LDL-c in these subjects may be related to various factors, such as the fact of *S. mansoni* be unable to synthesize cholesterol, getting it by endocytosis of LDL molecules, through surface receptors expressed by parasite [18, 19, 20]; decrease in activity of the LCAT resulted in a decrease in the circulation of lipids [04] and synthesis of natural antibodies to cholesterol in chronic inflammatory reactions, which are related to the metabolism of cholesterol by opsonization [21, 22]. Studies also report that during the course of chronic schistosomiasis occurs an increase in the population of macrophages and altering function to phagocytosis native LDL [23].

Despite schistosomiasis promote decreased levels of TC and LDL-c, is still controversial role of these reductions as a protective factor for atherosclerosis in these individuals. Work using Apo E deficient mice, infected by cercariae and getting fat diet showed a reduction of atherosclerotic plaque when the group bought uninfected that getting same diet [05]. On the other hand, another similar experiment with Apo E knockout mice and fed with a diet hyperlipidemic, but being infected by percutaneous application of eggs of *S. mansoni* weekly observed that infected individuals had atherosclerotic plaques similar to uninfected individuals [07].

Furthermore our infected patients showed diminished level of HDL-c that is considered atheroprotective factors. Others studies also were related the HDL-c reduction in

infection by *S. mansoni* in your study using primates of the species *Callithrix jacchus* as an experimental model by S. mansoni similar to humans. The author reported that the decreased levels of HDL-c may be related to reduce activity of LCAT [04]. Others works also reported that this reduction may be relationship with an overexpression of the enzyme phospholipase A2, which is responsible for increasing the catabolism of HDL particles from circulating in infection and inflammation states [24, 25].

In addition, the infected subject showed high LDL-ox plasma levels. In a study with animal model, has observed that animals submitted to inflammatory stimuli have high levels of LDL-ox in plasma and would be a response from the host organism [26]. The chemical and structural alteration present in the molecule of LDL-ox stimulates the phagocytic activity of macrophages, resulting in the formation of foam cells that trigger the atherosclerotic process. This makes the LDL-ox an independent risk factor to develop atherosclerosis [27].

The plasma levels of Apo A-I, Apo B and values Apo B/Apo A-I ratio had being with markers to evaluate risk of develop CVDs [28]. The Apo A-I, major component of HDL-c is considered a atheroprotective factor while Apo B is a major component of VLDL-c, IDL and LDL-c an atherogenic factor [29, 30].

In this study the infected patients presented decrease levels of both apolipoprotein. These finding that can be associated with decreased hepatic synthetic function observed in these patients. Furthermore, studies in subjects with severe hepatic disease noted that they had reduced levels of Apo B and these values are negatively correlated with the severity of steatosis presented [31]. Other work reported that in fibrotic disease occurs inhibition of triglycerides synthesis and accumulation of fatty free acids and consequent increased fibrosis [32]. In addition, the infected individuals presented a decreased in the hepatic synthesis function, demonstrated by lower levels of albumin. The liver is main site of Apo A-I and Apo B synthesis [33].

Thus, these results suggest that even presented lower levels of TC, LDL-c, VLDL-c and Apo B ours infected patients presented a potential risk for CVDs due mainly to high levels of current LDL-ox, an independent risk factor to develop atherosclerosis. Including these alterations observed in the lipid profile and Apo A-I and Apo B plasma

levels seems reduced the applicability of the ambulatory index to characterize predisposition to CVDs in these individuals. Thus this study opens a new perspective for investigation the development of CVDs in carriers infectious chronic diseases suggesting the existence of mechanisms independent to qualitative alterations of plasma lipids and lipoproteins.

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Table 01 Liver functions tests in HS Schistosomiasis patients and controls subjects.

Characteristics	Controls	Hepatosplenic Patients	
		Hepatosplenic	Hepatosplenic Splenectomized
Subjects (N)	30	30	30
AST (U/L)	22.7±1.6	54.5±7.8*	55.5±4.6*
ALT (U/L)	10.6±3.2	35.9±6.4*	49.7±6.1*
ALP (U/L)	70.7±5.1	150.9±14.4**	138.2±21.4*
GGT (U/L)	56.6±10.3	288.4±50.1**	130.5±21.1**
Albumin (g/dL)	4.61±0.08	4.01±0.11**	4.21±0.17*

Values expressed as mean±Standard error (SE). One-way ANOVA followed by Fisher's PLSD post test. *p<0.05, **p<0.01 *vs* Control.

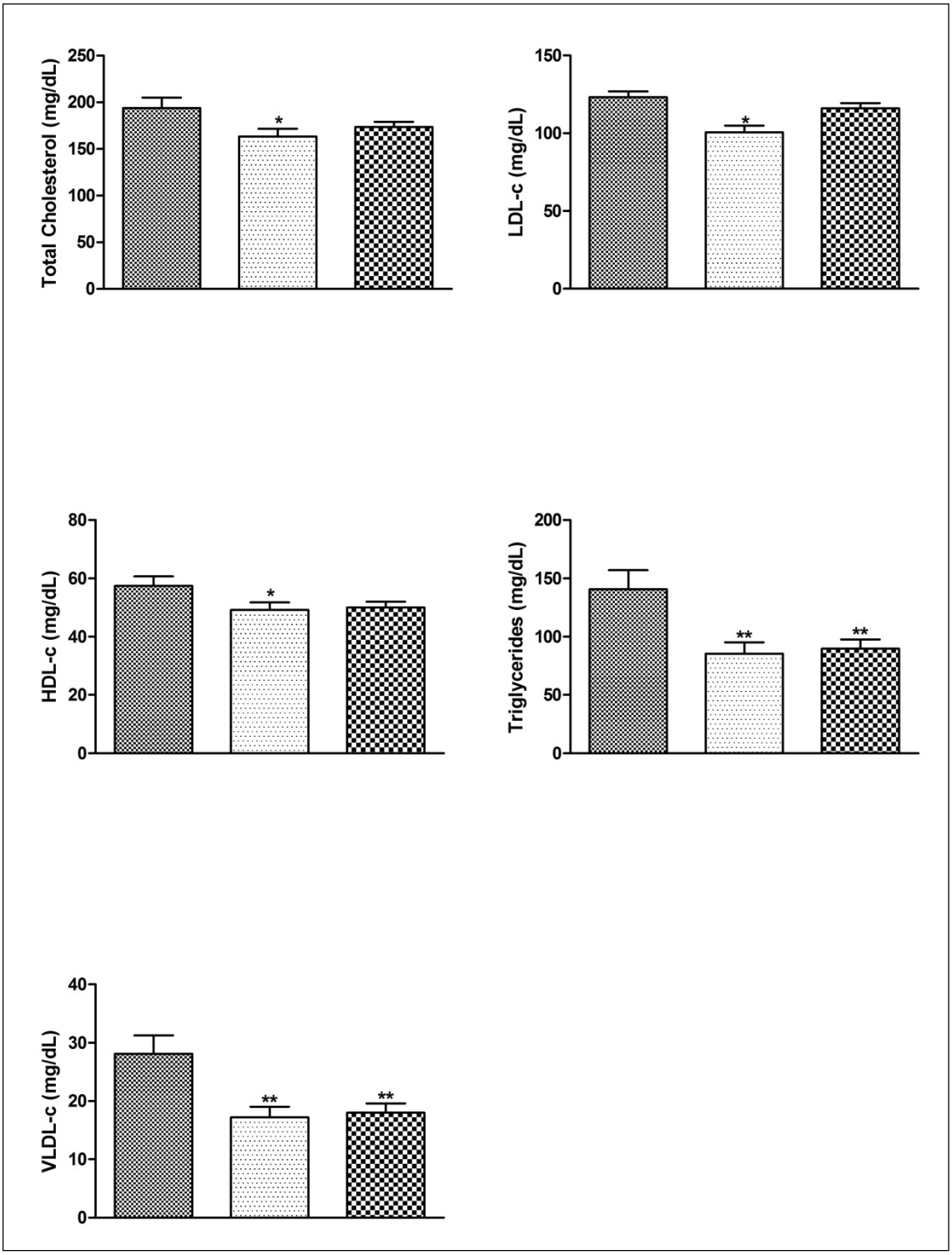

Figure 01. Plasma Levels of TC, LDL-c, HDL-c, Triglycerides, VLDL-c of the groups. Values expressed as mean±Standard error (SE). One-way ANOVA followed by Fisher's PLSD post test. *$p<0.05$, **$p<0.01$ *vs* Control.

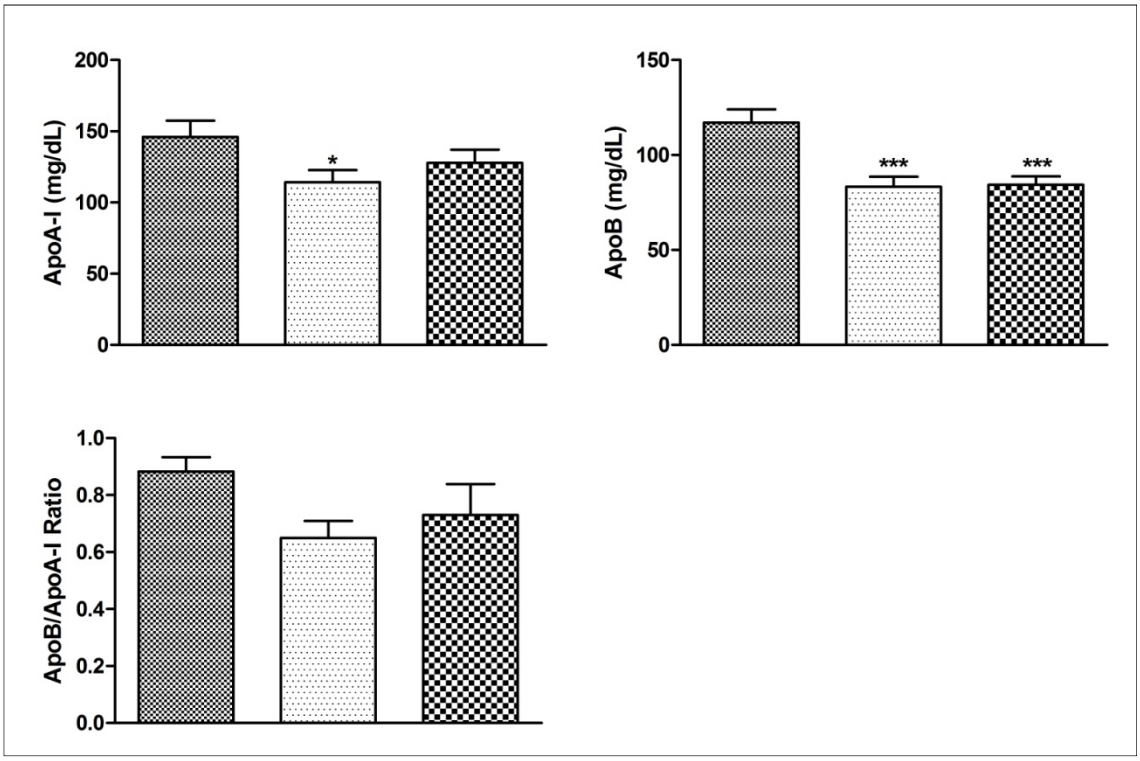

Figure 02. Plasma Levels of Apo A-I, Apo B and values of Apo B/Apo A-I ratio of the groups. Values expressed as mean±Standard error (SE). One-way ANOVA followed by Fisher's PLSD post test. *$p<0.05$ ***$p<0.001$ *vs* Control.

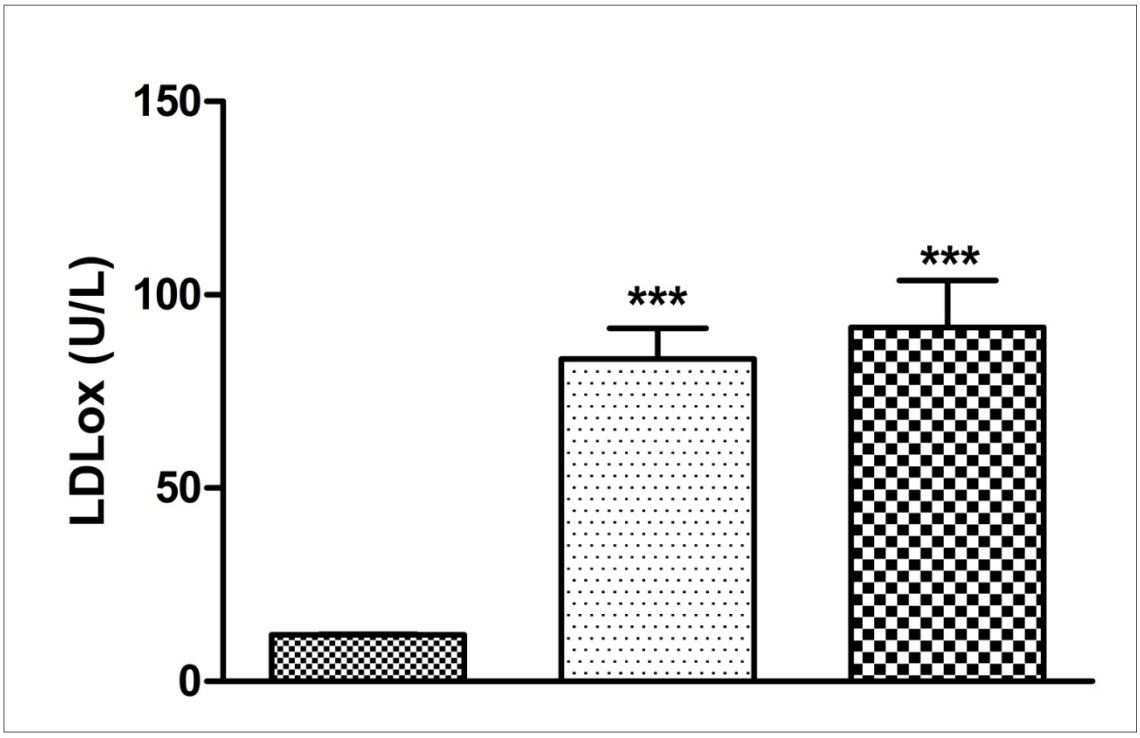

Figure 03. Plasma Levels of LDL-ox of the groups. Values expressed as mean±Standard error (SE). One-way ANOVA followed by Fisher's PLSD post test. ***p<0.001 *vs* Control.

