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HIGHLIGHT TOPIC

Unexplained Liver Damage, Cryptogenic Liver Cirrhosis, and Steatohepatitis May Be Caused by Latent Chronic Toxoplasmosis

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ABSTRACT

T. gondii is a globally distributed intracellular protozoan parasite affecting approximately 5-90% of human population and causing a variety of so far neglected diseases and clinical entities in both immunocompromised and immunocompetent individuals. Acute infection of the parasite in mice caused marked reduction in serum butyrylcholinesterase (BChE) activity and liver damage. BChE and acetylcholinesterase (AChE) are biomarkers of low-grade systemic inflammation and earlier it was suggested that the elevation of these two bioparameters may predict development of type 2 diabetes

mellitus and Alzheimer's disease. Serum BChE activity was found to be associated with overweight, obesity, and body fat distribution parameters. Recently, a positive association between *T. gondii* seropositivity and obesity has also been reported. Infection with the parasite was linked with the increase in the number of hepatic stellate cells known to play an important role in the development of fibrosis and its advancement to cirrhosis of any etiology. Moreover, several authors suggested that hepatitis with a clinical picture resembling acute viral hepatitis result from *T. gondii* infection. Abnormalities associated with cryptogenic liver cirrhosis markedly affect acquired immunity of the host and probably participate in triggering and persistence of several autoimmune diseases, especially that anti-*T. gondii* IgG antibodies have been found in the sera of both patients suffering from these clinical entities and in healthy individuals. Oxidative stress and immunosuppression due to the infection play an important role in these processes. It should be also noted that mammalian as well as the parasite cells, express two cholesterol ester-synthesizing enzymes, ACAT1 and ACAT2, which share 44-47% of amino acid homology. ACAT1 is present in various cells and tissues, while ACAT2 expression is restricted to hepatocytes and intestinal mucosal cells. The parasite enzymes *TgACAT1* and *TgACAT2* localized to endoplasmic reticulum can synthesize and store abundant esters of cholesterol and triglycerides. The lifelong persistence and resulting surplus of ACAT1 and ACAT2, and *TgACAT1* and *TgACAT2* enzymatic activities especially in hepatocytes and/or intestinal cells of the host may therefore at least in part be responsible for development of steatohepatitis, and generation of ballooning hepatocytes, foamy macrophages, and clear cells (foamy) colitis. These abnormalities can be explained by the suppressed autophagy (it regulates lipid metabolism) in the liver due to proliferation of *T. gondii*. In alcoholic steatohepatitis, ballooning degeneration of hepatocytes may also at least partly result from the fact that ethanol dose-dependently stimulate microneme secretion in *T. gondii* tachyzoites and parasite attachment to the host cells, thus facilitating infection of the hepatic cells. The increased serum iron levels and hepatic iron overload reported in the patients with nonalcoholic hepatitis may be caused by the excess of NO produced by activated macrophages and hepatic cells of the host, as a defense molecule against infection with the parasite. NO intercepts iron before incorporation into ferritin and directly mobilizes iron from the serum protein in a glutathione-dependent manner. Finally, several reports provided data suggesting that the generation of Mallory-Denk bodies is linked with latent chronic hepatic toxoplasmosis, and

T. gondii cathepsin L and B proteases play an important role in this process. These findings may be supported by the population-based studies demonstrating the link between infection with the parasite and development of liver abnormalities, and the increasing global burden of overweight and obesity in children and adults.

Key words: Toxoplasmosis; hepatic stellate cells; Butyrylcholinesterase; Cryptogenic liver cirrhosis; steatohepatitis; Hepatic fibrosis; Ballooned hepatocytes; Foamy cells; Mallory-Denk bodies; Cathepsin L and B proteases; Autoimmune hepatitis

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INTRODUCTION

T. gondii is the most prevalent chronic parasitic infection affecting about 6 billion people^[1,2] and suspected to be a global health threat^[1-3]. At present it is believed that the parasite causes asymptomatic infection in healthy persons, but severe clinical presentations can be found in congenital toxoplasmosis, ocular involvement, and in immunocompromised individuals, including patients with AIDS^[4-6]. The purpose of this work was to present and analyze available literature data indicating a possible link between acute and chronic infection with the parasite and development of unexplained liver damage, cryptogenic liver cirrhosis, steatohepatitis, and several other hepatic histopathological abnormalities of unknown etiology.

LIVER DAMAGE

In animals, experimental *T. gondii* infection was associated with liver and kidney injury manifesting with a significant increase in serum alanine (AIAT) and aspartate aminotransferase (AspAT) activities, blood urea nitrogen level, decreased serum albumin concentration and alkaline phosphatase activity, albumin to globulin ratio, and marked abnormalities in plasma triglyceride and cholesterol levels^[7-9]. Histopathology and histochemistry studies showed liver cells degeneration which progressed from hydropic to fatty, and marked cellular infiltration (granulomata) around the blood vessels^[9A]; the reticuloendothelial cells, Kupffer cells and phagocytes exhibited increased alkaline phosphatase activity^[9A]. In mice, treatment with azithromycin (a macrolide antibiotic with antitoxoplasmic effects^[9B,9C]) caused normalization of serum AIAT and AspAT activities and improvement of total serum protein, albumin and globulin concentrations, and albumin/globulin ratio^[7] (Tables 1-3).

It must be noted that acute *T. gondii* infection in mice caused marked reduction in serum BChE activity ($p < 0.01$) and liver damage, and positive significant correlations between BChE activity in serum and BChE activity in liver ($r = 0.89$)^[9]. In addition, serum albumin concentrations were markedly decreased (Table 1), and previously it was reported that this biomarker is strongly correlated with BChE activity^[10]. At necropsy, liver of the infected animals showed random necrosis foci combined with the presence of tachyzoites and cysts containing bradyzoites, as well as an increase in spleen size. These findings are very important because AChE and BChE are biomarkers of low-grade systemic inflammation^[11-13], and

it was suggested that the elevation of these two bioparameters may predict the development of type 2 diabetes mellitus and Alzheimer's disease^[14]. These proposed associations are in line with my previous reports suggesting an important role of *T. gondii* infection in development of these two clinical entities^[15,16]. BChE also serves as a marker predicting the prognosis of diseases^[11] because when the serum activity of the enzyme is low, a high-risk of death must be

Table 1 Serum markers of liver and kidney injury in BALB/c mice infected intraperitoneally with tachyzoites of *T. gondii* (RH strain) and examined after 5 days (acc. to Da Silva *et al* [9]; with own modification).

| Serum biochemical parameters | Controls (n = 10) | Infected animals (n = 10) |
|------------------------------|-------------------|-----------------------------|
| AIAT (U/L) | 35.21 ± 11.73 | 258.13 ± 84.37 ^a |
| Alkaline phosphatase (U/L) | 338.44 ± 120.12 | 190.87 ± 78.66 ^a |
| Albumin (g/dL) | 2.21 ± 0.03 | 1.51 ± 0.08 ^a |
| Total proteins (g/dL) | 5.80 ± 0.40 | 4.55 ± 0.44 ^a |
| Urea (mg/dL) | 69.28 ± 3.10 | 100.12 ± 25.75 ^a |
| Creatinine (mg/dL) | 0.96 ± 0.32 | 0.87 ± 0.24 |

Results represent means ± SD. ^aStatistically significant results compared with controls ($p < 0.05$). AIAT, alanine aminotransferase.

