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Review Article
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# *T. gondii* Infection Acquired during Pregnancy and/or after Birth may be Responsible for Development of both Type 1 and 2 Diabetes Mellitus

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## Abstract

Recently, it was suggested that maternal T and potentially B cells transferred during pregnancy and/or the breast milk feeding and their encounter with the antigen in mesenteric lymph nodes might play a role in development of type 1 diabetes mellitus (T1DM). *T. gondii* infection during gestation and/or after birth may be responsible for development of both T1DM and T2DM in children, adolescents and adults because: a) maternal microchimerism in peripheral blood was demonstrated to be significantly higher in patients with T1DM compared to unaffected siblings and healthy subjects, b) transmission of *T. gondii* as a Trojan horse in various types of eukaryotic cells, including T and B lymphocytes, c) swallowing by the fetus of amniotic fluid containing infected leukocytes and other cells, d) elimination of *T. gondii* in the breast milk during lactation, e) involvement of mesenteric lymph nodes after oral infection with the parasite, and f) damage of the myenteric neurons during infection with the parasite in both the animals with streptozotocin-induced diabetes and diabetic patients. Moreover, a significantly lower occurrence of antibodies against *T. gondii* found in the sera of patients with T1DM compared with their first-degree family members or healthy controls may be due to their T and/or B cell exhaustion perspective caused by chronic infection with the parasite. This suggestion may be supported by the finding that latent toxoplasmosis was associated with markedly reduced lymphocyte B-cell counts responsible for production of antibodies, markedly lower serum IgG, IgM, and IgA levels, and a significant suppression of IL-2. On the other hand, patients with T2DM had increased anti-*T. gondii* antibodies significantly more frequently than respective controls. Impaired vascular endothelial function characteristic for the patients with diabetes mellitus may be at least in part due to the preferential *T. gondii* infection of endothelial cells. Vitamin D and minocycline exerted beneficial effects on development and clinical course of diabetes mellitus probably because of their immunomodulatory and antitoxoplasmatic activities. These data strongly suggest that the parasite play an important role in development of both types of diabetes mellitus.

**Keywords:** Diabetes mellitus; *T. gondii* infection; Toxoplasmosis; Microchimerism; Diabetic ketoacidosis; Amyloid deposition; Vitamin D deficiency; Diabetes comorbidities; Diabetes termal therapy; Cold stress; Glucose-6-dehydrogenase deficiency; Indoleamine 2,3-dioxygenase; Minocycline

## Introduction

Diabetes mellitus is characterized by persistent hyperglycemia with disturbances of carbohydrate, fat and protein metabolism that results from abnormalities in insulin secretion, action or both [1]. About 350 million people across the globe are estimated to have diabetes [2], and type 2 diabetes mellitus (T2DM) accounts roughly 90% of all diagnosed cases [1]. Diabetes has a prevalence of 2-5% in most Western countries, and is rapidly increasing in Asiatic countries due to changes in dietary habits during the last years [3]. From 1980 through 2010, the number of Americans with diagnosed diabetes has more than tripled (from 5.6 to 20.9 million) [4], and in the UK insulin use in children and adolescents increased significantly from 1.08 per 1000 children in 1998 to 1.98 in 2005 ($p<0.001$) [5]. Type 1 diabetes (T1DM), previously called insulin-dependent diabetes mellitus or juvenile-onset diabetes, accounts for about 10% of all diagnosed cases, and usually affects children and young adults, although disease onset can occur at any age. Risk factors may be autoimmune, genetic, and/or environmental [6].

In T1DM, pancreatic islet β cells are destroyed as a result of autoimmune processes causing severe insulin deficiency. Without insulin, blood glucose concentration increase with glucose uptake into muscle (energy), and to the liver (storage of glycogen), and hepatic gluconeogenesis also continues unabated, while ketone bodies and keto-acids are accumulating, finally leading to acute metabolic crises [7]. In T2DM, increases in insulin resistance lead to enhanced demand for insulin generation, beta cell hypertrophy, beta cell damage and fibrosis caused by excessive ROS/RNI production and/or other molecular pathomechanisms, with further reductions in insulin secretion [7].

*T. gondii* is a protozoan parasite known to infect animals, birds and mammals, including about 30-50% of the world human immunocompetent population who have chronic asymptomatic infection and harbor parasite cysts especially in the central nervous system [8,9]. In Europe, North America, and Africa, there are three dominant clonal lineages of *T. gondii* called type I (e.g. RH and GT1), type II (ME49), and type III (VEG), which differ in prevalence, virulence, migratory capacity within the host, and ability to convert to the bradyzoite cyst phase [10]. Geoepidemiological prevalence of the parasite varies depending on the world region, ranging from as low as 4% in some areas of the Far East, through 10-30% in the US, and from 10% to up to 60% in European regions with high consumption of raw food, such as France [11-13].