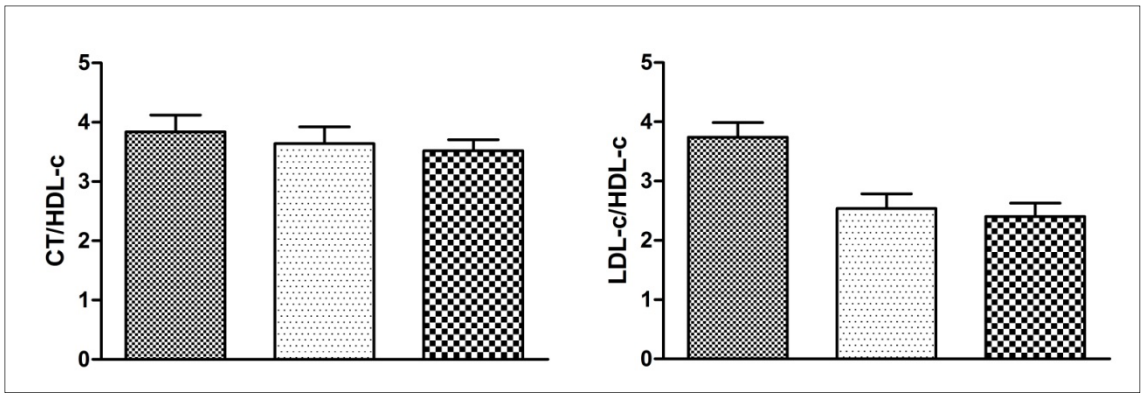

Figure 04. Values of TC/HDL-c (Castelli's index I) and LDL-c/HDL-c (Castelli's index II) there was no significance between groups.

5.2 Capítulo II: Artigo a ser submetido.

Hepatosplenic Mansonic Schistosomiasis, With Or Without Splenectomy, Is Related To Insulin Resistance, Despite Nonexistent Or Negative Correlations With Triglycerides and With The Lipid Ratio Triglycerides/HDL-Cholesterol.

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Insulin Resistance & *Schistosoma mansoni*

Key words: Hepatosplenic Mansonic Schistosomiasis, Insulin Resistance, Absence of Dyslipidemia, Lipid Ratios

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Abstract

Context. Schistosomiasis is a parasitic disease that causes serious injuries to the liver, and these may be associated with alterations in insulin and glycemic metabolism, even in absence of dyslipidemia feature of the state of insulin resistance. **Objective:** The main objective was to investigate insulin resistance in individuals with hepatosplenic mansonic schistosomiasis, splenectomised and non-splenectomised. **Design, Setting, and Participants:** A cross-sectional study was conducted in 129 patients with hepatosplenic mansonic schistosomiasis, from *Hospital das Clínicas*, Brazil, characterized by ultrasonography, subdivided into two groups – 83 Hepatosplenic (HS), and 46 Hepatosplenic Splenectomised (HSS) – plus 111 control individuals, in 2011-2012. **Main Outcome Measures:** Total Cholesterol (TC), HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, Triglycerides (TG), fasting plasma glucose and insulin, Homeostasis Model Assessment Insulin Resistance – HOMA-IR, and lipid ratios (TC/HDL-cholesterol, LDL-cholesterol/HDL-cholesterol and TG/HDL-cholesterol) were determined. χ^2 test, ANOVA and Pearson's correlation ($p<0.05$) were used. **Results:** HS patients had lower levels of TC and LDL-cholesterol. However HSS patients presented concentrations of these lipids similar to those found in control group. HS and HSS presented higher levels of HDL-cholesterol and lower levels of TG and VLDL-cholesterol, as well as, the values of the lipid ratios. HS and HSS patients did not present correlation or presented negative correlations with TG, VLDL-cholesterol, and TG/HDL-cholesterol, respectively, different from that found in control subjects, which presented positive correlations expected to insulin resistance and lipids. **Conclusion:** Despite nonexistent or negative correlations with TG and with the lipid ratio TG/HDL-cholesterol, hyperinsulinemia/insulin resistance state was present in these hepatosplenic mansonic schistosomiasis patients.

Key words: Hepatosplenic Mansonic Schistosomiasis, Insulin Resistance, Absence of Dyslipidemia, Lipid Ratios

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 noncommunicable 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, like the genus *Schistosoma*; 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).

Mansonic schistosomiasis 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 become to be 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 in mansonic schistosomiasis patients 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 feature with increase in triglycerides and decrease in HDL-cholesterol levels (11-14). It was suggested that infection with *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 rural Chinese (14, 15). On the other hand, it was 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, on the presence of treatment, if the patient was splenectomized or not, and

also depending on the population studied, and all this combined with the damage that hyperinsulinemia/insulin resistance may cause plus the lack of studies on the subject, it is essential that the possible dysfunction in glucose metabolism and insulinemic profile continues being evaluated in the chronic liver disease – schistosomiasis.

Therefore the present study aimed to investigate insulin resistance in patients with hepatosplenic mansonic schistosomiasis, splenectomised and non-splenectomised, 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, that 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. 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 committees on the ethics of human research of Center for Health Sciences of the *Universidade Federal de Pernambuco* (Protocol Nº. 359/09).

Study subjects

A hundred twenty nine patients that presented the HS form of mansonic schistosomiasis attended into the Gastroenterology Outpatient at the *Hospital das Clínicas* of the *Universidade Federal de Pernambuco* were recruited from 2011 to 2012. These patients were divided into two groups – HS without splenectomy surgery (n = 83; 34.6% of the total of participants); and HSS patients who suffered 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%) healthy 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 biochemichal parameters

Blood samples were collected into vacuum tubes in aseptic conditions (Vacutainer; Becton Dickinson, USA). The first tube contained EDTA-K3 (1 mg/mL) at a 1:9 ratio using 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 1500 xg for 15 minutes and the all samples were stored in 0.5 ml aliquots at -80º C.

Plasma concentrations of insulin were obtained 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, 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 χ^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 mansonic schistosomiasis 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 mansonic schistosomiasis than in healthy 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 mansonic schistosomiasis, 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 mansonic schistosomiasis, 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 healthy 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 mansonic schistosomiasis. 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 mansonic schistosomiasis, 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 mansonic schistosomiasis 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 mansonic schistosomiasis, even without changes in the glucose levels, may have insulin resistance despite the absence of dyslipidemia feature. We found that the patients with mansonic schistosomiasis 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 splenectomised 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 mansonic schistosomiasis, as suggested by Pimenta et al. (2003) (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, splenectomised 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 mansonic schistosomiasis, splenectomised or non-splenectomised, 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.

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

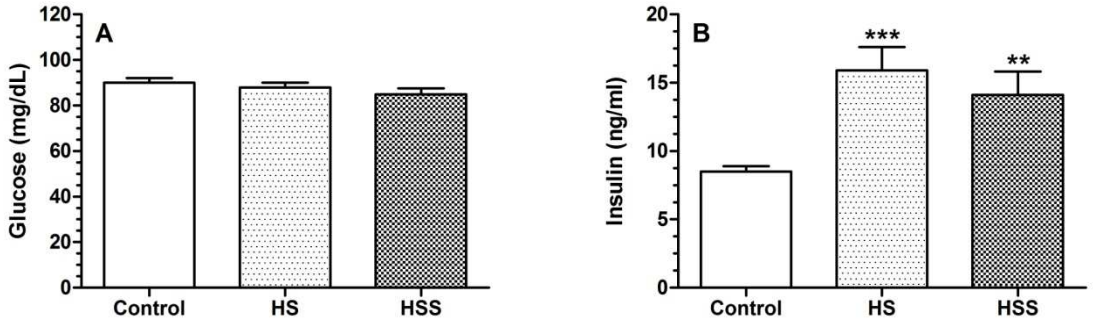

FIGURE 1. Plasma Levels of Glucose (A) and Insulin (B) in Individuals with Hepatosplenic form of Mansonic Schistosomiasis, Non- splenectomized (HS) and Splenectomized (HSS), and in Healthy Controls. Values expressed as mean±Standard error (SE). One-way ANOVA followed by Fisher's PLSD post test. **p≤0.001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

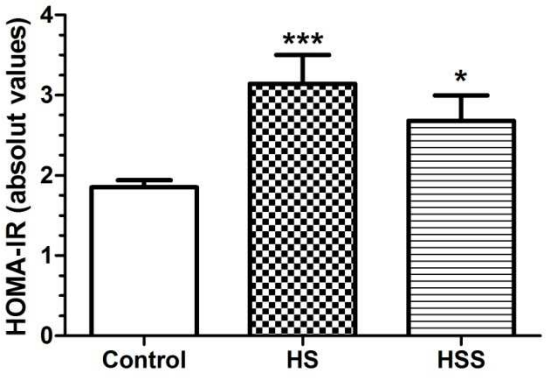

FIGURE 2. Values of the Homeostatic Model Assesment – Insulin Resistance (HOMA-IR) in Patients with Hepatosplenic Form of Mansonic Schistosomiasis, Non- splenectomized (HS) and Splenectomized (HSS), and in Healthy Controls. Values expressed as mean±Standard error (SE). One-way ANOVA followed by Fisher's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

TABLE 3. Correlation Between Insulin Resistance (HOME-IR Value and Serum Lipid Concentrations and Values of Lipid Ratios, in Patients with Hepatosplenic Form of the Mansonic Schistosomiasis, Non-splenectomized (HS) and Splenectomized (HSS), and in Healthy Controls.

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

Abbreviations: TC, Total Cholesterol; TG, Triglycerides. Pearson's Correlation.

5.3 Capítulo III: Artigo a ser submetido.

Human Plasma Lipids Modulation by *Schistosoma mansoni* Depends on Apolipoprotein E Polymorphism

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Abstract

Background: Schistosomiasis mansoni is a chronic liver disease which is related to some metabolic disturbances. Apolipoprotein E polymorphism has been reported to modulate lipid metabolism. The aim of this study was to evaluate the influence of APOE polymorphism on the lipid metabolism of schistosomiasis mansoni patients.

Methodology/Principal Findings: Blood samples were used for measurement of lipid parameters (Total Cholesterol, LDL-c, HDL-c and Triglycerides) and for APOE genotyping. APOE alleles frequency were similar between healthy (n=108) and schistosomiasis (n=84) individuals (p=0.3568). Schistosomiasis patients showed reduced levels of Total Cholesterol (25%), LDL-c (38%) and Triglycerides (32%) amid healthy individuals. However, among schistosomiasis patients, only ε2 and ε4 alleles account for such variation. Otherwise, HDL-c levels were significantly increased in schistosomiasis (10%) compared with control subjects, but it was caused only by the ε2 and ε4 carriers, not the ε3 carriers.

Conclusion/Significance: Plasma lipid modifications during schistosomiasis occur in a different manner according to APOE alleles. These findings open the way to identification of new metabolic pathways and molecular targets of treatment for morbidities associated to schistosomiasis and other lipid associated diseases, as cardiovascular disease and diabetes.

Short title: APOE Polymorphism in Schistosomiasis mansoni.

Keywords: Schistosomiasis, ApoE Polymorphism, Cholesterol, Triglycerides.

Author Summary

Schistosomiasis is a liver parasitic disease and affects lipid human metabolism. Our study aim was to evaluate the influence of APOE polymorphism on the lipid metabolism of schistosomiasis mansoni patients. We observed by analyzing blood samples and by APOE genotyping of 84 patients that relevant differences in the manner how hepatosplenic schistosomiasis affects plasma cholesterol and triglycerides levels are associated to APOE polymorphism. Our findings open the way to identification of new metabolic pathways and molecular targets of treatment for morbidities associated to schistosomiasis and other lipid associated diseases, as cardiovascular disease and diabetes.

Introduction

Schistosomiasis, caused by *Schistosoma mansoni* worms, is one of the most prevalent parasitic diseases. More than 200 million people are infected and at least 280,000 people dies because of schistosomiasis every year worldwide, mostly in developing countries (Van der Werf, 2003; Gryseels, 2012). *S. mansoni* causes severe hepatic fibrosis associated with portal blood hypertension. Around 5-7% of patients progress to the most severe form, hepatosplenic.

It is generally reported that schistosomiasis causes reduced plasma levels of cholesterol and triglycerides (Gillet, 1989; Doenhoff, 2002; Ramos, 2004). On the other hand, it is also reported that such reduction of cholesterol levels has no effect on atherosclerosis development (La Flamme, 2007). Intrinsic aspects of the host may be related to these different observations.

One possible factor that might affect host lipid metabolism is Apolipoprotein E (APOE, gene; ApoE, protein) polymorphism. There are three major alleles, ε2, ε3 and ε4, which correspond to three main protein isoforms, ApoE2, 3, and 4. ApoE3 has a cysteine residue at position 112 and an arginine residue at position 158, while the ApoE2 contains two cysteines and ApoE4 contains two arginines, at both sites (Papaioannou, Simons and Owen, 2012).

ApoE is widely distributed among lipoproteins in which orchestrate the regulation of cholesterol and triglycerides plasma levels variation and is involved in other functions not directly related to its role in lipid metabolism. APOE polymorphism is also related to influence susceptibility or severity of some infections (Wozniak et al, 2003; Tursen et al, 2004; Burt et al, 2008; Wang et al, 2009). However, it remains unclear whether the lipid modification induced by schistosomiasis is linked to the lipid modulation caused by APOE different alleles. Thus, the aim of this study was to evaluate the influence of APOE polymorphism on the lipid metabolism of schistosomiasis mansoni patients.

Methods

Ethical Statement

The whole study was planned and executed following the Ethical Guidelines of the Helsinki Declaration. 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, and signed an informed consent statement before inclusion in the study. Ethical approval for all procedures was obtained from the committees on the ethics of human research of Center for Health Sciences of UFPE (Protocol No. 359/09).

Study Area and Subjects

Eighty-four patients attended into the Gastroenterology Outpatient at the *Hospital das Clínicas* of Federal University of Pernambuco (UFPE) were recruited from 2009 to 2010. The control group was formed by 108 healthy individuals of the same age groups (18 to 65 years) from the same socioeconomic background without epidemiological history compatible with schistosomiasis and three negative stool examinations. We considered subjects excluded from the study if they presented the following clinical conditions: Hepatitis B or C virus infection, chronic kidney disease, thyroid dysfunction or cancer. Individuals who were taking lipid-lowering drugs anytime within the past year were also excluded.