Table 2 Damage of liver function in mice intraperitoneally infected with 3×10^6 *T. gondii* trophozoites and treated with azithromycin (250 mg/kg/day for 3 days, p.o.) (acc. to Al-Kaysi *et al* [7]; with own modification).

| Group of animals | Total serum protein concn (g/dL) | Serum albumin concn (g/dL) | AIAT (U/L) | AspAT (U/L) | Serum globulin concn (g/dL) | A/G ratio |
|--------------------------|----------------------------------|----------------------------|--------------------|--------------------|-----------------------------|-------------------|
| Controls | 7.42 | 4.86 | 9.6 | 10.6 | 2.56 | 1.89 |
| | ± 0.12 | ± 0.09 | ± 0.26 | ± 0.12 | ± 0.03 | ± 0.03 |
| Infected | 11.02 ^a | 3.50 ^a | 59.80 ^a | 24.20 ^a | 7.52 ^a | 0.47 ^a |
| | ± 0.22 | ± 0.26 | ± 5.71 | ± 1.33 | ± 0.19 | ± 0.12 |
| Infected and treated | 8.38 | 3.8 | 10.6 | 10 | 4.58 | 0.83 |
| | ± 0.06 | ± 0.05 | ± 0.12 | ± 0.16 | ± 0.03 | ± 0.02 |
| Not infected and treated | 7.44 | 4.88 | 9.2 | 10 | 2.56 | 1.96 |
| | ± 0.11 | ± 0.13 | ± 0.29 | ± 0.16 | ± 0.08 | ± 0.10 |

The values are means ± SE of 10 animals per group. ^aStatistically significant differences between respective groups were calculated using ANOVA test at $p < 0.05$. A/G, serum albumin to globulin concentration ratio.

Table 3 Serum biochemical markers of liver and kidney damage in ewes and does with toxoplasmosis (positive serum IgM antibodies directed against *T. gondii*) (acc. to Mahboub *et al* [8]; with own modification).

| Bioparameters | Controls (n = 20) | Sheeps with <i>T. gondii</i> infection (n = 15) | Controls (n = 20) | Goats with <i>T. gondii</i> infection (n = 15) |
|----------------------|-------------------|---|-------------------|--|
| Total protein (g/dL) | 4.20 ± 0.15 | 4.10 ± 0.37 | 5.44 ± 0.27 | 4.03 ± 0.80 ^a |
| Albumin (g/dL) | 1.81 ± 0.20 | 1.24 ± 0.13 ^a | 2.15 ± 0.23 | 0.84 ± 0.3 ^a |
| Globulin (g/dL) | 2.39 ± 0.07 | 3.11 ± 0.28 ^a | 3.47 ± 0.27 | 3.19 ± 0.63 |
| A/G ratio | 0.85 ± 0.08 | 0.41 ± 0.087 ^a | 0.72 ± 0.07 | 0.34 ± 0.23 ^a |
| AspAT (U/mL) | 58.87 ± 1.92 | 33 ± 2.69 ^a | 67.23 ± 5.09 | 47.33 ± 3.56 ^a |
| AIAT (U/mL) | 15.11 ± 0.43 | 22.86 ± 1.99 ^a | 15.071 ± 0.81 | 14.67 ± 2.04 |
| BUN (mg/dL) | 24.35 ± 2.07 | 32.26 ± 2.26 ^a | 42.50 ± 2.74 | 25.34 ± 5.64 ^a |
| Triglyceride (mg/dL) | 17.82 ± 1.29 | 51.49 ± 6.65 ^a | 25.28 ± 2.53 | 20.38 ± 5.95 |
| Cholesterol (mg/dL) | 41.61 ± 4.33 | 57.84 ± 5.17 ^a | 84.15 ± 5.85 | 51.79 ± 17.80 ^a |
| CRP (mg/dL) | 8.40 ± 1.70 | 10.71 ± 2.48 | 8.12 ± 2.37 | 12.00 ± 8.49 ^a |

Results are means ± SE. ^aStatistically significant results compared with respective control values ($p < 0.05$). AspAT, aspartate aminotransferase; AIAT, alanine aminotransferase. BUN, blood urea nitrogen. CRP, C-reactive protein.

taken into consideration^[11,17]. The individuals with the lowest serum BChE activity had significantly higher mortality than those with the highest values^[10]. In middle-aged and elderly men and women, Calderon-Margalit *et al*^[10] showed that serum BChE activity was inversely related to age and was positively correlated with the serum levels of albumin ($r = 0.35$, $p < 0.001$), cholesterol ($r = 0.31$, $p < 0.001$), and triglycerides ($r = 0.30$, $p < 0.001$). Moreover, the enzyme activity was strongly associated with overweight, obesity, and body fat distribution parameters (*e.g.*, body mass index, $r = 0.20$, $p < 0.001$), and recently, a positive association between *T. gondii* seropositivity and obesity has been found^[18].

Cholinesterases are enzymes present in cholinergic and noncholinergic tissues, as well as in blood and other body fluids. The AChE is a membrane-bound enzyme mainly found in the brain, muscles, erythrocytes, lymphocytes, and cholinergic neurons^[19,20], while BChE is present in the intestine, liver, kidney, heart, lung, brain, and serum^[21,22]. AChE inhibits activation of macrophages and release of proinflammatory cytokines, such as IL-6, TNF- α , IL-1, and IL-18^[23]. In rats, *T. gondii* infection was associated with a significant increase in the activity of AChE in whole blood and lymphocytes, and a positive correlation was found between the enzyme activity and number of lymphocytes ($p < 0.01$)^[20]. The BChE is responsible for cholinergic neurotransmission^[24], immune responses^[12], cardiovascular risk and lipid metabolism^[10]. Autonomic system dysfunctions, characterized by lower vagal and higher sympathetic tone have been documented in chronic inflammatory diseases and aging, associated certain level of systemic inflammation^[25-28]. A correlation between higher vagal tone as determined by the increased frequency component of heart rate variability and lower proinflammatory cytokine levels has been reported^[27,28]. Cholinergic modalities, acting through vagus nerve and/or $\alpha 7$ subunit-containing nicotinic acetylcholine receptor-mediated mechanisms have been shown to suppress excessive inflammation in several experimental models of disease^[27]. In this context, numerous comorbidities frequently observed in the patients with autoimmune diseases, associated with viral and other infectious agents, including *T. gondii*, may be at least in part explained by the liver damage caused by the parasite^[9]. The hepatic injury probably establishes a favorable local tissue and systemic environment in the body that facilitates growth and proliferation of these infectious agents further intensified by the impaired cholinergic modulation of systemic inflammation. In addition, such patients are usually subjected to many invasive procedures, including endoscopy, surgery, dental care, otorhinolaryngologic investigation, and frequently receive intravenous medications, fluids, blood and/or its components, that all increase risk of viral, bacterial and/or parasite infections^[29,30].

Several authors reported that hepatitis with a clinical picture resembling acute viral hepatitis may result from *T. gondii* infection^[31-33]. T cell function in patients with Australia antigen (AA)-associated hepatitis has shown that persistence of this antigen was related to impaired PHA-induced lymphocyte transformation^[34,35]. It was suggested that viral hepatitis appeared unlikely to be the sole causal agent in primary biliary cirrhosis, because AA has been demonstrated in the serum of only 15-20% of patients^[36-38]. Because development and clinical course of these comorbidities are often associated with an increased production of various pro- and anti-inflammatory cytokines which are overlapping with T_H1 and T_H2 type of cytokines generated by *T.gondii* infection, such an excess of various factors and released biomediators may exert beneficial or harmful effects in the host, depending on their final constellations in the body. These suggestions are in agreement with recent reports that

emphasized an important role of the parasite in development of liver damage and *T. gondii*-related hepatitis^[39-41].

The above-presented data suggest that liver damage caused by latent chronic *T. gondii* infection has an important impact on the innate and acquired immunity of the host, development of various comorbidities and their clinical course, and finally on improvement/worsening of the host's health. The seroprevalence of anti- *T. gondii* IgG antibodies in various autoimmune diseases, including primary biliary cirrhosis have been presented in **Tables 4 and 5**. In these patients, impaired cellular immunity due to the infection with the parasite may also play an important role in triggering, development and persistence of these clinical entities^[62].