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*T. gondii* can infect and replicate in virtually any nucleated host cells and specifically increase the levels of key host microRNAs [14]. IFN-γ-mediated immune responses control the parasite in both phagocytic and non-phagocytic cells through at least six different mechanisms depending on the types of cells responding to this cytokine. Such effector functions involve: 1) mechanisms mediated by IFN-γ responsive gene family proteins, including IGTP (an essential mediator of specialized antimicrobial activities of IFN-γ), which may be involved in the processing and trafficking of cytokines and/or antigens; 2) production of NO by inducible NO synthase (iNOS); 3) production of various cytokines (TNF-α, IFN-γ, IL-1β, etc.); 4) tryptophan degradation by indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase; 5) limiting the availability of intracellular iron to the parasite, and 6) production of reactive oxygen/nitrogen species/intermediates (ROS/RNI) [15,16].

Immunocompetent hosts infected with *T. gondii* must develop a powerful immune response that has to be under tight control [17] and persistently maintained during their lifetime in all infected tissues to avoid life-threatening toxoplasmic encephalitis after reactivation of latent parasites [18,19].

## Possible Association between Diabetes Mellitus, Autoimmunity and *T. gondii* Infection

A significantly lower occurrence of antibodies against *T. gondii* and some viruses was reported in the sera of patients with T1DM compared with their first-degree family members or healthy controls [20]. This very interesting finding may be caused by the impaired innate immune state capacity of those individuals caused by chronic infection with the parasite (probably acquired early during prenatal life), which is primarily responsible for the prolific production of autoantibodies due to various autoimmune condition. Host cell-mediated immune responses are suppressed during chronic infection with *T. gondii,* and this was associated with significant suppression of IL-2, IFN-γ, and markedly lower levels of IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM [21] (Tables 1 and 2). This reasoning may be supported by the fact that latent toxoplasmosis was associated with markedly reduced lymphocyte B-cell counts responsible for production of antibodies [22], and is consistent with the current studies on CD8 T cell deficiency and probable exhaustion perspective observed during chronic toxoplasmosis [23].

Gokce et al. [24] studied seropositivity rate of anti-*T. gondii* antibodies in 807 persons (351 men, 456 women; mean age 52.8 ± 14.01 SD, range 15-88 yrs) with T2DM and found that IgG antibodies were present significantly more frequently as compared with 250 healthy controls (110 men, 140 women; mean age 51.94 ± 13.44, range 18-75 yrs) ($p<0.001$). IgM antibodies were found in 19 of the patients with diabetes mellitus and only in 4 controls (2.4 vs. 1.6%, $p=0.3$) [24] (Table 3). These findings may be supported by the geoepidemiology of autoimmune diseases which demonstrates that genetic individual susceptibility interacted with lifestyle and environmental factors, such as socioeconomic status, nutritional habits, environmental pollutants, and viral, bacterial and parasitic infections, acting as triggering and/or protective agents [25-29]. For example, helminths were found to be protective in T1DM, autoimmune encephalitis, and ulcerative colitis probably via an induction of proinflammatory responses ($T_H1$ cytokines) and a concomitant development of type 2 Th cell line adaptive immunity [25,26]. Shapira et al. [25] suggested that *T. gondii* infection can initiate a pathogenic process that may eventually result in clinically overt autoimmunity because serum anti-toxoplasma antibodies IgG were positive in 42% of 1514 European patients with 11 different autoimmune diseases as compared with 29% of controls ($p<0.0001$). In addition, ATxA IgM were more prevalent in the patients with anti-phospholipid syndrome ($p<0.01$), systemic sclerosis ($p<0.05$), and inflammatory bowel disease ($p<0.05$) than in controls [25]. Maternal microchimerism was found in the peripheral blood of patients with T1DM and pancreatic islet beta cell microchimerism [30]. It was demonstrated that this event leads to the production of IL-2, a proinflammatory cytokin, in IL-2 knockout mice [31]. Several autoimmune diseases due to maternal/fetal microchimerism were presented in table 4. Some of these diseases were probably associated

| Infectious agent | T1DM | FM | Controls | p value |
|---|---|---|---|---|
| **T. gondii** | 5.4 | 24.4 | 40.0 | 0.001 |
| EBV-VCA (IgG anti-VCA) | 82.1 | 92.6 | 92.8 | 0.04 |
| EBV-EBNA (IgG anti-EBNA) | 71.4 | 89.3 | 90.7 | 0.001 |
| CMV | 69.6 | 79.7 | 92.9 | 0.001 |
| HP | 55.1 | 78.3 | 80.7 | 0.01 |

CMV: Cytomegalovirus; EBV: Ebstein-Barr Virus; EBV-VCA: EBV Viral Capsid Antigen; EBV-EBNA: EB Nuclear Antigen; HP: Helicobacter Pylori

**Table 1:** Percentages of T1DM patients, their first-degree family members (FM) and healthy controls with antibodies against *T. gondii* and some other infectious agents (according to Krause et al. [20]; with own modification).