All the volunteers lived in *Zona da Mata*, an endemic area in the state of *Pernambuco*, in the northeastern region of Brazil. Hepatosplenic Schistosomiasis was evaluated by physical examination and upper abdominal ultrasound, conducted by a qualified and experienced examiner, according to the WHO protocol for ultrasound assessment of schistosomiasis-related morbidity (Niamey Working Group, 2000). The hepatosplenic schistosomiasis patients (SM) had typical hepatosplenomegaly and were treated with praziquantel (50mg/Kg) prior to the study.

Sample collection and Processing

Venous blood samples were drawn into evacuated tubes containing EDTA (0.562M) after a 12-h fasting period. Plasma was separated within 2 h after collection by centrifugation at 1500x*g* (10 min at 4 °C), stored at -20°C and used for lipid analyzes within 24 h. Whole blood samples were stored at 2-8ºC and used for genotyping analysis within 7 days.

Biochemical Measurement

Plasma total cholesterol (TC) and triglyceride (TG) concentrations were assayed by routine enzymatic methods. Cholesterol of high density lipoprotein (HDL-c) was measured also using enzymatic method after precipitation of the plasma with phosphotungstic acid in the presence of magnesium ions. Cholesterol of low density lipoprotein (LDL-c) was calculated by the Friedewald formula in patients whose TG levels were $\leq$ 400 mg/dL (Santos et al, 2009).

Examination of APOE genotype

Genomic DNA was extracted from leukocytes in sample of whole blood, following a standard salting-out technique (Miller, Dykes & Polesky, 1998). Genotypes for APOE polymorphisms (rs7412 and rs429358) were detected by polymerase chain reaction (PCR) (Kim et al, 2010). The amplified fragments were then digested with the enzyme HhaI (5 Units) for three hours and the restriction fragments were identified with 4% agarose gel electrophoresis and ethidium bromide staining (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

Unpaired t-test or one-way ANOVA followed by Fisher's Protected Least Significant Difference (PLSD), when concerning to APOE allele groups, were used to compare differences among continuous variables of SM and control individuals. χ^2 goodness-of-fit test was used to test the deviation from Hardy-Weinberg equilibrium for each polymorphism and χ^2 test to compare categorical parameters among groups. Correlation Z test was used to estimate de 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 analysis were performed using Statview SAS Inc. (1998, NC, USA).

To evaluate the effect of APOE genotype schistosomiasis mansoni infection, 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 analysis because of the putative opposite effects of these two alleles.

Results

In this cross-sectional study, the two groups were matched by age and gender, as shown in Table 1. The frequency of APOE alleles among participants were: ε2 – 11.46%, ε3 – 71.35%, and ε4 – 17.19%. All SNPs were found to be in accordance with Hardy-Weinberg equilibrium for both SM (χ^2 = 3.4164, ϕ=3, p=0.3318) and control (χ^2 = 3.2518, ϕ=3, p=0,3544) subjects. The allele frequencies were not statistically different between healthy and SM groups (p=0.3568), indicating that APOE polymorphism was not able to affect the chance of schistosomiasis infection in this population.

Overall, the SM patients showed significantly ($p<0.05$) differences among the lipid markers of cardiovascular disease risk compared to the healthy controls with reduced levels of TC (25%), LDL-c (38%) and TG (32%) amid SM individuals. Otherwise, HDL-c levels were significantly increased in SM (10%) compared with control subjects (**Table 1**).

In order to assay the APOE polymorphism influence on lipid parameters, we performed the analysis differentiating them by APOE alleles. Lower TC and LDL-c levels were founded in the ε3/ε3 genotype of SM individuals, as observed without allele differentiation (**Figure 1A-B**). However, those disturbs founded for TG and HDL-c among overall SM patients has not been found after allele differentiation (**Figure 1C-D**). The ε3/ε3 genotype analysis allows us to access the influence of schistosomiasis to lipid metabolism without the possible interfering factors from the inclusion of ε2 and ε4 alleles in the evaluation.

Despite the decrease observed on TC level of ε3 SM group, both ε2 groups showed similar TC values, which may be correlated with plasma HDL-c markedly increase (77%), but not LDL-c, in SM group. Since a positive correlation between TC and HDL-c (R=0.724; p=0.0250) but not between TC and LDL-c (R=0.225; p=0.5750) among ε2 SM carriers were observed. We further observed that, among SM subjects, ε2-carriers showed increased TC levels than ε3-carriers. Unlike ε3 SM group, ε2 carriers showed slightly significant decreased TG levels.

The lipid improving effect caused by schistosomiasis persisted on ε4 healthy allele carriers. Although there has been no significant difference on TG and HDL-c levels of ε3-carriers, they occurred on ε4 SM individuals. This group showed increased HDL-c (39%) and reduced TC, LDL-c, and TG (65%) levels compared with healthy controls.

Discussion

The report of a host genetic factor influencing lipid parameters brings considerable progress to our understanding of what are the pathways of lipid metabolism modification induced by schistosomiasis mansoni and how host background can influence such progress.

Several studies have shown ApoE influence on infection susceptibility and damage in the case of certain diseases caused by bacteria (Wang et al, 2009), viruses (Burt et al, 2008), protozoa (Wozniak et al, 2003) and fungi (Tursen et al, 2004). Our data suggest that ApoE does not affect susceptibility to schistosomiasis infection, at least in hepatosplenic subjects. Gene studies with schistosomiasis infected individuals found significant association of some cytokines related to immune response against infection. However, resembling to our results, none of them observed relation between APOE gene locus and overall schistosomiasis (Marquet et al, 1996; Dessein et al, 1999).

Schistosomiasis infection was able to modify plasma lipid parameters in a manner similar to cardioprotective one. Decreased plasma cholesterol levels were also shown in previous studies from our and others laboratories using animal models (Lima et al, 1998; Ramos et al, 2004; Doenhoff et al, 2002; La Flamme et al, 2007; Stanley et al, 2009) and with humans (Silva et al, 2002). Doenhoff et al, 2002 associated decreased cholesterol levels with atheroma reduction in APOE-knockout mice. Despite them, La Flamme et al, 2007 observed that chronic exposure to schistosome eggs does not induce reduction of atherosclerotic lesion in APOE-knockout mice although there was cholesterol decrease.

Schistosomiasis may induce cholesterol reduction by an interaction of the LDL with the *S. mansoni* tegument, as observed by Tempone et al, (1997), suggesting a possible mechanism of LDL internalization and cholesterol remove from host serum by adult worms via proteins similar to LDLR. Otherwise, infection with worms of same sex was not able to induce cholesterol lowering in mice (Stanley et al, 2009), indicating that adult worms alone are not responsible for decreasing cholesterol levels and eggs are needed to induce such effect. Schistosomiasis may also provoke cholesterol reduction through egg-induced antibodies production against lipid epitopes carried by lipoproteins, leading them to endocytosis by neutrophils (Sprong et al, 2006). Similarly

to Stanley et al, 2009, we observed TC and LDL-c reduction although SM patients had been previously treated.

Besides TC and LDL-c levels reduction, we observed increased HDL-c levels. Previous studies with mice observed HDL-c reduction or no significant variation (Doenhoff, et al, 2002; La Flamme et al, 2007). There are some differences regarding the content of lipids into lipoproteins between humans and rodents. Humans exhibit LDL-c levels in a higher proportion of TC than mice and rats (Lima et al, 1998). In addition, acute infections are reported to inhibit ApoA-I synthesis and provoke low HDL-c levels (Pruzanski et al, 2000) similar to observed in those studies. This process has not been observed in states of chronic inflammation and fibrosis as SM patients in this study.

On the other hand, early studies showed that in chronic inflammation states, as some forms of arthritis or chronic renal disease, the reverse cholesterol transport and the antioxidant properties of HDL are impaired (Natarajan, Ray and Cannon, 2010; Saemann et al., 2010). Although patients studied here do not exhibit active infection, HDL dysfunction may be occurring due to oxidation. We had previously observed elevated levels of erythrocyte lipid peroxidation in chronic schistosomiasis patients treated before the study (Facundo et al, 2004).

SM patients also showed decreased TG levels. Ramos et al, 2002 also observed reduced TG plasma levels in primates reinfected by *S. mansoni*. Such effect may be caused by diminished hepatic expression of acetyl coenzyme A acyltransferase, one of the enzymes participants of fatty acid synthesis, as observed by Harvie et al, 2007 in *S. mansoni* infected mice. This effect may be occurring even in absence of active infection, non-infectious associated pulmonary fibrosis was reported to cause reduced plasma levels of TG (Ianello et al, 2002).

In humans, APOE polymorphism has been reported to be responsible for plasma cholesterol levels variation around 10%. Dissimilarities among alleles receptor affinities have been accounted for such variation. ApoE4 shows slightly greater receptor-binding capability than ApoE3, but ApoE2 has only 2% and 40% binding activity to the LDLR and LRP, respectively (Papaioannou, Simons and Owen, 2012). We observed relevant differences, regarding to APOE polymorphism, in the manner how hepatosplenic schistosomiasis affects plasma cholesterol and triglycerides levels.

The SM patients showed increased HDL-c levels, but it was caused only by the ε2 and ε4 carriers, not the ε3 carriers. The putative inhibitory effect of schistosomiasis on reverse cholesterol transport seems to only produces significantly effects on HDL-c levels among ε2 and ε4 carriers. Additionally, Hirata et al, 2012 observed higher HDL-c levels in transgenic mice carrying CETP after inhibition of this enzyme, such effect was positively related to the presence of ApoE on HDL particles. Since ApoE2 have higher affinity to HDL particles (Mahley, Weisgraber and Huang, 2009) and decreased activity of CETP has been related in acute inflammation states (Khovidhunkit et al, 2004), it is possible that exist decreased activity of CETP in chronic inflammation state observed in schistosomiasis patients, which may be related to high HDL-c values. In addition, the ApoE2 have recognized low affinity to LDLR, it is possible that the presence of ApoE2 on HDL may influence the cholesterol uptake by liver.

On the other hand, ApoE4 shows low affinity to HDL and high affinity to LDLR, features which refute in these individuals the putative mechanism of increased HDL-c levels reported to ε2 carriers. However, it has been reported that ApoE4 have lower antioxidant capability than the others isoforms (Miyata and Smith, 1996), which can lead to less lipoprotein protection against the oxidation state during the schistosomiasis. Oxidized HDL is related to decreased reverse cholesterol transport (Natarajan, Ray and Cannon, 2010; Saemann et al., 2010), which may cause increased HDL-c levels.

SM ε4 carriers showed lower TG plasma concentrations than healthy controls. Actually, there was no increase in TG levels in SM group as occurs to healthy individuals. Previous studies reported higher levels of TG among healthy ε4 carriers (Almeida et al, 2006; Carvalho-Wells et al, 2010). Such fact lead us to believe that the mechanism by which ApoE4 causes increased Tg levels is not present in schistosomiasis patients. The physiological basis of it remains relatively unknown, but recent studies observed that reduced ApoE hepatic recycling may be involved. ε4 carriers show lower ApoE mediated endocytosis and associated higher circulating TG levels (Heeren et al, 2004; Heeren, Beisiegel and Grewal, 2006).

In summary, our study points for the first time that plasma lipid modifications during schistosomiasis occur in a different manner according to APOE alleles. These findings open the way to identification of new metabolic pathways, new pathological processes and, additionally, new molecular targets of treatment of morbidities associated to schistosomiasis and other lipid associated diseases, as cardiovascular disease and diabetes that will enable better life quality to hundreds of millions of people worldwide.

Author Contributions

Fonseca CSM, Silva AC and Lima VLM designed the study protocol; Pimenta Filho AA and Domingues ALC carried out patient assessment; Fonseca CSM and Pimenta Filho AA carried out genotype and biochemical assays and were involved in statistical analysis and interpretation of all the data; Fonseca CSM, Pimenta Filho AA, Santos BS, Silva AC, Domingues ALC, Owen JS 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

General, Genotype and Lipid parameters of participants according to group.

Parameters*	Control	SM	*p*-value
Age (years)	47.0 ± 3.2	55.0 ±2.3	0.0541
Female sex	78	63	0.7309
N total	108	84	-
ε2	14 (13.0)	8 (9.5)	-
ε3	73 (67.6)	64 (76.2)	-
ε4	21 (19.4)	12 (14.3)	-
TC	194.4 ± 4.5	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	<0.0001

SM, Hepatosplenic schistosomiasis 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 (relative) frequencies and were compared by the Chi-square test. *Plasma Lipids were expressed in mg/dL.

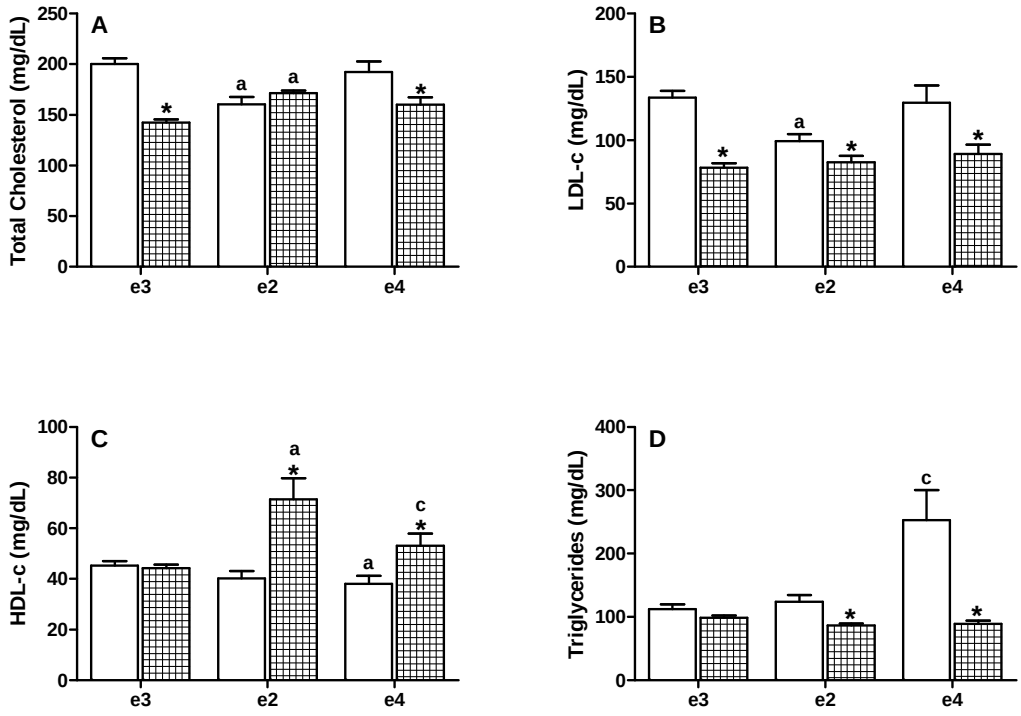

Figure 1 Effect of APOE polymorphism on levels of circulating Total Cholesterol (A), LDL-c (B), HDL-c (C), and Tg (D) in Control (▭) and SM (▦) individuals. ***=*p*≤0,05 *vs.* Control of the same allele; **a** = *p*≤0,05 *vs.* ε3 of same group; **b** = *p*≤0,05 *vs.* ε2 of same group; **c** = *p*≤0,05 *vs.* ε2 and ε3 of same group.