CRYPTOGENIC LIVER CIRRHOSIS (CLC)

This chronic liver disease with so far not established particular etiology accounts for 5-30% of cases of cirrhosis and about 10% of liver transplants^[63-65]. In Germany, presumed cryptogenic liver disease (probably an early phase of liver cirrhosis) was found to be associated with high prevalence of autoantibody-negative autoimmune hepatitis, low prevalence of nonalcoholic steatohepatitis (NASH), and no evidence for occult viral etiology^[66]. At present, it is believed that the leading causes of CLC include previously unrecognized NASH, silent autoimmune hepatitis, occult

Table 4 Serum IgG levels of anti-infectious agents antibodies more prevalent in primary biliary cirrhosis compared with controls (acc. to Shapira *et al*[42]; with own modification)

| Infectious agent | Patients with primary biliary cirrhosis | Controls | P value |
|--------------------------|---|----------------|----------|
| <i>T. gondii</i> (IU/mL) | 53.4 \pm 9.1 | 36.4 \pm 7.6 | < 0.001 |
| EBV-EA AI | 1.7 \pm 0.2 | 0.9 \pm 0.2 | < 0.0001 |

AI, antibody index. EBV-EA, Epstein-Barr virus - early antigen. Results are given as mean \pm SE. Anti-EBV-EA antibodies concentrations positively correlated with anti-*T. gondii* antibody levels in those patients (Spearman correlation $r = 0.35$, $p < 0.01$). A similar positive correlation was detected in PBC patients between anti-EBV-EA and CMV antibodies levels ($r = 0.32$, $p < 0.01$), which may suggest a secondary origin of their generation as compared with the primary and the same *T. gondii*-related IL-21 cytokine stimulus intensity required to control chronic viral infection^[43-45].

Table 5 Prevalence of anti-*T. gondii* IgG antibodies in 1514 serum samples of patients with various autoimmune diseases (acc. to Shapira *et al* [46]; with own modification)

| Disease/Clinical entity | Anti- <i>T. gondii</i> IgG positive | Geographical region | P-value ^c |
|--|-------------------------------------|---------------------|----------------------|
| Antiphospholipid syndrome (APS) ^a | 82/159 (52%) | Europe | < 0.0001 |
| Cryoglobulinemia ^b | 65/117 (56%) | Europe | < 0.0001 |
| ANCA-associated vasculitis | 45/68 (66%) | Europe | < 0.01 |
| Autoimmune thyroid diseases | 68/120 (57%) | Europe | < 0.0001 |
| Systemic sclerosis | 46/80 (58%) | Europe | < 0.0001 |
| Rheumatoid arthritis | 27/35 (77%) | Europe | < 0.0001 |
| Rheumatoid arthritis | 55/152 (36%) | Latin America | NS |

^a Including primary and secondary APS; ^b Including cryoglobulinemia and mixed cryoglobulinemia. ^c P values for the comparison with matched healthy controls of the same geographical region. In addition, the following specific autoimmune diseases were associated with markedly increased serum anti-*T. gondii* IgG antibodies/autoantibodies: polymyositis^[47], rheumatoid arthritis^[48-50], Hashimoto's and Graves' diseases^[51,52], Crohn disease^[30,53], antiphospholipid syndrome^[54], Wegener's granulomatosis and other vasculitides^[55], autoimmune bullous disease ^[56], systemic lupus erythematosus^[57], diabetes mellitus type 1 and 2^[16,53,58], optical nerve and retinal diseases with visual loss (the 22 kDa neuronal antigen found in the patients may represent *T. gondii* antigen^[59-61]).

non-B, non-C viral hepatitis (hepatitis X), and occult past ethanol exposure^[64]. Leite *et al*^[63] reported that in their 47 patients with CLC, the following prevalences were demonstrated: impaired fasting glycemia (68%), low HDL levels (58.1%), total hypercholesterolemia (27.9%), hypertriglyceridemia (16.3%), and obesity (27.5%). The results obtained in the patients with CLC showed statistical similarity with the data of the NASH group regarding fasting glycemia (62.8%) and HDL levels (53.8%, males)^[63]. Caldwell *et al*^[67] found that among their 70 consecutive patients with cryptogenic cirrhosis, both diabetes type 2 and obesity (73% of individuals) were significantly more common compared with the cirrhotic patients with primary biliary cirrhosis or hepatitis C. It must be noted that among 567 patients with chronic liver disease evaluated by Kodali *et al*^[68], 28 individuals (4.9%) had no established a definite etiology, and nearly half of the patients with presumed CLC (13 of them, 46%) had a history of previous blood transfusion, thus supporting the hypothesis of a non-A, non-B, and non-C hepatitis virus. This may suggest that latent *T. gondii* infection associated with blood transfusion plays at least in part an important role in development of cryptogenic liver disease, and support the indication of several authors for screening blood donors for serum AIAT as well as *T. gondii* IgG activities to reduce posttransfusion hepatitis^[68-73]. Moreover, it was found that the infection with the parasite significantly affected glycolysis, gluconeogenesis and tricarboxylic acid cycle metabolic processes^[74], and probably was responsible for development of type 1 and 2 diabetes, and obesity^[16,68,75]. These findings are therefore well in line with the suggestion that cryptogenic liver disease in children represents a novel congenital disorder of glycosylation^[76].

In a study of 354 patients with unexplained abnormal liver function tests and in the absence of diagnostic serology, liver biopsy showed some degree of fibrosis in 26% of individuals, and 6% were cirrhotic^[77]. Thirty four and 32% of biopsies suggested NASH or fatty liver, respectively. Other diagnoses included cryptogenic hepatitis, drug toxicity, secondary biliary cirrhosis, and autoimmune hepatitis^[77]. NASH was considered the commonest cause of cryptogenic cirrhosis^[67,78,79], also associated with diabetes and obesity in some individuals^[78]. In two other studies, cirrhosis of unknown etiology was found in 3-30% of individuals hospitalized because of this clinical entity^[80,81]. The so-called residual histological findings, such as foci of autoimmune-like inflammatory infiltrates versus NASH-like foci of steatosis, cellular ballooning, and glycogenated nuclei may finally help in defining the underlying cause of cryptogenic cirrhosis^[82-84].

Interestingly, subclinical liver involvement is frequent in systemic lupus erythematosus (SLE). Chowdhary *et al*^[85] identified 40 SLE patients with liver enzyme abnormalities, and biopsies performed in 20 individuals showed changes characteristic for nonalcoholic fatty liver disease ($n = 8$), autoimmune hepatitis (6), primary biliary cirrhosis (3), hepatitis C (3), and cryptogenic cirrhosis (2). Systemic sclerosis (SSc) is another autoimmune disease characterized by vascular obliteration, excessive extracellular matrix deposition, and fibrosis of the connective tissues of the skin, lung, gastrointestinal tract, heart, and kidneys. Endothelial cell dysfunction and damage represent one of the first disturbances in the pathogenesis of SSc, and chronic inflammation plays an important role in the development of atherosclerotic plaques^[86]. A 60-kDa protein belonging to the heat shock proteins (HSPs) was suggested to be involved in the pathomechanism of these abnormalities^[87]. It was demonstrated that Hsp60 bound lipopolysaccharide (LPS) tightly and it was the Hsp60-bound LPS, not Hsp60 itself, that was responsible for the observed cytokine effects of Hsp60 preparations^[88]. In addition, Hsp60-bound LPS was more

potent than LPS alone in inducing cytokine production^[88-91]. It must be noted that *T. gondii* HSPs, particularly Hsp60 and Hsp70 (Hsp70 provides cellular protection against oxidative stress and functions as a B cell mitogen) released mainly during bradyzoite transition are highly immunogenic, capable of inducing antibody production and T-cell activation^[61,92]. Each of these HSPs was found to bound a distinct set of peptides, including antigen peptides and its precursors^[93], and various HSPs have different efficiency to activate monocytes and dendritic cells^[94], as well as to release NF- κ B, NO and other pro- and antiinflammatory cytokines^[95,96]. Specifically, Hsp60 exerted immune stimulatory effects on vascular endothelium (upregulation of adhesion molecules), smooth muscle cells (IL-6)^[97], monocyte/macrophages inducing NO, IL-6, IL-12 and TNF- α production^[94,98,99], dendritic cells (IL-1 β , IL-6, IL-12, TNF- α maturation)^[94,100], B cells (proliferation, IL-6, IL-10, upregulation of MHCII, CD69, CD40, CD86^[101]). In this context, it should be emphasized that the proteins with molecular weights of 58 and 60 kDa have been demonstrated amongst excretory/secretory protein antigens released from purified tachyzoites/bradyzoites of *T. gondii* and various components of the parasite in samples of human and animal sera^[61].

Tweezer-Zaks *et al*^[102] found nine patients with cryptogenic cirrhosis comprising 0.15% of the familial Mediterranean fever (FMF) patient population. This rate was significantly higher than the rate of 0.015% of cirrhosis of all types expected in the total population of Israel ($p < 0.000$). FMF is a disorder presenting in childhood or adolescence with 1- to 3-day episodes of fever often accompanied by severe abdominal pain, pleurisy, monoarticular arthritis, or an erythematous rash on the ankle or foot known as erysipeloid erythema^[103]. Importantly, targeted disruption of pyrin, the FMF protein, caused heightened sensitivity to endotoxin and a defect in macrophage apoptosis^[104]. In addition, pyrin appeared to have both inhibitory and potentiating effects on IL-1 β production and played a role in regulating NF- κ B activation and apoptosis^[105,106]. It was suggested that in patients with FMF *T. gondii* infection plays an important role in the pathogenesis of Mollaret meningitis^[107]. Moreover, in human astroglia cells and in murine mast cells infected with *T. gondii* tachyzoites, the parasite induced upregulation of profibrotic factors, such as matrix metalloproteinase-2 and -9 (MMPs) via Erk1/2-NF- κ B pathway^[108], and MMPs are a family of the extracellular matrix-degrading enzymes that are generally synthesized and secreted as latent soluble enzymes requiring activation in the extracellular space^[109].

In summary, these data suggest that latent chronic *T. gondii* infection may be responsible for development of CLC. Abnormalities associated with CLC markedly affect acquired immunity of the host and probably participate in triggering and maintaining several autoimmune diseases, especially that anti- *T. gondii* IgG antibodies have been found in the sera of both patients suffering from many of these clinical entities and in healthy individuals^[46,110], which is in line with a cosmopolitan distribution of the pathogen.

MOLECULAR PATHOBIOLOGY OF HEPATIC FIBROSIS

Chronic inflammatory response is sometimes associated with the accumulation of extracellular matrix components including collagens, fibronectin and hyaluronic acid, and numerous fibrotic diseases affecting the liver, lung, skin, heart, and kidney, are believed to have an infectious etiology with bacteria, viruses, *T. gondii*, and fungi^[111]. Also, hepatic fibrosis may be associated with autoimmune liver disorders, including autoimmune hepatitis, primary biliary cirrhosis

or primary sclerosing cholangitis. Chemokines, such as for example, a profibrotic factor CXCL 10, promote hepatic inflammation and liver cirrhosis by prevention of NK cell mediated hepatic stellate cells (HSCs) inactivation, because the cytokine is responsible for the recruitment and localization of inflammatory cells to sites of tissue damage or infection^[112]. It must be noted that HSCs secrete CXCL 10 when stimulated with IFN- γ ^[112], and during *T. gondii* infection the cell numbers markedly increased^[40]. Moreover, Furtado *et al*^[113] found that human monocyte-derived dendritic cells infected with *T. gondii* tachyzoites transmigrated in larger numbers across stimulated human retinal endothelium than noninfected dendritic cells ($p < 0.004$). Antibody blockade of ICAM-1, VCAM-1, and activated leukocyte cell adhesion molecule inhibited transmigration, while chemokines CCL21 or CXCL 10 increased this process^[113].

Ustun *et al*^[114] investigated etiology of liver cirrhosis and frequency of *T. gondii* seroprevalence in 108 patients and found that toxoplasma IgG and IgM seropositivity was present in 74 (68.5%) individuals compared with 24 (48%) of 50 participants in the control group ($p < 0.05$) (Tables 6 and 7). A case-control epidemiological study demonstrated a link between *T. gondii* infection with liver cirrhosis^[115]. Atmaca *et al*^[40] showed that infection with the parasite in mice was associated with the increase in the number of HSCs. The number of these cells was highest on day 6 after inoculation of animals as compared with the numbers found on days 4 and 2 after inoculation, and in controls (all $p < 0.05$). There was also a significant relationship between the number of GFAP-positive HSCs when they were compared with *T. gondii* antigen immunostaining ($p < 0.05$), and the amount of immunostaining markedly increased with the increase in the number of HSCs^[40] (Table 8). HSCs are known to play an important role in development of fibrosis and its advancement to cirrhosis as indicated by their close proximity to collagen fibers in injured livers^[116], and finding as the main matrix-producing cells in the process of liver injury and fibrosis of any etiology^[41]. In the activation process of these cells, TGF- β was believed to be the main mediator of fibrogenesis, while platelet-derived growth

Table 6 Etiology of liver cirrhosis in 108 analyzed patients (acc. to Ustun *et al*[114]; with own modification).

| Etiology of liver cirrhosis | Number of patients | % |
|-----------------------------|--------------------|------|
| Hepatitis B | 37 | 34.3 |
| Hepatitis C | 27 | 25 |
| Alcoholic cirrhosis | 18 | 16.7 |
| Primary biliary cirrhosis | 12 | 11.1 |
| Unknown etiology | 9 | 8.3 |
| Autoimmune hepatitis | 5 | 4.6 |

Table 7 Seroprevalence of *T. gondii* IgG and IgM in patients with liver cirrhosis (acc. to Ustun *et al* [114]; with own modification).

| | No of pts | Age (yrs) | Sex (W/M) | IFAT and ELISA IgG(+) | IFAT and ELISA IgM(+) | Active disease significant with respect to | |
|-----------------|-----------|-----------------|-----------|-------------------------|-----------------------|--|-----------|
| | | | | | | IgG(+) | IgM(+) |
| Liver cirrhosis | 108 | 51.5 \pm 10.2 | 38/70 | 74 (68.5%) ^a | 2 | 31 (28.7%) ^a | 2 (1.85%) |
| Controls | 50 | 40 \pm 6.7 | 19/31 | 24 (48%) | 0 | 4 (8%) | 0 |

Results represent means \pm SD. ^a Statistically significant results compared with controls.

Table 8 Virulent *T. gondii* RH strain antigen expression in the hepatic stellate cells (HSCs) of Swiss albino mice infected intraperitoneally with 10⁵ tachyzoites and examined 2, 4, and 6 days after inoculation (DAI) (acc. to Atmaca *et al*[40]; with own modification)

| Antibody | Controls (n = 5) | 2 DAI (n = 5) | 4 DAI (n = 5) | 6 DAI (n = 5) | P |
|---|-------------------|-------------------|-------------------|-------------------|--------|
| No of GFAP-positive HSCs | 0.902 \pm 0.080 | 2.515 \pm 0.383 | 3.090 \pm 0.171 | 3.609 \pm 0.316 | < 0.05 |
| Amount of anti- <i>T. gondii</i> antigen immunostaining | 0 | 3.538 \pm 0.440 | 4.136 \pm 0.218 | 4.632 \pm 0.337 | < 0.05 |

Results represent means \pm SD; n, number of animals. GFAP, glial fibrillary acidic protein. The Mann-Whitney U-test was used to compare GFAP-immunoreactive cells and anti-*T. gondii*-immunopositive areas in the liver between the parasite-infected groups and controls. The relationship between HSCs and the parasite antigens were assessed using the Kruskal-Wallis test.

factor (PDGF) was the major inducer of HSCs proliferation^[117-119]. Also leptin has been reported as a novel profibrogenic cytokine in HSCs^[120]. [Nb. in rats *T. gondii* infection caused a significant increase of plasma leptin concentration ($p < 0.01$), with no change in body weight of animals^[121]; in obese individuals, leptin exerted a proinflammatory activity, and its structure is similar to that of IL-2, an important proinflammatory cytokine (Table 9)].