6 Conclusão

- Contrastando com a diminuição dos níveis colesterol total e LDL-c, a esquistossomose promove aumento dos níveis de LDL-oxidada, um fator de independente para desenvolvimento de DCV.

- Esquistossomose crônica pode ser um fator predisponente para resistência insulínica/hiperinsulinemia nos indivíduos infectados.

- As alterações lipídicas ocorridas durante a esquistossomose estão relacionadas com o polimorfismo da Apo E.

7 ANEXOS

Normas para redação de artigos para a revista "Clinica Chimica Acta - CCA" a ser submetido o artigo I.

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- **Clinical Case Seminars** are descriptions of a case or small number of cases revealing novel and important insights into a condition's pathogenesis, presentation, and/or management. The case report is to be accompanied by a concise scholarly review of the literature regarding relevant aspects of the disorder. These manuscripts should be 2400 words or less, with no more than four figures and tables and 30 references.
- **Extensive Clinical Experiences** are learned descriptions of substantial clinical experience with a specific endocrine or metabolic disorder, or class of disorders, by a single clinical endocrinologist or facility. This experience should expose novel aspects of the condition's presentation, diagnosis, natural history, and/or treatment. These manuscripts should be no longer than 3600 words and include no more than four figures and tables and 40 references.
- **Position** and **Consensus Statements** related to the endocrine and metabolic health standards and healthcare practices may be submitted by

professional societies, task forces, and other consortia. All such submissions will be subjected to peer review, must be modifiable in response to criticisms, and will be published only if they meet the Journal's usual editorial standards. These manuscripts should typically be no longer than 3600 words and include no more than six figures and tables and 120 references.

- **Controversies in Clinical Endocrinology** describe and justify different approaches to diagnosis and/or management of patients with an endocrine or metabolic condition. This feature typically consists of a pair of manuscripts authored by two individuals who thoughtfully describe their respective clinical perspectives on a problem, their related practices, and the rationale and evidence supporting them. The entire manuscript should be no longer than 2400 words and include no more than two figures and tables and 30 references.
- **Images in Endocrinology** are to be comprised of a single figure or two closely related figures that illustrate the value of visual information in clinical diagnosis of endocrine and metabolic disorders, with a caption that is 50 words or less, an accompanying commentary that is 250 words or less, and five or fewer references.
- **Commentaries** are essentially uninvited editorials, which should concisely address and take a well-reasoned position on a timely issue of importance to clinical endocrinologists and/or endocrine clinical investigators. These manuscripts should be no longer than 1200 words with no more than 10 references; no figures or tables are permitted.
- **Letters to the Editor** may be submitted in response to work that has been published in the Journal. **Letters**should be short commentaries related to specific points of agreement or disagreement with the published work.**Letters** are not intended for presentation of original data unrelated to a published article. **Letters** can only be submitted electronically via the Journal website, by clicking on the link entitled "Submit a Letter to the Editor" on the abstract page or the article itself. Letters should be no longer than 500 words with no more than five complete references, and may not include any figures or tables.

Manuscript Submission Procedures

JCEM only uses electronic manuscript submission at Editorial Manager (http://jcem.edmgr.com).

If this is your first submission to an Endocrine Society Journal, click on "Register Now" to create an author account. If you already have an account from a previous submission to any of The Endocrine Society's Journals, enter your username and password to submit a new or revised manuscript. If you have forgotten your username and/or password, e-mail the editorial office (sherman@endo-society.org) for assistance.

Note that your author account is the same for *JCEM*, *Endocrinology*, *Molecular Endocrinology*, and *Endocrine Reviews*. Authors should be aware that in submitting a manuscript for consideration by *JCEM*, they are submitting their paper to The

Endocrine Society Central Journals Office database, which is accessible by the Editors-in-Chief of all the Society's journals.

All submissions must include:

- A cover letter requesting that the manuscript be evaluated for publication in *JCEM* and any information relevant to your manuscript. Elsewhere on the submission form authors may suggest up to five specific reviewers and/or request the exclusion of up to three others.
- Assignment of Copyright and Disclosure of Potential Conflict of Interest is part of the online submission process. At the time of submission all co-authors will receive authorship verification emails to which they must respond. It is imperative that all co-authors are listed on the submission forms and their email address be correct.
- At least three key terms.
- Completed Disclosure Summary on the title page. For instructions on preparing the summary, see the following page (http://jcem.endojournals.org/site/author/RequiredForms.pdf).
- Authors are encouraged to submit a PDF for the initial submission. See the instructions on the JCEM homepage. If you do submit original files, Editorial Manager will create a PDF of your files, but it may take some time depending on the size of the files.

Manuscript Preparation

General Format

The Journal requires that all manuscripts be submitted in a single-column format that follows these guidelines:

- All text should be double-spaced with 1-inch margins on both sides using 11-point type in Times Roman font.
- All lines should be numbered throughout the entire manuscript and the entire document should be paginated.
- All tables and figures must be placed after the text and must be labeled. Submitted papers must be complete, including the title page, abstract, figures, and tables. Papers submitted without all of these components will be placed on hold until the manuscript is complete.
- Authors are encouraged to cite primary literature rather than review articles in order to give credit to those who have done the original work.
- Any supplemental data intended for publication must meet the same criteria for originality as the data presented in the manuscript.

Title Page

The title page should include the following:

- Full title (a concise statement of the article's major contents)
- Authors' names and institutions. At least one person must be listed as an author; no group authorship without a responsible party is allowed. A group can be listed in the authorship line, but only on behalf of a person or persons. All group members not listed in the authorship line must be listed in the Acknowledgments.
- Abbreviated title of not more than 40 characters for page headings
- At least three key terms for indexing and information retrieval
- Word count (excluding abstract, figure captions, and references)
- Corresponding author's e-mail and ground mail addresses, telephone and fax numbers
- Name and address of person to whom reprint requests should be addressed
- Any grants or fellowships supporting the writing of the paper
- Disclosure summary (see Disclosure of Potential Conflict of Interest form for instructions)
- Clinical Trial Registration Number, if applicable

Structured Abstracts

All Original Articles, Brief Reports, Clinical Reviews, Clinical Case Seminars, Consensus and Position Statements, Controversies in Endocrinology, and Extensive Clinical Experiences should be submitted with structured abstracts of no more than 250 words. All information reported in the abstract must appear in the manuscript. The abstract should not include references. Write the abstract with a general medical audience in mind. Please use complete sentences for all sections of the abstract. Detailed instructions on writing Structured Abstracts are at http://jcem.endojournals.org/site/misc/Structured_Abstracts.xhtml.

Introduction

The article should begin with a brief introductory statement that places the work to follow in historical perspective and explains its intent and significance.

Materials and Methods

These should be described and referenced in sufficient detail for other investigators to repeat the work. The source of hormones, unusual chemicals and reagents, and special pieces of apparatus should be stated. For modified methods, only the modifications need be described.

Results and Discussion

The Results section should briefly present the experimental data in text, tables, and/or figures. For details on preparation of tables and figures, see below. The Discussion should focus on the interpretation and significance of the findings with concise objective comments that describe their relation to other work in that area. The Discussion should not reiterate the Results.

Acknowledgments

The Acknowledgments section should include the names of those people who contributed to a study but did not meet the requirements for authorship. The corresponding author is responsible for informing each person listed in the acknowledgment section that they have been included and providing them with a description of their contribution so they know the activity for which they are considered responsible. Each person listed in the acknowledgments must give permission - in writing, if possible - for the use of his or her name. It is the responsibility of the corresponding author to collect this information.

References

References to the literature should be cited in numerical order (in parentheses) in the text and listed in the same numerical order at the end of the manuscript on a separate page or pages. The author is responsible for the accuracy of references. The number of references cited should be limited, as indicated above for each category of submission. Appropriate recent reviews should be cited whenever possible.

Examples of the reference style that should be used are given below. Further examples will be found in the articles describing the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (Ann Intern Med.1988; 108:258-265, Br Med J. 1988; 296:401-405). The titles of journals should be abbreviated according to the style used in the *Index Medicus*.

Journal articles and abstracts: List all authors. The citation of unpublished observations, of personal communications, and of manuscripts in preparation or submitted for publication is not permitted in the bibliography. Such citations should be inserted at appropriate places in the text, in parentheses and without serial number, or be presented in the footnotes. The citation of manuscripts accepted for publication but not yet in print is permitted in the bibliography provided the DOI (Digital Object Identifier) and the name of the journal in which they appear are supplied. Listing a manuscript as "in press" without a DOI and journal title is not permitted. If references to personal communications are made, authors are encouraged to keep written proof of the exchange. If it is necessary to cite an abstract because it contains substantive data not published elsewhere, it must be designated at the end of the reference [e.g., 68:313 (Abstract)].

Books: List all authors or editors.

Sample References

1. **Binoux M, Hossenlopp P** 1986 Insulin-like growth factor (IGF) and IGF-binding proteins: comparison of human serum and lymph. J Clin Endocrinol Metab 67:509-514
2. **MacLaughlin DT, Cigarros F, Donahoe PK** 1988 Mechanism of action of Mullerian inhibiting substance. Program of the 70th Annual Meeting of The Endocrine Society, New Orleans, LA, 1988, p 19
3. **Bonneville F, Cattin F, Dietemann J-L** 1986 Computed tomography of the pituitary gland. Heidelberg: Springer-Verlag; 15-16
4. **Burrow GN** 1987 The thyroid: nodules and neoplasia. In: Felig P, Baxter JD, Broadus AE, Frohman LA, eds. Endo crinology and metabolism. 2nd ed. New York: McGraw-Hill; 473-507

For general aid in the preparation of manuscripts, authors should consult: CBE Style Manual: A Guide for Authors, Editors and Publishers. 5th ed. Bethesda, MD: Council of Biology Editors; 1983.

Tables

Tables must be constructed as simply as possible and be intelligible without reference to the text. Each table must have a concise heading. A description of experimental conditions may appear together with footnotes at the foot of the table. Tables must not simply duplicate the text or figures. The width of the table must be designed to occupy one or two journal columns, with no more than four table columns or 8-10 table columns, respectively.

Figures and Legends

Please review the detailed instructions for preparing digital art at http://art.cadmus.com/da/index.jsp. E-mail queries can be sent todigitalart@cadmus.com. All figures must display the figure number.

Sizing the figure: The author is responsible for providing digital art that has been properly sized, cropped, and has adequate space between images. Plan the size of the figure to fill 1, 1.5, or 2 columns in the printed journal (see chart below for dimensions). In most cases, figures should be prepared for 1-column width. Produce original art at the size it should appear in the printed journal. (Note for PowerPoint users: The sizing instructions do not apply if you are submitting PowerPoint files for print production in Editorial Manager. On the submission page, check boxes to indicate that the figures are the correct size and resolution.)

1 column	=	18 picas,	7.5 cm,	3.0 in
1.5. columns	=	30 picas,	12.5 cm,	5.0 in
2 columns	=	38 picas,	16.0 cm,	6.5 in

Lettering: At 100% size, no lettering should be smaller than 8 point (0.3 cm high) or larger than 12 point (0.4 cm high). Use bold and solid lettering. Lines should be thick, solid, and no less than 1-point rule. Avoid the use of reverse type (white lettering on a

darker background). Avoid lettering on top of shaded or textured areas. Titles should be clear and informative. Keep wording on figures to a minimum, and confine any explanation of figures to their separate-page legends. Label only one vertical and one horizontal side of a figure. *Freehand lettering or drawing is unacceptable.*

Color Figures: Figures should now be submitted as RGB (red, green, blue) format. Saving color figures to this format will be more convenient for authors as RGB is the standard default on most programs. Color images will be preserved as RGB up until the time of printing and will be posted online in their original RGB form. Using RGB color mode for online images will be a significant improvement for figures that contain fluorescent blues, reds, and greens. Therefore the online journal will accurately reflect the true color of the images the way the author intended. For print, the images will be converted to CMYK through an automated color conversion process.

Shading: Avoid the use of shading, but if unavoidable, use a coarse rather than a fine screen setting (80-100 line screen is preferred). Avoid 1-20% and 70-99% shading; make differing shades vary by at least 20%, *i.e.*, 25%, 45%, 65%. Instead of shading, denote variations in graphs or drawings by cross-hatching; solid black; or vertical, horizontal, or diagonal striping. Avoid the use of dots.

Grouped figures: For grouped figures, indicate the layout in a diagram. Place grouped figures so that they can be printed in 1 column width with uniform margins. Indicate magnification in the legends and by internal reference markers in the photographs. Their length should represent the fraction or multiple of a micrometer, appropriate to the magnification.

Graphs: Graphs with axis measures containing very large or small numbers should convert to easily readable notations. *Example*: For an ordinate range of "counts per minute" values from 1,000 to 20,000, the true value may be multiplied by 10^{-3} (scale would read from 1 to 20) and the ordinate axis display "cpm ($x10^{-3}$)." Similarly, for a Scatchard plot with values ranging from 0.1 to 2 femtomolar (10^{-15} m), the scale may run from 0.1 to 2 with the abscissa labeled "m ($x10^{-15}$)." *Three-dimensional bar graphs will not be published if the information they refer to is only two-dimensional.*

Supplemental Data

Supplemental Data allows authors to enhance papers in *JCEM* by making additional substantive material available to readers. Supplemental Data may take the form of figures, tables, datasets, derivations, or videos, and is published only in *JCEM* online; it does not appear in the printed version of the journal. Authors who wish to include Supplemental Data should state so in the cover letter when the manuscript is submitted.

Supplemental Data files should be submitted through Editorial Manager at the time of manuscript submission, and will be reviewed along with the manuscript. The files should be uploaded in the field marked "Upload Supplemental Data Files", and should NOT be attached with the manuscript and figure files. Authors should refer to the

Supplemental Data in the manuscript at an appropriate point in the text or figure/table legend.

The file formats listed below may be used for Supplemental Data. Provide a brief description of each item in a separate HTML or Word file (*i.e.*, figure or table legends, captions for movie or sound clips, etc.). Do not save figure numbers, legends, or author names as part of an image. File sizes should not exceed 5 MB. Images should not exceed 500 pixels in width or height. Do not use tabs or spaces for Word or WordPefect tables; please use the table functions available within these word processing programs to prepare tables. For web pages, provide a complete list of files and instructions for creating directories.

.htm,				HTML*
.jpg,		JPEG		image*
.gif,		Graphical		image
.pdf,	Adobe	Portable	Document	Format
.xls,	MS		Excel	Spreadsheet
.mov,		Quick		Time
.wav,				Sound
.doc,	MS	Word	6	documents**
.txt,		Plain		ASCII*

*These files can be viewed directly on standard web browsers.
**MS Word may be used for text only.

Units of Measure

Results should be expressed in metric units. Systeme Internationale (SI units) must be added in parentheses. Temperature should be expressed in degrees Celsius (*e.g.*, 28 C) and time of day using the 24-hour clock (*e.g.*, 0800 h, 1500 h).

Standard Abbreviations

All nonstandard abbreviations in the text must be defined immediately after the first use of the abbreviation. The list of **Standard Abbreviations** is given in the link.

Editorial Policies and Guidelines

Prior Publication

Failure to notify the editor that some results in the manuscript are being or have been previously published will result in placement of a notice in the journal that the authors have violated the Ethical Guidelines for Publication of Research in The Endocrine Society Journals. The journal publishes original research and review material. Material previously published in whole or in part shall not be considered for publication. This

includes materials published in any form of mass communication. At the time of submission, authors must divulge in their cover letter all prior publications or postings of the material in any form of media. Abstracts or posters displayed for colleagues at scientific meetings need not be reported. Other postings of any part of the submitted material on web pages, as well as those essential for participation in required registries will be evaluated by the Editor-In-Chief, who shall determine if those postings are material enough to constitute prior publication.

Authorship Criteria

An author should have participated in either the conception, planning, or execution of the work, the interpretation of the results and the writing of the paper. An acknowledgment accompanying the paper is appropriate recognition for others who have contributed to a lesser extent, e.g., provision of clones, antisera or cell lines, or reading and reviewing manuscripts in draft. The signature of each author on the Affirmation of Originality and Copyright Release form that must be submitted with the manuscript indicates that all authors have had a part in the writing and final editing of the report, all have been given a copy of the manuscript, all have approved the final version of the manuscript, and all are prepared to take public responsibility for the work, sharing responsibility and accountability for the results. Medical writers can be legitimate contributors, and their roles, affiliations, and potential conflicts of interest should be described when submitting manuscripts. These writers should be acknowledged on the byline or in the Acknowledgments section in accord with the degree to which they contributed to the work reported in the manuscript. Failure to acknowledge these contributors would mean that the manuscript could have been "ghost-written," which is not allowed.

Guidelines for considering authors of non-research articles who have a potential COI

The editors of The Endocrine Society's journals appreciate the importance of assuring unbiased authorship of editorials, reviews, and other non-research features involving selection of evidence to be discussed and perspectives to be presented. Consequently, special care is taken in choosing authors for such articles to assure their views are balanced and unencumbered, and that the Society's policies on disclosure of conflicts of interest are implemented.

Obligations of Reviewers

The critical and confidential review of manuscripts is an essential element of research publications. Every scientist has an obligation to contribute to the peer review process by serving as a reviewer. Among the obligations of reviewers is the commitment to providing an expert, critical, and constructive scientific and literary appraisal of research reports in their fields of knowledge, skills, and experience in a fair and unbiased manner. In order to facilitate the prompt sharing of scientific results, it is also the obligation of each reviewer to complete their assignments promptly, within the editor's deadline. Should a delay in their review occur, the reviewer has the obligation to notify the editor at once. Reviewers should not review a manuscript if: 1) they do not

think that they are competent to assess the research described, 2) they believe there is a conflict of interest or personal or professional relationship with the author(s) that might bias their assessment of the manuscript, or (3) there is any other situation that could bias their review. Employment at the same institution as one of the authors does not automatically represent a conflict. Having previously reviewed the article for another journal does not disqualify a reviewer, although the editor should be informed so the reviewer's perspective can be considered. In circumstances when reviewers need to recuse themselves, they should notify the editor promptly, preferably with an explanation. If reviewers are uncertain whether they should recuse themselves, they should consult with the editor.

The reviewer should strive to provide accurate, detailed, and constructive criticisms, and the review should be supported by appropriate references, especially if unfavorable. The reviewer should also note whether the work of others is properly cited. If the reviewer notes any substantial resemblance of the manuscript being reviewed to a published paper or to a manuscript submitted at the same time to another journal, they should promptly report this to the editor.

No part of the manuscript under review should ordinarily be revealed to another individual without the permission of the editor. If a reviewer consults a colleague on a particular point, this fact, and the name of the collaborator or consultant, should be reported to the editor, preferably in advance. With these exceptions, a reviewer must obtain through the editor written permission from the authors to use or disclose any of the unpublished content of a manuscript under review.

Experimental Subjects

To be considered, all clinical investigations described in submitted manuscripts must have been conducted in accordance with the guidelines in The **Declaration of Helsinki** and must have been formally approved by the appropriate institutional review committees or its equivalent. All manuscripts must indicate that IRB approval was acquired; and that when informed consent was required by the IRB, that this was obtained from subjects in experiments involving humans. Investigators must disclose potential conflict of interest to study participants and should indicate in the manuscript that they have done so. The study populations should be described in detail. In many studies details of age, race, and sex are important. However, subjects must be identified only by number or letter, not by initials or names. Photographs of patients' faces should be included only if scientifically relevant. Authors must obtain written consent from the patient for use of such photographs. For further details, see the Ethical Guidelines.

Experimental Animals

A statement confirming that all animal experimentation described in the submitted manuscript was conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines, should be included in the manuscript.

Clinical Trials Registration

For clinical trial reports to be considered for publication in the Journal, the Endocrine Society requires their prospective registration, as endorsed by the International Conference of Medical Journal Editors. We recommend use of www.clinicaltrials.gov. The Society's full Position Statement on Clinical Trials Registration is at the following web site:http://jcem.endojournals.org/site/misc/ClinicalTrials.pdf. All trials beginning after January 1, 2007 must have been prospectively registered before enrollment of the first subject. All trials begun before that date must be retroactively registered before submission. Please note that the Clinical Trial Registration number should be provided clearly on the title page of the manuscript.

Genetic and Genome-Wide Association Studies

To ensure rigor in genetic and genome-wide association studies and permit readers to assess their biological and clinical significance, submitted manuscripts describing such work should generally conform to the following study design criteria, which will be applied by the Journal's reviewers and editors in their evaluations.

Sample Size and Multiple Testing: Studies should include sufficient samples to have the power to detect an effect. In addition, since multiple hypotheses are often tested (*e.g.*, multiple SNPs, substratification, and multiple phenotypes), analyses and interpretations should account for the influence of such multiple testing on the findings' biological and clinical significance.

Validation Samples: The most rigorous association studies should include both a testing (or training) sample set and an independent validation series.

Functional Data: Functional data strengthen association data if the functional assay(s) have demonstrable relevance to the associated phenotype. In some instances, association studies with a single testing sample set and highly relevant functional data may be acceptable without an independent validation series.

Single Genetic Marker (e.g., SNP) versus Whole Gene/Genome Studies: Single SNP studies are acceptable when the particular SNP has strong prior claims for involvement in the phenotype of interest. However, it is desirable to examine genetic variation at least across and flanking the gene of interest when this is feasible.

Negative Association Studies: Well-designed and executed association studies that demonstrate significant negative findings will be considered if the gene in question has clear relevance to disease pathogenesis or has been implicated in prior published association studies.

Microarray Expression Studies

Genome-wide expression studies require both technical validation and an independent validation series. Technical validation entails application of a different technique (*e.g.*, RT-PCR of single genes or immunohistochemistry) to confirm the differential expression detected by genome-wide expression. An independent validation series of samples should be utilized to confirm the differential expression noted by genome-wide analysis of the initial testing sample set.

Nomenclature and Technical Requirements

The value of study data is enhanced if, where relevant, manuscripts:

- Use standard terminology for variants, providing rs numbers for all variants reported. These can be easily derived for novel variants uncovered by the study. Where rs numbers are provided, the details of the assay (primer sequences, PCR conditions, etc.) should be described very concisely.
- Describe measures taken to ensure genotyping accuracy, *e.g.*, percentage of genotype calls, number of duplicate samples that were genotyped, and percentage concordance.
- Provide approved GDB/HUGO approved gene names, in the appropriate cases and italics.
- Provide linkage disequilibrium (LD) relationships between typed variants.
- Provide information and a discussion of departures from Hardy-Weinberg equilibrium (HWE). The calculation of HWE may help uncover genotyping errors and impact on downstream analytical methods that assume HWE.
- Provide raw genotype frequencies in addition to allele frequencies. It is also desirable to provide haplotype frequencies.
- Provide the criteria they have used to select tagSNPs.
- Denote the boundaries considered when studying SNPs within a gene of interest. For example, "gene X and 100 kb upstream of the first translational start site and 150 kb downstream of the stop codon."

Manuscripts Reporting New Amino Acid or Nucleotide Sequence

Manuscripts reporting amino acid or nucleotide sequences of proteins with sequences already known from other tissues or species will be considered only if they provide new biological insight. Manuscripts dealing with partial sequence data are not likely to be considered. The Endocrine Society has established policy that deals with submission of new protein or nucleic acid sequences. When a manuscript is accepted that contains novel sequences, such sequences must be deposited in the appropriate database (such as GenBank) and an accession number obtained before the manuscript is sent to the printer. It is recommended that the following statement containing the assigned accession number be inserted as a footnote: "These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number Ul2345."

Standards for Steroid Nomenclature

The 3 major classes of mammalian sex hormones - androgens, estrogens, and progestins (or progestagens or gestagens) - are defined by their biological activities, which are mediated via the well-defined androgen, estrogen and progesterone (or progestin) receptors. The principal bioactive sex steroid and natural ligand for each class is testosterone (or 5α-dihydrotestosterone), estradiol and progesterone, respectively. Androgen(s), estrogen(s) and progestin(s) are classes of compounds with hormonal activity, and not the names of individual steroids. Synthetic steroids or extracts can be considered as members of a generic steroid class (androgens, estrogens, progestins), but are distinct from the natural cognate ligand itself. Synthetic hormones or extracts of biological origin of each class may also have agonist, antagonist or mixed bioactivity in one or more classes. Therefore, the terms androgens, estrogens and progestins (or progestagens or gestagens) should be used when referring to the class of hormones, whereas when a specific natural or synthetic steroid is being used or assayed, the particular compound must be specified.

Apart from accepted trivial names, steroids should be named according to the systematic nomenclature of the IUPAC convention on Nomenclature of Steroids (Moss et al Pure & Applied Chemistry 61:1783-1822, 1989) at first mention in a single footnote defining all letter abbreviations. Subsequently, generic or trivial names or letter abbreviations, but not trade-names, should be used.

Examples of accepted trivial names include: cholesterol, estrone, 17α and 17β estradiol (estradiol is also acceptably used as the trivial name for 17β estradiol), estriol, aldosterone, androsterone, etiocholanolone, dehydroepiandrosterone, testosterone, 5α dihydrotestosterone, 5β dihydrotestosterone, androstenedione, pregnenolone, progesterone, corticosterone, deoxycorticosterone, cortisone, and cortisol.

Trivial names may be modified by prefixes or suffixes indicating substituents (as in 17-hydroxyprogesterone for 17-hydroxy-4-pregnene-3,20-dione), double bonds (as in 7-dehydrocholesterol for 5,7-cholestadien-3-ol) and epimeric configurations of functional groups provided the locus of epimerization is indicated (as in 11-epicortisol for 11α21-trihydroxypregn-4-en-3-one).

Manuscripts Reporting Novel Compounds

Manuscripts describing experiments with new compounds must provide their chemical structures. For known compounds, the source and/or literature reference to the chemical structure and characterization must be provided.

Validation of Data and Statistical Analysis

Assay validation: Bioassay and radioimmunoassay potency estimates should be accompanied by an appropriate measure of the precision of these estimates. For bioassays, these usually will be the standard deviation, standard error of the mean, coefficient of variation, or 95% confidence limits. For both bioassays and radioimmunoassays, it is necessary to include data relating to within-assay and between-assay variability. If all relevant comparisons are made within the same assay, the latter may be omitted. Authors should be aware that the precision of a measurement depends upon its position on the dose-response curve.

In presenting results for new assays, it is necessary to include data on the following: 1) within-assay variability; 2) between-assay variability; 3) slope of the dose-response curve; 4) mid-range of the assay; 5) least-detectable concentration (concentration resulting in a response two standard deviations away from the zero dose response); 6) data on specificity; 7) data on parallelism of standard and unknown and on recovery; and 8) comparison with an independent method for assay of the compound. When radioimmunoassay kits are utilized or hormone measurements are conducted in other than the authors' laboratories and the assay is central to the study, data regarding performance characteristics should be included.

Pulse analysis: Data from studies of pulsatile hormone secretion should be analyzed using a validated, objective pulse detection algorithm. The algorithm used should require that false-positive rates of pulse detection be defined in relation to the measurement error of the data set being analyzed, and the methods used to determine the measurement error should be described. The author(s) also should describe the methods used: 1) to deal with missing or undetectable values; 2) to determine peak frequency, interpeak interval, and pulse amplitude; and 3) for statistical comparisons of peak parameters.

Data analysis: It is the author's responsibility to document that the results are reproducible and that the differences found are not due to random variation. No absolute rules can be applied, but in general quantitative data should be from no fewer than three replicate experiments. Appropriate statistical methods should be used to test the significance of differences in results. The term "significant" should not be used unless statistical analysis was performed, and the probability value used to identify significance (*e.g.*, $P > 0.05$) should be specified.

When several *t* tests are employed, authors should be aware that nominal probability levels no longer apply. Accordingly, the multiple*t* test, multiple range test, or similar techniques to permit simultaneous comparisons should be employed. Also, in lieu of using several *t* tests, it is often more appropriate to utilize an analysis of variance (ANOVA) to permit pooling of data, increase the number of degrees of freedom, and improve reliability of results. Authors should use appropriate nonparametric tests when the data depart substantially from a normal distribution. Analysis of variance tables should not be inserted in manuscripts. F values with the degrees of freedom as subscripts together with the *P* values are sufficient.