Activated HSCs differentiate from vitamin-A-storing cells to contractile myofibroblastic cells responsible for extracellular matrix production in the damaged liver^[116]. HSCs may also interact with the sinusoidal space, thus affecting function of the antigen presenting cells, such as dendritic cells, liver macrophages or Kupffer cells^[126]. It must be noted that in several patients HSCs activation and tissue inhibitor of metalloproteinase overexpression persisted even after eradication of the underlying known cause of liver injury, preventing resolution of fibrosis, such as for example HCV infection^[127,128]. This observation is in a full agreement with the recent case-control study demonstrating that high serum anti-*T. gondii* IgG concentrations significantly correlated with high HCV load levels among chronic hepatitis C patients ($p = 0.026$)^[129], and with my reasoning suggesting an important role of chronic latent *T. gondii* infection in several such unresolved cases, especially that both PDGF and TGF- β also have been reported to play an important role in the pathobiology of the parasite^[130-132]. For example, *T. gondii* increased production of TGF- β , activated hypoxia-inducible factor 1 (HIF1) already at physiologically relevant oxygen levels, and required HIF1 for growth and survival^[133,134]. The human platelet contribution against

Table 9 Serum proinflammatory cytokines and leptin concentrations in obese children at prepubertal age compared with healthy children of the same age (acc. to Aygun *et al*. [122] and Kapiotis *et al*. [123]; with own modification)

| Parameters | Obese children | Controls | P |
|-----------------------------|-----------------|----------------|---------|
| Leptin (ng/mL) ^a | 19.9 \pm 7.4 | 7.9 \pm 5.1 | < 0.001 |
| IL-1 β (pg/mL) | 33 \pm 8.9 | 3.6 \pm 1 | < 0.001 |
| IL-2 (U/L) | 0.4 \pm 0.1 | 0.9 \pm 0.1 | < 0.01 |
| IL-6 (pg/mL) | 45.2 \pm 11.8 | 13.1 \pm 3.9 | < 0.001 |
| TNF- α (pg/mL) | 9.2 \pm 2.3 | 3.9 \pm 1 | < 0.001 |
| E-selectine (ng/mL) | 78 \pm 38 | 59 \pm 29 | < 0.01 |
| hsCRP (mg/L) | 4.1 \pm 4.8 | 0.9 \pm 1.5 | < 0.001 |

Results are mean \pm SD; CRP, C-reactive protein; hs, high-sensitivity. ^a Leptin has the structure similar to that of IL-2 and may activate the innate immune system and shift the cognate immune system toward a predominance of a proinflammatory T_H1 T cell population while reducing the regulatory T_H2 phenotype [124]. Leptin treatment was found to increase energy expenditure (oxygen consumption), as well as increased thermogenic marker uncoupling protein-1 and type II deiodinase mRNA levels 1.7- and 3-fold, respectively, in mice [125].

the intracellular growth of *T. gondii* *in vitro* in human pulmonary fibroblasts showed that tachyzoites of the parasite induced activation of human platelets, and PDGF mediated inhibition of the intracellular growth in a virulent *T. gondii* strain^[135]. It was found that human platelet to the parasite ratios as low as 1:3 were toxic to the protozoan with distinct cell-cell contact essential for platelet-mediated cytotoxicity^[135]. Adherence of platelets to *T. gondii* and disruption of surface membranes and cytoplasmic contents of the organisms were documented ultrastructurally^[136]. Thromboxane (TX) played an important role in these processes because both the TXA₂-generating platelet microsome system and a stable TXA₂ analog-induced damage to the cellular membranes of the parasite, as noted by transmission electron microscopy^[136]. Recently, Hendersan *et al.*^[137] showed that *T. gondii*-stimulated platelets released oxygenated products of both (13-hydroxyoctadecadienoic acid - 12.3%), that also may be of importance for the cytotoxic host defense response against infection with the parasite.

BALLOONED HEPATOCYTES AND MALLORY-DENK BODIES (MDBs)

Hepatocellular “ballooning” is an important histological hallmark of liver pathology found particularly in steatohepatitis manifested by degeneration of hepatocytes associated with enlargement, swelling, rounding, and characteristic reticulated cytoplasm^[138,139]. These changes in hepatocyte morphology may be observed also in many other acute and chronic liver diseases, including autoimmune hepatitis, neonatal giant hepatitis, acute viral hepatitis, both alcoholic and nonalcoholic steatohepatitis, and ischemia/reperfusion injury of liver allografts^[138-141]. The intermediate keratin filaments (IF) play an important role in the stabilization and topographical localization of a cell and its organelles, and a pronounced diminution or even less of keratin 8/18 was found in the IF cytoskeleton of ballooned hepatocytes^[138]. MDBs are an intracellular deposition of misfolded proteins (keratins, chaperones Hsp90 and 25 families, α -B crystallin, phosphoepitopes/kinases, protein degradation machinery, and others, such as ubiquitin, tubulin, as well as intracytoplasmic hyaline inclusions, glycogen bodies, ground glass inclusions (hallmarks of chronic HBV infection reflecting hypertrophic endoplasmic reticulum), pale bodies, α_1 -antitrypsin inclusions and megamitochondria^[142]. Sometimes MDBs appear as numerous small globular structures distributed throughout the cytoplasm, near nucleus, or localized at the cell periphery (early MDBs = small cytoplasmic globular structures; mature MDBs = large paranuclear inclusions; old MDBs = involution stage present at the cell periphery)^[142]. Interestingly, IF cytoskeleton damage was shown to result from oxidative stress in various human liver diseases and in the mouse models of steatohepatitis^[142-144]. It seems that *T. gondii* infection may be at least partly responsible for development of these abnormalities because Yang *et al.*^[145] showed that in mice injected intraperitoneally with 2.5×10^3 *T. gondii* tachyzoites, the serum level of the oxygen free radicals (NO, *OH, O²⁻) increased along with the days of infection, and superoxide dismutase concentration reached a peak on the 3rd day of the investigation. In asymptomatic *T. gondii*-seropositive blood donors oxidative stress and immunosuppression also have been demonstrated^[70,71]. Moreover, it was found that oxidative stress with markedly increased serum malondialdehyde and decreased glutathione levels is characteristic for *T. gondii* seropositive patients^[69,146] and chickens orally infected with tissue cysts of the protozoan^[147] (Tables 10 and 11).

Hepatic steatosis is caused by the abnormal accumulation of predominantly triglycerides in the liver^[148]. Histological examinations of liver parenchyma revealed presence of triglyceride droplets in the cytoplasm of hepatocytes and their accumulation in small and large vesicles which impairs liver function and makes this organ more susceptible to other injuries^[148]. Immunochemical analysis of steatohepatitis performed by Lackner *et al.*^[138] showed that in macrovesicular steatosis the IF cytoskeleton was only pushed to the cell periphery by the accumulated triglycerides, as it was also observed in all their cases of steatosis. In alcoholic steatohepatitis, ballooning degeneration of hepatocytes can at least partly be explained by the finding that ethanol and its metabolite acetaldehyde elevated intracellular concentration of Ca²⁺ by mobilizing the ion from intracellular stores^[149]. Moreover, ethanol dose-dependently stimulated microneme secretion in *T. gondii* tachyzoites and parasite apical attachment to host cells, thus facilitating infection of hepatic cells^[149]. Ethanol is known to have several targets in a variety of vertebrate cells by affecting signal transduction^[150], such as for example activation of phosphatidylinositol-specific phospholipase C in hepatocytes^[151], and triggered amylase secretion from pancreatic acini by increasing Ca²⁺ levels^[152], and therefore together with oxidative stress could be the cause of marked disturbances in function and morphology of the host cells. Furthermore, liver microsomal fatty acid oxidizing enzymes, such as cytochrome P450 CYP2E1 expression are increased in both alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD). Since CYP2E1 also metabolizes ethanol^[153], fatty acid-related induction of this enzyme contributes to generation of acetaldehyde, which forms immunogenic adducts^[154] with various intra-, and extracellular biomolecules and exacerbates reactive oxygen species production in AFLD^[155]. In this context, the prevalence of non-organ-specific autoantibodies (NOSAs) and chronic liver disease in general population^[156,157], NOSAs as the hallmark of a subclinical autoimmune disease^[156,158,159], the exacerbation of autoimmune chronic hepatitis by interferon therapy^[160], the presence of autoantibodies to cytoskeletal filaments, actin and intermediate filaments in chronic active hepatitis^[158], as well as the evidence against hepatitis viruses as important causes

Table 10 Levels of malondialdehyde (MDA) and glutathione (GSH) in aorta and heart of broiler chickens infected intraperitoneally with 50 tissue cysts of *T. gondii* (acc. to Al-Kennany[147]; with own modification).

| Groups | MDA (nmol per gm wet tissue) | | GSH (mmol per gm wet tissue) | |
|----------|------------------------------|---------------------------|------------------------------|---------------|
| | Aorta | Heart | Aorta | Heart |
| Controls | 125 ± 1.05 | 105 ± 0.33 | 0.622 ± 0.022 ^a | 0.773 ± 0.026 |
| Infected | 529.8 ± 2.32 ^a | 455.3 ± 0.43 ^a | 0.273 ± 0.01 | 0.421 ± 0.031 |

Results are expressed as means ± SD of 10 broiler chickens per group. ^aSignificant differences at $p \leq 0.05$.