In presenting results of linear regression analyses, it is desirable to show 95% confidence limits. When data points are fitted with lines (as in Scatchard or Lineweaver-Burk plots), the method used for fitting (graphical, least squares, computer program) should be specified. If differences in slopes and/or axis intercepts are claimed for plotted lines, these should be supported by statistical analysis.

Authors should include in the manuscript a list of the software used for statistical analyses.

Digital Image Integrity

When preparing digital images, authors must adhere to the following guidelines as stated in the CSE's White Paper on Promoting Integrity in Scientific Journal Publications:

- No specific feature within an image may be enhanced, obscured, moved, removed, or introduced.
- Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the entire image and as long as they do not obscure, eliminate, or misrepresent any information present in the original.
- The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (e.g., dividing lines) and in the figure legend.

Deviations from these guidelines will be considered as potential ethical violations.

Note that this is an evolving issue, but these basic principles apply regardless of changes in the technical environment. Authors should be aware that they must provide original images when requested to do so by the Editor-in-Chief who may wish to clarify an uncertainty or concern.

[Please see paper of Rossner and Yamada (Journal of Cell Biology, 2004, 166:11-15), which was consulted in developing these policy issues, for additional discussion, and the CSE's White Paper on Promoting Integrity in Scientific Journal Publications, published by the Council of Science Editors, 2006.]

Publication and Production Guidelines

Proofs and Reprints

Proofs and a reprint order form are sent to the corresponding author unless the Editorial Office is advised otherwise. The author should designate by footnote on the title page of the manuscript the name and address of the person to whom reprint requests should be directed. Questions about reprints should be directed to June Billman, Account Manager at Cenveo Publisher Services, (June.Billman@cenveo.com) (preferred), 410-943-3086 (direct) or 1-866-487-5625 (toll-free).

Publication and Color Costs

There is no submission fee for The Endocrine Society journals.

There will be a charge of $95 per page for members of The Endocrine Society and $115 per printed page for non-members. There will be a charge of $235 per color figure for members of The Endocrine Society and $735 per color figure for non-members. For more information on the benefits of membership in The Endocrine Society, please visit the Member Benefits page of The Endocrine Society's website. Authors must submit

usable digital art that passes Cadmus's Rapid Inspector. Queries on page charges may be directed to June Billman at Cenveo Publisher Services, (June.Billman@cenveo.com) (preferred), 410-943-3086 (direct) or 877-705-1373 (fax).

NIH Deposits

For articles that were funded by NIH, accepted manuscripts will be submitted to PubMed Central. These manuscripts will be made freely available online twelve months after print publication. NIH will contact the author to confirm submission.

Open Choice Option

The Endocrine Society's Open Choice program was developed to allow researcher authors the ability to provide immediate, open and free access to their work. For a growing number of our authors, providing open access is a condition of funding. For others, they simply want to have their latest findings available to the scientific domain without delay. Still others believe that paying to make their article free in the first 12 months of publication is not a worthwhile use of their grant monies.

The Endocrine Society offers authors an Open Choice option for $3,000 per article, in addition to other publication charges. Upon receipt of payment, the article will be made openly available on the journal site and the final print version will be deposited in PubMed Central for immediate public access.

Corresponding authors can indicate on the invoices included with their proofs if they wish to exercise this option. All articles will be licensed using the Creative Commons, Attribution, Non-commercial license 2.0.

Institutional Repositories and Other Archives

Authors may deposit the final PDF version of their manuscript in their institutional repository or other archive 1 year following the date of print publication. Any deposits to be made prior to 1 year following the date of print publication must be approved by the Publications Department of The Endocrine Society.

Normas para redação de artigos para a revista "*PLOS Neglected Tropical Diseases*" a ser submetido o artigo III.

PLOS Neglected Tropical Diseases Guidelines for Authors

Detailed below are guidelines for authors about the journal, open access, the editorial process, and the production process. We also provide a checklist for submitting manuscripts for the first time, a checklist for submitting revised manuscripts, and detailed guidelines for figure and table preparation. PLOS Neglected Tropical Diseases also offers several means of support to authors in developing countries.

pontents:

1. About ***PLOS Neglected Tropical Diseases***

PLOS Neglected Tropical Diseases is an open-access journal devoted to the pathobiology, epidemiology, prevention, treatment, and control of the neglected tropical diseases (NTDs), as well as public policy relevant to this group of diseases. The NTDs are defined as a group of poverty-promoting chronic infectious diseases, which primarily occur in rural areas and poor urban areas of low-income and middle-income countries. They are poverty-promoting because of their impact on child health and development, pregnancy, and worker productivity, as well as their stigmatizing features. The major NTDs that are within the scope of*PLOS Neglected Tropical Diseases* can be found in the description of the journal's scope.

Original Research Papers

All aspects of the NTDs will be considered, including their pathogenesis, clinical features, pharmacology and treatment, diagnosis, epidemiology, vector biology, and vaccinology and prevention. Demographic, ecological and social determinants, public health, and policy aspects of these diseases (including cost-effectiveness analyses) will also be a priority. *PLOS Neglected Tropical Diseases* is pleased to publish relevant in vitro and animal studies as well as human investigations. The journal is organized to provide additional support for authors from endemic countries, and such authors are particularly encouraged to submit their research to *PLOS Neglected Tropical Diseases*. Academic editors, supported by expert peer-reviewers, will select for publication those studies that drive their respective fields forward. We encourage papers that cross disciplines. If your study addresses an infection that is outside our detailed scope, you must first send a pre-submission inquiry indicating why you consider the infection to be a neglected tropical disease.

Magazine Section

In addition to publishing original research papers, *PLOS Neglected Tropical Diseases* will have an engaging magazine section with dedicated editors. Articles in the magazine section will mostly be commissioned, but we welcome your ideas for articles. If you would like to write a magazine-section article, please send a brief article proposal (up to 150 words) to **plosntds [at] plos.org**.

Open Access

PLOS applies the Creative Commons Attribution License (CCAL) to all works we publish. Under the CCAL, authors retain ownership of the copyright for their article, but authors allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles in PLOS journals, so long as the original authors and source are cited. No permission is required from the authors or the publishers.

Publication Charges

To provide open access, PLOS journals use a business model in which our expenses—including those of peer review, journal production, and online hosting and archiving—are recovered in part by charging a publication fee to the authors or research sponsors for each article they publish. The fees vary by journal.

PLOS is committed to the widest possible global participation in open access publishing. To determine the appropriate fee, we use a country-based pricing model, which is based on the country that provides 50% or more of the primary funding for the research that is being submitted. Research articles funded by Upper Middle and High Income Countries incur our standard publication fees. Corresponding authors who are affiliated with one of our Institutional Members are eligible for a discount on this fee. Such authors will be informed of the discount applicable after submission of their manuscript.

Fees for Low and Lower Middle Income Countries are calculated according to the PLOS Global Participation Initiative pricing program for manuscripts submitted after 9am Pacific Time on September 4, 2012 (this program is not retroactive).

- Group One: Countries from this list will not be charged for publishing
- Group Two: Countries from this list will be charged a flat $500

Our fee waiver policy, whereby PLOS offers to waive or further reduce the payment required of authors who cannot pay the full amount charged for publication, remains in effect. Editors and reviewers have no access to whether authors are able to pay; decisions to publish are only based on editorial criteria.

Criteria for Publication

Manuscripts should represent a substantial advance in medical science or medical practice in terms of:

- Originality
- Importance and relevance to researchers, practitioners, or policy makers in the field of NTDs
- Interest for researchers or practitioners outside the field
- Rigorous methodology with conclusions justified by the evidence presented
- Adherence to the highest ethical standards

Presubmission Inquiries

We strongly encourage authors to send a presubmission inquiry before making a full submission. To see if a manuscript is appropriate for full submission, please login or register at http://www.editorialmanager.com/pntd, click the link labeled, "Submit New Manuscript" and select Presubmission Inquiry as your article type. You will be asked for a cover letter explaining why you feel that the work is appropriate for *PLOS Neglected Tropical Diseases*, along with a referenced abstract of the paper (fewer than 500 words) that describes the background, aims and methodology, key results, and major conclusions of the work. We aim to provide responses to these inquiries within 48 hours. Authors who receive an invitation to submit their manuscripts will then enter the regular editorial process.

7.1 6. Overview of Editorial Process

Our aim is to provide all authors with an efficient, courteous, and constructive editorial process. To ensure the fairest and most objective decision-making, the editorial process is run as a partnership between the *PLOS Neglected Tropical Diseases* Editor-in-Chief, the eight Deputy Editors, and a team of academic experts who act as Associate Editors (AEs). These individuals, all of whom are members of the *PLOS Neglected Tropical Diseases* Editorial Board, are leaders in their fields and represent the full breadth of research on NTDs.

Submitted manuscripts are assigned to a Deputy Editor, who then assigns it to an appropriate AE. The AE promptly evaluates the paper and decides whether it is likely to

meet the requirements of providing enough of an advance in a particular field and describing a sufficient body of work to support that claim. If so, the paper is sent out for peer review.

Expert reviewers will be asked to assess the technical and scientific merits of the work. Where relevant, work presented in a manuscript will be subject to a rigorous review of the statistical methods used. *PLOS Neglected Tropical Diseases* encourages open (non-anonymous) peer-review. As a default, we will pass a reviewer's name on to the authors along with the comments. If reviewers do not wish to have their name revealed, they can request to stay anonymous and we will honor that request.

Upon submission of a manuscript, authors are asked if they wish to exclude any specific academic editors or reviewers from the peer review of their article. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the article. See the relevant guidelines for reviewers and more general information on PLOS' policy regarding competing interests.

Once all reviews have been received and considered by the professional and academic editors, a decision letter to the author will be drafted.

There are several types of decision possible:

- Accept without revision
- Minor revision
- Major revision
- Reject, typically because the paper does not meet the criteria for publication outlined above

Revised manuscripts will be assessed by the same academic editor. Sometimes, re-review or additional statistical review will be required, but in general we aim to make decisions without involving multiple rounds of review.

Upon acceptance, the manuscript is checked by PLOS staff to ensure that it is in a format that can be efficiently handled by our production system. The authors will be queried and allowed to make any final minor revisions that are needed.

This is the final stage at which authors will see their manuscript before publication. The authors' files will be carefully tagged to generate XML and PDF files, but will not be subject to detailed copyediting (see Overview of the Production Process). It is therefore essential that authors provide a thoroughly proofread and checked manuscript, following the manuscript checklist and any comments from PLOS staff.

7.1.1 Appeals of Decisions

PLOS Neglected Tropical Diseases encourages input from the community regarding editorial and publishing policies. However, appeals against manuscript decisions must be a) limited to the specific manuscript in question, b) made only by the corresponding author, and c) sent by e-mail to **plosntds [at] plos.org**. Telephone calls or other informal appeals are discouraged and will not be considered. Appeals will only be

considered when a reviewer or editor is thought to have made a significant factual error or when his/her objectivity is compromised by a documented competing interest, *and* when a reversal based on either of these grounds would change the original decision. The journal staff will ask for confirmation of the reason(s) in the first instance. If the authors proceed, the original editor(s) will usually be asked to consider the appeal. Additional editorial board members may also be consulted. Each appeal is treated on its merits, and the journal cannot make any guarantees about the turnaround time or outcome.

7.2 7. Supporting Information and Materials Required at Submission

PLOS Neglected Tropical Diseases is committed to the highest ethical standards in medical research. Accordingly, we ask authors to provide specific information regarding ethical treatment of research participants, patient consent, patient privacy, protocols, authorship, and competing interests. We also ask that reports of certain specific types of studies adhere to generally accepted standards. Our requirements are based on the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, issued by the International Committee for Medical Journal Editors and are enumerated below.

7.2.1 Human and Animal Research

All research involving humans and animals must have been approved by the authors' institutional review board or equivalent committee(s), and that board must be named by the authors in the manuscript. For research involving human participants, informed consent must have been obtained (or the reason for lack of consent explained, e.g. the data were analyzed anonymously) and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. It must be stated in the Methods section of the paper whether informed consent was written or oral. If informed consent was oral, it must be stated in the paper: (a) why written consent could not be obtained, (b) that the IRB approved the use of oral consent, and (c) how oral consent was documented.

Authors should be able to submit, upon request, a statement from the research ethics committee or institutional review board indicating approval of the research. We also encourage authors to submit a sample of a patient consent form, and may require submission on particular occasions.

All animal work must have been conducted according to relevant national and international guidelines. In accordance with the recommendations of the Weatherall report, "The use of non-human primates in research" we specifically require authors to include details of animal welfare and steps taken to ameliorate suffering in all work involving non-human primates. The institution that approved the study must be named, and it must be stated in the paper that the study was conducted adhering to the institution's guidelines for animal husbandry.

7.2.2 Patient Privacy and Informed Consent for Publication

Our human participant policy conforms to the Uniform Requirements of the International Committee of Medical Journal Editors:

"Patients have a right to privacy that should not be infringed without informed consent. Identifying information should not be published in written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Informed consent for this purpose requires that the patient be shown the manuscript to be published.
Complete anonymity is difficult to achieve, and informed consent for publication should be obtained if there is any doubt. If data are changed to protect anonymity, authors should provide assurance that alterations of the data do not distort scientific meaning.
When informed consent has been obtained it should be indicated in the published article."
For papers that include identifying information, or potentially identifying information, authors must download the Consent Form for Publication in a PLOS Journalfrom our site, which the patient, parent or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. (This license means that the images and text we publish online become available for any lawful purpose). Once authors have obtained the signed consent form, it should be filed securely in the patient's case notes and the article submitted to PLOS journal should include this statement indicating that specific consent to publication was obtained. "The patients in this manuscript have given written informed consent (as outlined in PLOS consent form) to publication of their case details."

Download "Consent Form for Publication":

- English
- French
- Portuguese
- Spanish

7.2.3 Material Required for the Submission of Specific Study Types

7.2.3.1 a. Clinical Trials

We follow the 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"

PLOS supports the position of the International Committee of Medical Journal Editors (ICMJE) on trial registration. All trials initiated after 1 July 2005 must be registered prospectively in a publicly accessible registry (i.e., before patient recruitment has begun), or they will not be considered for publication. For trials initiated before 1 July 2005, all trials must be registered before submission to our journals. See the ICMJE faq on trial registration for further details. The WHO's list of approved registries is listed here http://www.who.int/ictrp/network/primary/en/index.html.