Table 11 Serum glutathione, malondialdehyde, and NO concentrations in *T. gondii*-seropositive patients and healthy controls (acc. to Karaman *et al.*[69]; with own modification).

| Bioparameter | Group | No of participants | Serum levels (mean ± SD) | P values |
|-----------------|----------|--------------------|--------------------------|----------|
| Glutathione | Patients | 37 | 3.96 ± 0.10 | 0.001 |
| | Controls | 40 | 10.37 ± 0.13 | |
| Malondialdehyde | Patients | 37 | 41.32 ± 2.05 | 0.001 |
| | Controls | 40 | 9.18 ± 1.21 | |
| NO | Patients | 37 | 47.47 ± 1.00 | 0.001 |
| | Controls | 40 | 39.18 ± 1.29 | |

Serum glutathione and NO levels are expressed as $\mu\text{mol/L}$, and malondialdehyde concentrations represent nmol/L . Results statistically significant at $p < 0.05$.

of autoimmune hepatitis^[161], are all well in line with worldwide dissemination of the parasite and modulation of the host cell proteome by the intracellular pathogen (*e.g.* cytoskeleton-associated protein 4, actin filament, actin beta, actin gamma 1 propeptide, actinin-1 alpha, etc.^[74]). A similar profuse generation of antibodies and autoantibodies directed against brain proteins in patients with autism and their families probably associated with *T. gondii* infection^[61] also seems to further support the above-presented reasoning.

Studies in animals showed that oral infection with *T. gondii* resulted in different quantitative and morphologic changes in myenteric plexus neurons. For instance, in rats no changes in the population or density of these cells were observed^[162,163], while there was an intense myenteric plexus neuronal cells death and atrophy reported in chicken^[164]. On the other hand, in pigs orally infected for 30 days with oocysts of *T. gondii* III (M7741 strain) Odorizzi *et al.*^[165] showed that the number of nitrergic NADPHd-p neurons per ganglion markedly increased, and the cells became hypertrophic through the augmentation of the cell body by 12.8% ($p < 0.0001$), and specifically through the increase of the nuclear area by 24.8% ($p < 0.0001$) (no change was observed in the distribution among different classes of neuronal cell sizes) (Tables 12 and 13). Because NO play an important role in resistance of the host cells against *T. gondii* infection^[166,167], it was suggested^[165] that the cell hypertrophy may be indicative of the increased generation of biochemical mediator(s) with antiparasitic activity that may simultaneously exert a detrimental effect on the tissue cells containing parasites^[164,168]. This reasoning is in line with the above argumentation that the oxidative stress associated with *T. gondii* infection play an important role in development of disturbances of the host cell morphology.

Finally, one may suggest that MDBs and the intracellular hyaline bodies observed in neoplastic and nonneoplastic hepatocytes^[142-144] are linked with latent chronic intracellular *T. gondii* infection. These peculiar abnormalities probably represent the remnants of tachyzoites/bradyzoites/sporozoites/oocysts, such as for example amylopectin, lipid droplets, PV membranes, etc., biotransformed by changing (especially acidic) pH of the inflamed liver tissue and various enzymes of both the host cell and the parasite itself, like it was suggested for the amyloid plaques produced in the brain cells of patients with Alzheimer's disease^[15]. Interestingly, Mayer *et al.*^[169] also suggested that MDBs resemble inclusions bodies associated with chronic neurodegenerative diseases, including Alzheimer's and Parkinson's diseases.

STEATOHEPATITIS

T. gondii is a sterol-auxotrophic organism incapable of sterol synthesis and therefore scavenges cholesterol from mammalian host cells^[170]. Similarly as in mammalian cells, most of this lipid is concentrated at the plasma membrane of the parasite^[171]. Cholesterol is a vital part of membranes but its overabundance in cells leads to inflammation and oxidative damage^[170]. Mammalian cells as well as the parasite, express two cholesteryl ester (CE)-synthesizing enzymes, ACAT1 and ACAT2, which share 44-47% of amino acid homology^[170,172]. ACAT1 is a ubiquitous and is responsible for CE formation in brain, adrenal glands, macrophages, and kidneys, while ACAT2 is expressed in the hepatocyte as the major cholesterol esterifying enzyme in human liver^[173] and in the small intestine villi^[174]. Free cholesterol (a toxic polar lipid) is also esterified by ACAT2 to CEs (nonpolar compounds)^[172]. ACAT2 plays a pivotal role in dietary cholesterol absorption^[175]. In mammalian cells, the main stored neutral lipids are triacylglycerol and CEs, which are produced by two related enzymes, acyl-CoA: diacylglycerol acyltransferase (DGAT)

Table 12 Median and P25, P75 percentiles of the cell body, nucleus, and cytoplasm areas, and the nucleus/cell body area ratio of NADHD-p myenteric neurons of the jejunum in pigs orally infected with oocysts of *T. gondii* type III (M7741 strain) and examined 30 days post inoculation (acc. to Odorizzi *et al.* [165]; with own modification)

| Cell components | NADPHd-p | | NADHD-p | |
|------------------------------------|-------------------------|--------------------------------------|-------------------------|--------------------------------------|
| | CG | EG | CG | EG |
| Cell body area (μm^2) | 633.1 (475.0; 837.4) | 714.2 (543.9; 921.2) ^a | 392.1 (265.1; 557.5) | 388.9 (270.3; 566.4) |
| Nucleus area (μm^2) | 144.8 (99.5; 207.5) | 180.1 (130.4; 263.1) ^a | 101.7 (78.3; 126.1) | 94.3 (73.1; 120.0) ^a |
| Cytoplasm area (μm^2) | 473.9 (342.3; 643.5) | 516.5 (393.2; 669.9) ^a | 282.0 (174.5; 441.8) | 286.4 (183.7; 459.8) ^a |
| Nucleus/cell body area ratio | 0.24 (0.17; 0.31) | 0.27 (0.21; 0.32) ^a | 0.26 (0.19; 0.35) | 0.24 (0.17; 0.32) ^a |

CG, control group; EG, experimental group. The animals from the EGs started to present diarrhea and had positive serum antibodies against *T. gondii* from day 13 to day 30 post infection. NADHD-p, dihydronicotinamide adenine nucleotide diaphorase positive; NADPHd-p, dihydronicotinamide adenine dinucleotide phosphate diaphorase positive. ^a Values significantly different compared with respective controls ($p < 0.05$).

Table 13 Degree of correlation between the cell body, nucleus and cytoplasm areas of NADPHd-p and NADHD-p myenteric neurons of the jejunum in pigs orally infected with oocysts of *T. gondii* type III (M7741 strain) and examined 30 days post inoculation (acc. to Odorizzi *et al.* [165]; with own modification)

| Cell components | NADPHd-p | | NADHD-p | |
|-----------------------|----------|------|---------|------|
| | CG | EG | CG | EG |
| Cell body x nucleus | 0.56 | 0.73 | 0.62 | 0.54 |
| Cell body x cytoplasm | 0.93 | 0.95 | 0.98 | 0.98 |
| Nucleus x cytoplasm | 0.29 | 0.51 | 0.48 | 0.4 |

CG, control group; EG, experimental group. NADHD-p, dihydronicotinamide adenine nucleotide diaphorase-positive; NADPHd-p, dihydronicotinamide adenine dinucleotide phosphate diaphorase-positive.

and acyl-CoA:cholesterol acyltransferase (ACAT), respectively, and both can contribute to membrane biogenesis^[176,177]. Both enzymes ACAT1 and ACAT2 utilize cholesterol or oxysterols but different acyl-CoA (ACAT1 - 20:4; ACAT2 - 16:0,18:1 18:2)^[157].

T. gondii enzymes also can synthesize and store both abundant esters of cholesterol (*TgACAT1* and *TgACAT2*)^[170,178] and triglycerides (*TgDGAT1*)^[176].

Cholesteryl oleate C18:1 (42%) and palmitate C16:0 (26%) are the main esters in mammalian cells, but the parasite also has uniquely large amounts of cholesteryl eicosanoate C20:1 (7%), and lesser amounts of cholesteryl palmitoleate C16:1, stearate C18:0, linoleate C18:2, arachidonate C20:4, and some polyunsaturated C22 fatty acids^[170]. It should be noted that both these enzymes (ACAT1 and ACAT2) are endoplasmic reticulum (ER)-resident proteins. Interestingly, the MDBs were reported to contain ground glass inclusions described as the hallmarks of chronic HBV infection reflecting hypertrophic ER^[142], whereas ACAT2 produced CEs that were assembled just in the ER^[170]. One cannot therefore exclude that CEs accumulated in the hypertrophic ER of the patients with HBV infection^[142], at least in part were the result of abundant *TgACAT2* activity in the hepatic cells of those individuals who had a concomitant subclinical *T. gondii* infection.

The fact that mammalian ACAT2 expression is restricted to hepatocytes and intestinal mucosal cells^[170,178], is very important because it strongly supports our earlier suggestion that peroral *T. gondii* infection plays an active role in the pathogenesis of "clear cell" colitis with foamy macrophages present in intestinal tissues^[53]. Moreover, the ACAT2 expression in the hepatocytes probably is also involved in the development of nonalcoholic steatohepatitis because *TgACAT2* produces more CEs than *TgACAT1* and has broader fatty

acid specificity^[170].

T. gondii scavenges cholesterol from plasma low density lipoproteins (LDL) by rerouting host lysosomes to its PV, and further internalizes cholesterol using membrane-associated transport proteins^[180]. Host LDL and fatty acids scavenged by the parasite can serve as ACAT activators, resulting in stimulation of CEs synthesis and lipid droplet biogenesis in the body of the protozoan^[170]. It was demonstrated that *T. gondii* strain with genetic ablation of ACAT2 exhibited large deposits of osmophilic material, likely lipids, both in the PV lumen and in the cytoplasm, thus reflecting gross lipid disorders as a consequence of perturbation of the balance between CEs and free cholesterol^[170]. A similar disturbed balance can therefore occur during chronic latent *T. gondii* infection because in this biosituation both ACAT1 and ACAT2 enzyme activities in the infected host hepatocyte and intestine cells will be overlapping with the activity of *TgACA T1* and *TgACAT2*, thus accumulating a significant surplus of their activities that finally lead to the development of steatohepatitis, as well as generation of foam cells (Table 14).

It was demonstrated *in vitro* that profusion of LDL in the culture medium lead to massive uptake of cholesterol by the parasite, suggestive of uncontrolled uptake of this lipid by *T.gondii*^[170]. The parasite was competent to synthesize CEs using host cell-derived cholesterol and fatty acid, and the production was proportional to the amount of lipid taken up by the protozoan over the time^[178,181]. It appeared that several FFAs were incorporated into parasite cholesteryl ester, suggestive of broad fatty acid specificity, but nearly twofold higher incorporation of palmitate into cholesteryl ester as compared with oleate, arachidonate, stearate, and linoleate was observed^[176,178] (Tables 15 and 16). This preferential palmitate incorporation specificity by *T. gondii* acyl-CoA: diacylglycerol acyltransferase (*TgDGA T1*) has already been found also for triacylglycerol synthesis in the parasite^[182], and *TgDGAT1* is an integral membrane protein localized to the parasite cortical and perinuclear endoplasmic reticulum^[176]. The protozoan was able to take up both oleate and diacylglycerol and incorporate them into the triacylglycerol fraction, and oleate was incorporated into diacylglycerol (Table 17).

Lige *et al*^[170] suggested that the parasite scavenges LDL-cholesterol by uncontrolled uptake mechanisms, and engorges itself with cholesterol proportionally to the amount of the lipid in the environment. One may therefore speculate that this abnormality finally manifest histopathologically as steatohepatitis, and morphologically represent cholesterol ester-laden macrophages/foam cells, because the number and the size of lipid bodies in *T. gondii* vary between the different parasite developmental stages (tachyzoites, bradyzoites, oocysts), environmental conditions^[170,183], infected cells/tissues of the host, and intensity of oxidative stress associated with the infection. Moreover, proliferation of the parasite in the liver cells suppresses host cell autophagy in that organ^[184] (as well as in other cells^[185]), leading to abnormally high levels of cholesterol along with aberrant triglycerides deposition because of defective clearance of lipid droplets^[186,187].

Interestingly, the activities of ACAT1 and ACAT2 can be stimulated in mutant parasites in response to accumulated free cholesterol. Concomitant to the increase in CEs content, the number and size of lipid bodies are dramatically enhanced in mutant parasites, probably to collect excess CE^[188], and thereby providing a “safety sink” for toxic free cholesterol^[170]. It should however be added that in healthy individuals lipid bodies found in the cytoplasm of a variety of cells, including leukocytes, macrophages, play an important role in modulating innate immune defense response

Table 14 Uptake of cholesterol by MEF ACAT1^{-/-} cells transfected with *TgACAT1* or *TgACAT2*, and incorporation into cholesteryl esters (acc. to Lige *et al* [170]; with own modification)

| MEF ACAT ^{-/-} transfected with | Cholesterol uptake (cpm x mg cell protein ⁻¹) | Cholesterol ester levels (cpm x mg cell protein ⁻¹) |
|--|---|---|
| Vector | 3991 ± 601 | ND |
| <i>TgACAT1</i> | 4003 ± 510 | 898 ± 65 ^a |
| <i>TgACAT2</i> | 3758 ± 436 | 1514 ± 110 ^b |

MEF ACAT^{-/-} were transfected with vector alone, or plasmids containing either *TgACAT1* or *TgACAT2*. Cells were then incubated with [³H] cholesterol-LDL. Cholesterol uptake was monitored by scintillation counting. Cholesterol ester detection was performed by thin-layer chromatography analysis. The results are mean values ± SD from three incubations (^a vs ^b is statistically significant, *p* < 0.01). MEF, mouse embryonic fibroblasts. ND, not detected. CPM, counts per minute.

Table 15 Comparison of free fatty acids (FFAs) uptake by *T. gondii* and CHO cells (acc. to Quittnat *et al* [176]; with own modification).

| | Oleate | Palmitate | Stearate | Linoleate | Arachidonate |
|------------------|------------|-------------------------|------------|-----------|--------------|
| <i>T. gondii</i> | 780 ± 99 | 1833 ± 105 ^a | 810 ± 69 | 750 ± 91 | 767 ± 77 |
| CHO cells | 1230 ± 142 | 1156 ± 101 | 1467 ± 151 | 1350 ± 98 | 1058 ± 103 |

CHA, Chinese hamster ovary. Extracellular parasites or CHO cells were incubated for 1 h at 37°C with the indicated 0.3 mM radioactive FFAs. After washing, the total FFAs uptake was determined by measuring the cell-associated radioactivity and expressed in nmol per mg cell protein/h. Results are means ± SD of four separate experiments. Differences between values of the uptake of palmitate vs. other FFAs in *T. gondii* are statistically significant. ^a *p* < 0.005.

Table 16 Fatty acid specificity for triacylglyceride (TAG) synthesis in *T. gondii* (acc. to Quittnat *et al* [176]; with own modification)

| | Relative activities | | | | |
|------------------|----------------------------------|-------------------------------------|------------------------------------|-------------------------------------|--|
| | [³ H]oleate + oleate | [³ H]oleate + palmitate | [³ H]oleate + stearate | [³ H]oleate + linoleate | [³ H]oleate + arachidonate |
| <i>T. gondii</i> | 1 | 0.18 | 0.69 | 0.5 | 0.61 |
| CHO cells | 1 | 0.52 | 0.62 | 0.55 | 0.59 |

CHO, Chinese hamster ovary. Extracellular *T. gondii* and CHO cells were incubated for 1 h at 37°C with 10 nM of radioactive oleate (~50,000 dpm per nmol) previously mixed with 0.3 mM of the indicated unlabeled FFAs to measure its incorporation into TAG. The production of TAG with oleic acid alone was 45,950 cpm per mg cell protein for *T. gondii*. The values expressed as relative activities, are means of three separate experiments.