Authors of trials must adhere to the CONSORT reporting guidelines appropriate to their trial design. Please check the CONSORT statement Web site for information on the appropriate guidelines for specific trial types. Before the paper can enter peer review authors must: 1) name in the paper trial registry, trial registration number, and IRB and 2) provide a copy of the trial protocol and a completed CONSORT checklist as supporting files (these documents will also be published alongside the paper, if accepted). The CONSORT flow diagram must be included as Figure 1. Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and PLOS reserves the right to ask for a copy of the patient consent form. Information on statistical methods or participants beyond what is indicated in the CONSORT statement should be reported in the Methods section.

7.2.3.2 b. Systematic Reviews and Meta-Analyses

Reports of systematic reviews and meta-analyses should use the PRISMA statement as a guide, and include a completed PRISMA checklist and flow diagram to accompany the main text. Blank templates of the checklist and flow diagram can be downloaded from the PRISMA Web site.

7.2.3.3 c. Diagnostic Studies

Reports of studies of diagnostic accuracy should conform to the STARD requirements.

7.2.3.4 d. Epidemiological Studies

For reports of epidemiological studies, you should consult the STROBE initiative.

7.2.3.5 e. Microarray Experiments

Reports of microarray experiments should conform to the MIAME guidelines, and the data from the experiments must be deposited in a publicly accessible database.

7.2.4 Author Status

All authors will be contacted via e-mail at submission to ensure that they are aware of and approve the submission of the manuscript, its content, authorship, and order of authorship. Articles will not be published unless all authors have provided their assent to publication.

The involvement of any professional medical writer in publication must be declared. We encourage authors to consult the European Medical Writers' Association Guidelines on the role of medical writers. For all PLOS journals, the corresponding author must submit the manuscript, related files, and all required data and information. From the point of submission through to publication, all communication related to that manuscript will be directed to and received from the corresponding author only.

PLOS Neglected Tropical Diseases bases its criteria for authorship on those outlined in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals,which are

summarized below. The contributions of all authors must be described. Contributions that fall short of authorship should be mentioned in the acknowledgements.

"Authorship credit should be based on
1) substantial contribution to conception and design, or acquisition of data, or analysis and interpretation of data;
2) drafting the article or revising it critically for important intellectual content; and
3) final approval of the version to be published.
Authors should meet conditions 1, 2, and 3.
When a large, multi-center group has conducted the work, the group should identify the individuals who accept direct responsibility for the manuscript (3). These individuals should fully meet the criteria for authorship defined above and editors will ask these individuals to complete journal-specific author and competing interests disclosure forms. When submitting a group author manuscript, the corresponding author should clearly indicate the preferred citation and should clearly identify all individual authors as well as the group name.
Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship. All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content."
PLOS journals follow the COPE guidelines covering changes in authorship. Please note that if any changes to the list of authors of a manuscript are necessary after the initial submission of a manuscript to a PLOS journal but before its publication, the corresponding author may be asked to provide written confirmation that all authors consent to the change(s). The journal also reserves the right to request written confirmation from all authors (including those added, removed, or moved in the author order). Such written consent may be required before the revised submission is sent to the editors.

7.2.5 Prior Publication

When submitting an article, all authors are asked to indicate that they have not submitted a similar manuscript for publication elsewhere. If related work has been submitted elsewhere, then a copy must be included with the article submitted to PLOS. Reviewers will be asked to comment on the overlap between related submissions.

7.3 8. Preparation of Research Articles

PLOS Neglected Tropical Diseases publishes original research articles of importance to the NTDs community and the wider health community. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a more limited range of experiments.

The writing style should be concise and accessible, avoiding jargon so that the paper is understandable for readers outside a specialty or those whose first language is not English. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our

aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions. Although we encourage submissions from around the globe, we require that manuscripts be submitted in English. Authors who do not use English as a first language may contact us for additional information. As a step towards overcoming language barriers on acceptance of the paper, we encourage authors fluent in other languages to provide copies of their full articles or abstracts in other languages. We will publish these translations as supporting information and list them, together with other supporting information files, at the end of the article text.

7.3.1 Cover Letter

Please include a cover letter explaining why this manuscript is suitable for publication in *PLOS Neglected Tropical Diseases*. Why will your research paper inspire the NTDs community, and how will it drive the understanding of NTD pathobiology, epidemiology, prevention, treatment, control, or policy?

If your study addresses an infection that is outside our detailed scope, you must first send a pre-submission inquiry indicating why you consider the infection to be a neglected tropical disease.

7.3.2 Electronic Formats

Our submission system supports a limited range of formats for text and graphics. The following file formats/types and manuscript information are required before submission. If you are concerned about the suitability of your files, please contact us at **plosntds [at] plos.org**.

7.3.2.1 Manuscript and Table Files

Articles can be submitted for review in DOC, DOCX, RTF, or PDF. Any articles that have been prepared in LaTeX will be accepted for review, but only in PDF format. After acceptance, only text files (RTF or DOC) of the revised manuscript and tables can be accepted for use in the production process.

7.3.2.2 Math Equations and DOCX

If your manuscript is or will be in DOCX and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.

If you have not yet composed your article, you can ensure that the equations in your DOCX file remain editable in DOC by enabling “Compatibility Mode” before you begin. To do this, open a new document and save as Word 97-2003 (*.doc). Several features of Word 2007/10 will now be inactive, including the built-in equation editing tool. You can insert equations in one of the two ways listed below.

If you have already composed your article as DOCX and used its built-in equation editing tool, your equations will become images when the file is saved down to DOC. To resolve this problem, re-key your equations in one of the two following ways.

1. Use MathType to create the equation. MathType is the recommended method for creating equations.
2. Go to Insert > Object > Microsoft Equation 3.0 and create the equation.
   If, when saving your final document, you see a message saying “Equations will be converted to images,” your equations are no longer editable and PLOS will not be able to accept your file.

7.3.2.3 LaTeX

Articles prepared in LaTeX may be submitted in PDF format for use during the review process. After acceptance, however, .tex files and formatting information will be required as a zipped file. Please consult our LaTeX Guidelines for a list of what will be required.

7.3.2.4 Tables

Tables must conform to our Guidelines for Figure and Table Preparation and placed at the end of the article DOC or RTF file. Accepted LaTeX submissions only should have table files—which must also conform to these guidelines—uploaded individually into the online submission system.

7.3.2.5 Figure Files

Graphics files can only be submitted in EPS or TIF format. For the article to be accepted for publication, the author will need to supply high-resolution versions of the figures. When preparing your figures, please ensure that the files conform to our Guidelines for Table and Figure Preparation.

If you are uploading your files in EPS format, please use the "create outlines" option under the type menu in Illustrator so that all text and fonts appear as intended in print. If you need additional help with figure preparation, please contact **figures [at] plos.org**.

Authors are encouraged to provide a striking image to accompany their article, if one is available. This image may be chosen to highlight the article on our journal Web site.

PLOS does not accept vector EPS figures generated using LaTeX. We only accept LaTeX generated figures in TIFF format. Export your LaTeX files as PDFs, and then open them in GIMP or Photoshop and save as TIFF. In general, Figures must be generated in a standalone graphics application such as Adobe Illustrator, InkScape, PyMol, MatLab, SAS, etc. Please see our Figure Guidelines for more information.

All figures will be published under a Creative Commons Attribution License. Upon publication they will be made available online without cost to anyone, anywhere—to download, redistribute, include in databases, and otherwise use—

subject only to the condition that the original authorship is properly attributed. Please do not submit any figures that have been previously copyrighted unless you have express written permission from the copyright holder to publish under this license.

7.3.3 Financial Disclosure

This section should describe sources of funding that have supported the work. Please include relevant grant numbers and the URL of any funder's Web site. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any sponsors or funders, and amend the aforementioned sentence as needed.

7.3.4 Competing Interests

The submitting author is asked at submission to declare, on behalf of all authors, whether there are any financial, personal, or professional interests that could be construed to have influenced the paper. The information entered here will appear in the published version, so please do not include the same in the manuscript file.

Reviewers are also asked to declare any interests that might interfere with their objective assessment of a manuscript. Any relevant competing interests of authors must be available to editors and reviewers during the review process and will be stated in published articles. Read more about PLOS's Competing Interests Policy.

7.3.5 Abbreviations

Please keep abbreviations to a minimum and define them upon first use in the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

7.3.6 Nomenclature

The use of standardized nomenclature in all fields of science and medicine is an essential step toward the integration and linking of scientific information reported in published literature. We will enforce the use of correct and established nomenclature wherever possible:

- We strongly encourage the use of SI units. If you do not use these exclusively, please provide the SI value in parentheses after each value.
- Species names should be italicized (e.g., *Homo sapiens*) and the full genus and species must be written out in full, both in the title of the manuscript and at the first mention of an organism in a paper; after that, the first letter of the genus name, followed by the full species name may be used.
- Genes, mutations, genotypes, and alleles should be indicated in italics. Use the recommended name by consulting the appropriate genetic nomenclature database,

e.g., HUGO for human genes. It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman: v-fes, c-MYC, etc.

- The Recommended International Non-Proprietary Name (rINN) of drugs should be provided.

7.3.7 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.

7.3.8 Organization of the Manuscript

Most articles published in *PLOS Neglected Tropical Diseases* are organized into the following sections: Title, Authors and Affiliations, Abstract, Author Summary, Introduction, Methods, Results, Discussion, Acknowledgments, References,

Figure Legends, and Tables. Uniformity in format facilitates the experience of readers and users of the journal. To provide flexibility, however, the Results and Discussion can be combined into one Results/Discussion section. All manuscripts must contain line numbers. Although we have no firm length restrictions for the entire manuscript, we urge authors to present and discuss their findings concisely.

7.3.8.1 Templates for Specific Study Types

- Clinical Research article
- Clinical Trial article
- Systematic Review / Meta-Analysis article

These manuscript templates will help to prepare your manuscript in the standard format. The templates consist of the standard headings along with body text explaining what to include in each section. You should overwrite (or copy and paste) the body text with the corresponding section text for your article.

7.3.8.2 Title (150 characters)

The title should be specific to the study yet concise, and should allow sensitive and specific electronic retrieval of the article. It should be comprehensible to readers outside your field. Avoid specialist abbreviations if possible. Titles should be presented in title case, meaning that all words except for prepositions, articles, and conjunctions should be capitalized. If the paper is a randomized controlled trial or a meta-analysis, this description should be in the title.

Examples:

- Climate Change and Spread of Lymphatic Filariasis in Sub-Saharan Africa
- A Cluster-Randomized Controlled Trial of a Nurse-Led Deworming Program for Soil-Transmitted Helminths

Please also provide a brief Short Title of no more than 50 characters (including spaces).

7.3.8.3 Authors and Affiliations

Provide the first names or initials (if used), middle names or initials (if used), surnames, and affiliations—department, university or organization, city, state/province (if applicable), and country—for all authors. One of the authors should be designated as the corresponding author. It is the corresponding author's responsibility to ensure that the author list, and the summary of the author contributions to the study are accurate and complete. If the article has been submitted on behalf of a consortium, all consortium members and affiliations should be listed after the Acknowledgments.

(For authorship criteria, see Supporting Information and Materials Required at Submission)

7.3.8.4 Abstract

The abstract succinctly introduces the paper. We advise that it should not exceed 250 – 300 words. It should mention the techniques used without going into methodological detail and summarize the most important results with important numerical results given. The abstract is conceptually divided into the following three sections with these headings: Background, Methodology/Principal Findings, and Conclusions/Significance. Please do not include any citations in the abstract. Avoid specialist abbreviations.

7.3.8.5 Author Summary

We ask that all authors of research articles include a 150- to 200-word non-technical summary of the work, immediately following the Abstract. Subject to editorial review and author revision, this short text is published with all research articles as a highlighted text box.

Distinct from the scientific abstract, the author summary should highlight where the work fits in a broader context of life science knowledge and why these findings are important to an audience that includes both scientists and non-scientists. Ideally aimed to a level of understanding of an undergraduate student, the significance of the work should be presented simply, objectively, and without exaggeration.

Authors should avoid the use of acronyms and complex scientific terms and write the author summary using the first-person voice. Authors may benefit from consulting with a science writer or press officer to ensure that they effectively communicate their findings to a general audience.

Examples are available at:

Pseudogenization of a Sweet-Receptor Gene Accounts for Cats' Indifference toward Sugar

A Hybrid Photoreceptor Expressing Both Rod and Cone Genes in a Mouse Model of Enhanced S-Cone Syndrome

Life in Hot Carbon Monoxide: The Complete Genome Sequence of Carboxydothermus hydrogenoformans Z-2901

7.3.8.6 Introduction

The introduction should discuss the purpose of the study in the broader context. As you compose the introduction, think of readers who are not experts in this field. Include a brief review of the key literature. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

7.3.8.7 Methods

This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. Detailed methodology or supporting information relevant to the methodology can be published on our Web site.

This section should also include a section with descriptions of any statistical methods employed. These should conform to the criteria outlined by the Uniform Requirements, as follows: "Describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty (such as confidence intervals). Avoid relying solely on statistical hypothesis testing, such as the use of P values, which fails to convey important quantitative information. Discuss the eligibility of research participants. Give details about randomization. Describe the methods for and success of any blinding of observations. Report complications of treatment. Give numbers of observations. Report losses to observation (such as dropouts from a clinical trial). References for the design of the study and statistical methods should be to standard works when possible (with pages stated) rather than to papers in which the designs or methods were originally reported. Specify any general-use computer programs used."

7.3.8.8 Results

The results section should include all relevant positive and negative findings. The section may be divided into subsections, each with a concise subheading. Large datasets, including raw data, should be submitted as supporting files; these are published online alongside the accepted article. The results section should be written in past tense.

As outlined in the Uniform requirements, authors that present statistical data in the Results section, should "...specify the statistical methods used to analyze them. Restrict tables and figures to those needed to explain the argument of the paper and to assess its support. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid nontechnical uses of technical terms in statistics, such as "random" (which implies a randomizing device), "normal," "significant," "correlations," and "sample." Define statistical terms, abbreviations, and most symbols."

7.3.8.9 Discussion

The discussion should be concise and tightly argued. It should start with a brief summary of the main findings. It should include paragraphs on the generalisability, clinical relevance, strengths, and, most importantly, the limitations of your study. You may wish to discuss the following points also. How do the conclusions affect the existing knowledge in the field? How can future research build on these observations? What are the key experiments that must be done?

7.3.8.10 Acknowledgments

People who contributed to the work, but do not fit the criteria for authors should be listed in the Acknowledgments, along with their contributions. You must also ensure that anyone named in the acknowledgments agrees to being so named.

Details of the funding sources that have supported the work should be confined to the funding statement provided in the online submission system. Do not include them in the acknowledgments.

7.3.8.11 References

Only published or accepted manuscripts should be included in the reference list. Papers that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only as "unpublished data." All "personal communications" citations should be supported by a letter from the relevant authors.

Style information:

- PLOS uses the numbered citation (citation-sequence) method and first five authors, et al.
- References are listed and numbered in the order that they appear in the text.
- In the text, citations should be indicated by the reference number in brackets.
- The parts of the manuscript should be in the correct order *before* ordering the citations: body, boxes, figure captions, tables, and supporting information captions.
- Abstracts and author summaries may not contain citations.
- Journal name abbreviations should be those found in the NCBI databases: http://www.ncbi.nlm.nih.gov/nlmcatalog/journals.
  Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial. For convenience, a number of reference software companies supply PLOS style files (e.g., Reference Manager, EndNote).

Published Papers
1. Hou WR, Hou YL, Wu GF, Song Y, Su XL, et al. (2011) cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res 10: 1576-1588.

Note: Use of 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 papers
Same as above, but "In press" appears instead of the page numbers.

Electronic Journal Articles
1. Huynen MMTE, Martens P, Hilderlink HBM (2005) The health impacts of globalisation: a conceptual framework. Global Health 1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14. Accessed 25 January 2012.

Books
1. Bates B (1992) Bargaining for life: A social history of tuberculosis. Philadelphia: University of Pennsylvania Press. 435 p.

Book Chapters
1. Hansen B (1991) New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health. pp. 21-28.

7.3.8.12 Figure Legends

The aim of the figure legend should be to describe the key messages of the figure, but the figure should also be discussed in the text. An enlarged version of the figure and its full legend will often be viewed in a separate window online, and it should be possible for a reader to understand the figure without switching back and forth between this window and the relevant parts of the text. Each legend should have a concise title of no more than 15 words that can stand alone, without the use of figure part labels. The overall legend itself should be succinct, while still explaining all figure parts, symbols, and abbreviations. Avoid lengthy descriptions of methods.

7.3.8.13 Tables

All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables should not occupy more than one printed page; larger tables can be published as online supporting information. Tables must be cell-based; do not use picture elements, text boxes, tabs, or returns in tables. Please ensure that the files conform to our Guidelines for Figure and Table Preparation when preparing your tables for production.

Tables should be placed at the end of the manuscript file, rather than uploaded separately into the submission system.

7.3.8.14 Multimedia Files and Supporting Information

We encourage authors to submit essential supporting files and multimedia files along with their manuscripts. All supporting material will be subject to peer review, and should be smaller than 10 MB in size because of the difficulties that some users will experience in loading or downloading files of a greater size.

Supporting files should fall into one of the following categories: Dataset, Figure, Table, Text, Protocol, Audio, or Video. All supporting information should be referred to in the manuscript with a leading capital S (e.g., Figure S4 for the fourth supporting information figure). The numbered title and caption for each supporting information file

should be included in the main article file, after the titles and captions for the main figures.

Supporting files may be submitted in a variety of formats, but should be publication-ready, as these files are not copyedited. Carefully consider whether your supporting information needs to be searchable and/or editable, and choose the most suitable format accordingly. See the Figure Guidelines for more detail about our requirements for multimedia files and the file formats we accept.

7.4 9. Submission of Research Manuscripts

7.4.1 Are You Ready to Submit Your Manuscript?

We have provided an author checklist to help you prepare your materials for submission and to make the online submission process as straightforward as possible. Please take the time to look through the list before submitting your article.

If you are submitting a revised manuscript, you will have been given substantial guidance by the editors. We have provided a checklist for revised manuscripts.

7.4.2 Electronic Submission

Detailed instructions for submission can be found on the *PLOS Neglected Tropical Diseases* Manuscript Submission and Peer Review Web site. Files are uploaded individually and are combined into a single PDF file, which must be approved by the author at the end of the submission process.

Text files can be submitted in DOC or RTF format. Please convert LaTeX files to one of the acceptable formats.

Graphics files can only be submitted in EPS or TIF format. If possible, please label all figures with a standard font such as Arial or Times New Roman. Please read the Guidelines for Figure Preparation before submitting figures.

7.5 10. Other Types of Articles

7.5.1 Articles for the Magazine Section

In addition to publishing original research papers, *PLOS Neglected Tropical Diseases* will have an engaging magazine section with dedicated editors. Articles in the magazine section will mostly be commissioned, but we welcome your ideas for articles. If you would like to write a magazine-section article, please submit a presubmission inquiry or a full submission. If you wish to submit a full submission, please note that you must submit your manuscript as a "Research Article" - please kindly make a note in the "Comments" box of your submission form and we will change the article categorization for you.

Word counts for magazine-section articles are given in the descriptions below. Very long documents can be hosted as supplementary files (Supporting Information) with the magazine-section articles.

7.5.1.1 Editorial

These 600- to 800-word articles are written in-house by the Editor-in-Chief or a member of the Editorial Board.

7.5.1.2 Viewpoints

Viewpoints are opinion pieces grounded in evidence. The word limit is 1,500 words. Authors are encouraged to cite up to 15 references in support of their key assertions, and to use a logical structure for their piece. We encourage all authors to include a display item (a figure, photo, or illustration), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.3 Debate

The Debate highlights topical, emerging, or controversial issues in the NTDs field, such as controversies about the best treatment or prevention approach. Debates will be commissioned from two or more authors with differing points of view. Each author has up to 800 words and 10 references to outline their initial viewpoint, and then 400 words and 5 references to respond to the opposing viewpoint. We encourage each author to include a display item (a figure, photo, or illustration), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.4 Policy Platform

These articles provide a platform to discuss specific policies that could improve the lives of those at risk of, or affected by, the NTDs. New and specific policy proposals that arise from high-level national or international meetings will be considered for this section, but we will not publish traditional "meeting reports." These articles are usually 2,000 words, with up to 25 references. In very exceptional circumstances (i.e., when the article is of particular public-health importance), we will give authors a higher word limit, but this must be negotiated with the editors ahead of writing the article. We encourage all authors to include 3-5 display items (figures, photos, illustrations), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.5 Review

In these articles, the author reviews the best available evidence on a topic relevant to the NTDs community. Authors must include a short abstract and a brief "Methods" section that tells readers how they searched and appraised the literature in preparing the review. The word limit is 3,000 words, with 50-80 references. In very exceptional circumstances (i.e., when the article is of particular public-health importance), we will give authors a higher word limit, but this must be negotiated with the editors ahead of writing the article. Authors must include two boxes:

- A box that lists the 3-5 key learning points in their review

- A box that lists the 5 key papers in the field

We encourage all authors to include 3-5 display items (figures, tables, photos, or illustrations), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.6 Expert Commentary

In this article, we commission an expert to comment on a Research Article published in *PLOS Neglected Tropical Diseases*. The author will usually be the Academic Editor who oversaw the peer review of the Research Article, or one of the peer reviewers. The word limit is 1,000 words, with up to 15 references. We may also commission expert commentaries on research papers in other journals, provided that these papers are freely available online. We encourage all authors to include a display item (a figure, photo, or illustration), which will be published under the Creative Commons Attribution License. Please seeGuidelines for Table and Figure Preparation.

7.5.1.7 From Innovation to Application

These short articles (1,000 words, 10 references) discuss new technologies, such as drugs, vaccines, and diagnostics, relevant to NTDs. Authors are asked to take an objective and critical view, and they should include a box that lists up to 3 advantages and 3 disadvantages of the new technology. We will ask for a second box or table depending on what kind of tool is described (for example, if the tool is a new diagnostic tool, we will ask for a table that gives the sensitivity and specificity of the new tool compared with the existing gold standard). Authors with competing interests related to the technology (e.g., financial ties) will not be allowed to write for this section. We encourage all authors to include a display item (a figure, photo, or illustration), which will be published under theCreative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.8 Photo Quiz

These articles provide question-and-answer challenges that illustrate a key clinical issue in the diagnosis, management, or prevention of a neglected tropical disease. Submissions should follow this format:

- Case Discussion and Question

1. Initial brief presentation of a clinical case with key images that invite a diagnosis from the reader.
2. The question portion may state the history of the case and note the findings and the outcome, but it should not provide the diagnosis. The case presentation and question should be written in a single paragraph of no more than 150 words and should be accompanied by no more than 2 images/figures. Please see Guidelines for Table and Figure Preparation.

3. Similar to the Clinical Symposium manuscripts, authors must obtain written consent from the patient using our consent form (also available inFrench, Portuguese, and Spanish).

- Answer/Discussion. The Answer section should give the diagnosis, followed by a discussion of the most relevant clinical issues (no more than 1,200 words).
- Key Learning Points. Authors must include a box that lists 3-5 key learning points of the case, similar to other clinical sections of *PLOS Neglected Tropical Diseases*.
- References. No more than 10 references.

7.5.1.9 Symposium

This section has four sub-types:

- Laboratory Symposium
- Clinical Symposium
- Control Symposium
- Social, Cultural, Economic Symposium

In each case, the article begins by presenting a short "real-world" problem or challenge, and then uses this problem as the basis for an educational piece of up to 2,000 words, with 25 references. Further details for each type of symposium are given below:

Laboratory Symposium

These are problem-based learning articles, up to 2,000 words long. They begin with a description of a "real-world" problem (not a hypothetical one), which will be in the form of a set of laboratory results (e.g., microscopy, hematology results, drug susceptibility tests, alternative diagnoses) that are interesting, illuminating, or unusual and that will appeal to the journal's wider audience. This is then followed by a tutorial in the form of a series of questions and answers that help readers make sense of, and learn from, this set of laboratory results. Authors must include a box that lists the 3-5 key learning points of the article. We cannot publish any data that would identify a patient unless we have the patient's written consent, using our consent form (also available in French,Portuguese, and Spanish). We encourage all authors to include 3-5 display items (figures, photos, illustrations), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

Clinical Symposium

There are two types of article that we will publish in the Clinical Symposium section:

- Case-based learning articles, up to 2,000 words long. These begin with a description of how the patient presented, under the heading "Description of Case." This is then followed by a tutorial in the form of clinical questions and answers interspersed with further details of the case. An example of how this type of article is structured is at http://dx.doi.org/10.1371/journal.pmed.0020229. The title should succinctly describe

the problem but should not reveal the diagnosis (e.g., "A 17-Year-Old with Gradual Onset Blindness" or "A 45-Year-Old Woman with Chronic Itching"). Authors must obtain written consent from the patient using our consent form (also available in French, Portuguese, and Spanish). Authors must include a box that lists the 3-5 key learning points of the article. We strongly recommend that authors include examples of the patient's investigations (e.g., imaging, electrocardiograms, a video of the patient's clinical signs), all of which will be published under the Creative Commons Attribution License.

- Case reports, up to 1,000 words long. Case reports will not be commissioned. To inquire about submitting a case report, please e-mail **plosntds [at] plos.org**. Authors must obtain written consent from the patient using our consent form (also available in French, Portuguese, and Spanish). We will publish only cases that contain a valuable lesson or clinical reminder, and authors must include a box that lists the 3-5 key learning points of the article. An example of how case reports in *PLOS Neglected Tropical Diseases* should be structured is athttp://dx.doi.org/doi:10.1371/journal.pmed.0010015. We strongly recommend that authors include examples of the patient's investigations (e.g., imaging, electrocardiograms, a video of the patient's clinical signs), all of which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

Control Symposium

These are problem-based learning articles, up to 2,000 words long. They begin with a description of a "real-world" disease control challenge (i.e., at the community level, not the individual level). This is then followed by a tutorial in the form of a series of questions and answers that help readers understand how to tackle this type of control problem. Authors must include a box that lists the 3-5 key learning points of the article. We cannot publish any data that would identify a patient unless we have the patient's written consent, using our consent form (also available in French, Portuguese, and Spanish). We encourage all authors to include 3-5 display items (figures, photos, illustrations), which will be published under the Creative Commons Attribution License. Please seeGuidelines for Table and Figure Preparation.

Social, Cultural, Economic Symposium

These are problem-based learning articles, up to 2,000 words long. They begin with a description of a "real-world" scenario with social, cultural, or economic implications. Examples include: the case of a woman with lymphatic filariasis whose family is too afraid to touch her; an African community that declines to allow mass drug administration because of culturally based suspicions of "Western" medicine; the case of a man blinded by trachoma or onchocerciasis who can no longer provide for his family; or the case of a boy with chronic hookworm infection with chronic stunting and

cognitive difficulties. The description of the scenario is then followed by a tutorial in the form of a series of questions and answers that help readers understand how to approach such social, cultural, and economic concerns. Authors must include a box that lists the 3-5 key learning points of the article. We cannot publish any data that would identify a patient unless we have the patient's written consent, using our consent form (also available in French, Portuguese, and Spanish). We encourage all authors to include 3-5 display items (figures, photos, illustrations), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.10 Historical Profiles and Perspectives

These articles look back in history to discuss a notable figure or a control program that worked or failed. Articles should be up to 1,500 words, with 15 references. We encourage all authors to include a display item (figure, photo, illustration), which will be published under the Creative Commons Attribution License. Please see Guidelines for Table and Figure Preparation.

7.5.1.11 Interviews

These articles are up to 1,000 words long, and the author interviews a person who has made an important contribution to the fight against NTDs. We encourage the author to include a photo of the interviewee, which will be published under the Creative Commons Attribution License.

7.6 11. Overview of the Production Process

Before formal acceptance, the manuscript will be checked by PLOS staff to ensure that it complies with all essential format requirements. The authors' files are then carefully tagged to generate XML and PDF files, but will not be subject to detailed copyediting.

Once an article has been accepted for publication, the manuscript files are transferred into our production system and will be published in PDF and HTML formats, with an XML download option. Articles will also be archived in PubMed Central.

7.7 12. Embargoes and the Media

Authors are of course at liberty to present and discuss their findings ahead of publication: at medical or scientific conferences, on preprint servers, and in blogs, wikis and other informal communication channels. We recommend, however, that authors not contact the media or respond to such contact unless an article has been accepted for publication and an embargo date has been established. Respect for press embargoes will help to ensure that your work is reported accurately in the popular media, and that the full peer-reviewed paper is freely available to any interested reader when the news item is published. If a journalist has covered a piece of work ahead of publication, this will not affect consideration of the work for publication. See also our embargo guidelines for journalists.