Table 17 Lipid acquisition and substrate incorporation into n-acylglycerides of *T. gondii* (acc. to Quittnat *et al* [176]; with own modification).

| | Lipid uptake (cpm per mg cell protein) | Lipid synthesis (cpm per mg cell protein) | |
|-------------------------|--|---|--------------|
| | | DAG | TAG |
| [³ H]Oleate | 78589 ± 190 | 10444 ± 1874 | 32400 ± 3880 |
| [¹⁴ C]DAG | 58430 ± 3209 | 39650 ± 4705 | 23664 ± 2099 |

DAG, diacylglycerol; TAG, triacylglycerol; cpm, counts per minute. Extracellular *T. gondii* were incubated for 1 h at 37°C with radioactive oleate or DAG before measurement of total lipid associated to parasites and lipid incorporation into DAG or TAG. The values are means ± SD of three separate experiments.

to bacterial and parasitic infections^[189], because they are sites of enzymes localization, cytokine storage, eicosanoid production, etc., and function as intracellular domains of biomediators synthesis and secretion during inflammation^[190,191].

HEPATIC IRON OVERLOAD IN STEATOHEPATITIS

Patients with nonalcoholic steatohepatitis and chronic viral hepatitis had increased serum iron levels and hepatic iron overload^[194]. This finding is consistent with cytotoxic actions of NO produced by activated macrophages and a model of glucose-dependent

NO-mediated iron mobilization from cells proposed by Watts & Richardson^[193]. They showed that NO intercepted iron before incorporation into ferritin and directly mobilized iron from ferritin in a glutathione-dependent manner. It should be emphasized that NO is produced in excess by the host cells as an important defense molecule directed against *T. gondii* infection^[132,194,195]. On the other hand, iron is also necessary for multiplication of tachyzoites^[132], and therefore overloading with iron is associated with persistent generation of a *circulus viciosus*, detrimental for the host. Moreover, the excessive iron accumulation in liver causes hepatocellular injury and fibrosis^[196-198], because reactive iron species produced in hepatocytes result in iron-dependent microsomal peroxidation of polyunsaturated fatty acids^[199,200] and lipocyte activation/lipocyte fibrogenesis^[192,201,202]. Similar abnormalities in iron and copper metabolism play a crucial role also in the pathogenesis of various neurodegenerative diseases^[203,204], and this may further support an important role of *T. gondii* infection in development of these disturbances.

FOAMY MACROPHAGES AND FOAMY COLITIS

Characteristic foamy macrophages demonstrated in histopathological specimens are an important diagnostic factor in foamy colitis, and confirm mild and moderate course of that type of inflammation in children^[205-207]. The induction of foamy macrophages packed with lipid bodies have been reported in several clinical pathologic states associated with chronic proinflammatory stimuli, including *T. gondii*^[208] and another intracellular human pathogen, *Mycobacterium tuberculosis* infections^[209]. Development of foamy colitis (microscopic colitis)^[205] may be due to oral infection with *T. gondii*. The parasite employs host low-density lipoproteins (LDL) receptor to acquire cholesterol^[207] and diverts it for cholesteryl ester synthesis and storage in lipid bodies^[178,189,210], leading to the formation of foam cells^[208]. Macrophages convert into foam cells through a dysregulation in the balance between the influx and efflux of LDL particles (containing cholesterol, triacylglycerides and phospholipids) from the serum. Foamy macrophages are not only the product of an inflammatory response but amplify that response through production of PGE₂ and leukotrienes^[211,212], and appeared to be a key player in both sustaining persistent bacteria and contributing to development of human tuberculosis granuloma and cavitation^[209]. Furthermore, in various types of cells, such as for example Kupffer cells, hepatocytes, vascular smooth muscle cells, the increased generation of PGE₂ inhibited cytokine-stimulated NOS type 2 mRNA expression and NO synthesis in a concentration-dependent manner^[213-215]. The effect of the prostaglandin was also associated with the number of macrophages^[216]. All these disturbances could therefore affect innate and acquired immune responses of the host directed against oral prenatal or postnatal *T. gondii* infection, and result in generation of foamy macrophages and development of foamy colitis^[53].

Frequent occurrence of a cryptogenic hypertransaminasemia in celiac patients (celiac hepatitis) reverting to normal after a few months of gluten withdrawal, as well as the presence of celiac disease in patients with primary biliary cirrhosis, autoimmune hepatitis, and primary sclerosing cholangitis^[217], are in agreement with the above-presented reasoning^[53]. Beneficial effects of diet with no gluten in patients with celiac disease may be explained by the fact that gluten induces a profuse intestinal cytokine response markedly predominated by IFN- γ (about 1000-fold in untreated disease)^[218,219], and simultaneously there is also a significantly increased production

of IFN- γ and other proinflammatory cytokines representing the host defense against concomitant latent *T. gondii* infection^[166,167]. This overlapping excess of generation of interleukins in specific tissues of the host may finally be very destructive.

It must be emphasized that the presence of anti-tissue transaminase antibodies in patients with abnormal liver tests may not always solely suggest celiac disease^[220,221]. Such comorbidities may be potentially harmful/beneficial for both humans and experimental animals because infecting pathogens can modulate inflammatory responses and final clinical outcome^[3,53,222,223]. Moreover, certain medicaments, such as for example disodium cromoglycate used in the patients with allergy, decrease activation of mast cells, and therefore attenuate parasite burden and decrease tissue lesions because its antihistaminic properties affect T_H1/T_H2 cytokine balance in the host^[3,224]. Moreover, in animals infected with RH strain of the parasite this drug decreased *T. gondii* tachyzoite surface antigen 1 gene expression in spleen and liver tissues^[225].

In summary, literature data suggest that latent chronic *T. gondii* infection plays an important role in triggering and maintaining disturbances of so far unexplained liver function and histopathologic abnormalities observed in many clinical entities and healthy subjects. It seems therefore that individuals with clinical symptoms of liver disease and associated laboratory irregularities that are not helpful in establishing proper diagnosis, should have tests for the parasite routinely performed and receive specific pharmacologic treatment. Hypocholesterolemic properties of citrus flavonoids, especially naringenin and hesperetin, may help in reducing activity and expression of ACAT1, ACAT2 and microsomal triglyceride transfer proteins, thus limiting harmful effects of triglyceride accumulation in the endoplasmic reticulum lumen of the host cells^[226-229]. In addition, cysteine protease inhibitors that block *T. gondii* microneme protein secretion, gliding motility, and host cell invasion may represent potent anti-parasitic agents capable of curing infections *in vivo*, including the chick embryo model of toxoplasmosis^[230-232]. Moreover, the parasite cathepsin B and L proteins may represent potential novel DNA vaccine antigens against toxoplasmosis^[233], especially that cathepsin B has recently been identified as β -secretase for the production of β -amyloid peptides that accumulate in Alzheimer's disease^[234] (this is in line with the above-mentioned suggestion that similar biochemical processes participated in the generation of amyloid present in MDBs). Interestingly, phenytoin (an antiepileptic drug) and cyclosporine A (an immunosuppressant) suppress the expression and activity of cathepsin L and B in cultured gingival fibroblasts^[235,236]. Moreover, these two drugs stimulated TGF- β and cathepsin B production and inhibited expression of matrix metalloproteinase-1 by fibroblasts^[236,237]. These are very important findings because they represent a novel mechanism of action of these two medicaments, and the above-presented reasoning is supported by the fact that chronic *T. gondii* infection was suggested to be the etiologic agent in cryptogenic epilepsy^[238,239]. Recently, two groups^[240,241] published clinical data supporting my suggestions that toxoplasmosis is a critical risk factor responsible for development of liver cirrhosis. In addition, an important role of *T. gondii* in the generation of nonalcoholic fatty liver disease has been suggested in a population-based studies^[242]. The increasing prevalence of unexplained liver abnormalities^[243], and overweight and obesity growing dramatically all over the world^[244], are well in line with wide geographical distribution of the parasite. High burden of liver diseases and low awareness of toxoplasmosis amongst healthcare providers reported even in the United States require more effective public health actions^[245,246].

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