| Autoantibody | T1DM | FM | Controls | p value |
|---|---|---|---|---|
| Antigliadin IgG | 31.0 | 8.2 | 1.4 | 0.001 |
| Antitissue transglutaminase IgG | 3.5 | 0 | 0 | 0.03 |
| Anticentromere | 3.6 | 0.8 | 0 | 0.06 |

**Table 2:** Percentages of autoantibodies associated with various autoimmune condition in T1DM patients,their first-degree Family Members (FM) and healthy controls (according to Krause et al. [20]; with own modification).

| Duration of T2DM (yrs) | Number of IgG-positive individuals | Number of IgG-negative individuals |
|---|---|---|
| 0-5 | 39 (16.8) | 193 (83.2) |
| 6-10 | 149 (51.6) | 140 (48.4) |
| > 11 | 269 (94.1) | 17 (5.9) |

Values in parentheses denote percentages

**Table 3:** Relationship between the seropositivity rate of anti-*T. gondii* antibodies analyzed in 807 individuals with T2DM and duration of the disease (according to Gokce et al. [24]; with own modification).

| Disease | Female/male ratio | Tissue source | References |
|---|---|---|---|
| Systemic sclerosis | ≥ 8:1 | Peripheral blood cells | [33,34] |
| Juvenile idiopathic inflammatory myopathy | 3:1 | Sorted $CD4^+$ or $CD8^+$ peripheral blood cells | [35,36] |
| Systemic lupus erythematosus | 5:1 | Peripheral blood nucleated cells | [37] |
| Sjögren syndrome | 9:1 | Peripheral blood whole nucleated cells | [37] |
| Primary biliary cirrhosis | 14:1 | Peripheral blood nucleated cells | [38] |
| Hashimoto's thyroiditis | 20:1 | Thyroid tissue | [39] |
| Graves' disease | 8:1 | Thyroid tissue | [40] |
| Lichen planus | 2:1 | Peripheral blood nucleated cells | [41] |
| Polymorphic eruptions of pregnancy | pregnancy | | [42] |

Interestingly, maternal microchimerism was found in the peripheral blood of patients with T1DM and pancreatic islet beta cell microchimerism [43]. It was demonstrated that this bioevent also leads to the production of IL-2,a proinflammatory cytokine,in IL-2 knockout mice [44]

**Table 4:** Autoimmune diseases associated with fetal and/or maternal microchimerism (according to Klonisch and Drouin [32]; with own modification).

with transmission of the parasite in various nucleated cells as a Trojan horse, because the percentage of *T. gondii* positive persons increases with age (Table 5).

It should be emphasized that insulin and D-glucose had a dose-responsive mitogenic effect on intracellular *T. gondii* replication and development in 3T3-L1 cells. *In vitro* insulin concentrations between $10^{-2}$ and $10^{-1}$ μg/ml combination of 4.5 g/l D-glucose in DMEM (Dulbecco's Modified Eagle Medium) gave maximum stimulus to *T. gondii* replication [44]. In the absence of D-glucose, insulin had comparably less effect on the parasite growth than two of their combination. D-glucose markedly affected the tachyzoite replication and appeared to be indispensable for maintaining the host 3T3-L1 cells [44]. Thus, the additive/synergistic effect of insulin plus D-glucose on multiplication of the parasite in pancreatic islet beta-cells may be at least in part responsible first for inducing insulitis, and then progressing to diabetes, as well as for triggering development of various autoimmune defense reactions of the host.

In summary, there is a strong laboratory, pathophysiologic and clinical evidence supporting suggestion about the association between chronic latent *T. gondii* infection and both development of diabetes mellitus and several concomitant autoimmune diseases.

## Similarities between Genetic Contribution of HLA-DQ Molecules to the Development of Diabetes and Genetically-Dependent Outcome of *T. gondii* Infection

### Diabetes

In humans, T1DM is associated with genes encoding the MHC, particularly the class II molecule DQA1*0301/DQB1*0302 [47-50]. About 90% of patients with T1DM express HLA-DQ8/DR4 or HLA-DQ2/DR3 biomolecules [48]. Geogenetically, in Chinese population DQA1*0501, DQA1*0501, DQB1*0201, and DQB1*0302 were the susceptible alleles (all $p<0.005$) are relevant to T1DM, which is not totally the same as non-Chinese populations [51]. It must be emphasized that in the patients with the T1DM-associated DQB1*0302-DRB1*04 haplotype, maternal microchimerism was found more often when the haplotype was paternally (70%) rather than maternally transmitted (14%) [30]. This finding is in agreement with the suggestion that not only the inherited but also non-inherited maternal HLA haplotypes may influence the risk for development of T1DM [52].

In mice, Wen et al. [50] provided direct *in vivo* evidence for the contribution of HLA-DQ molecules to the development of diabetes. They found that substitution of HLA-DQA1*0301/DQB1*0302 for murine MHC class II provoked autoimmune diabetes in non-diabetes-prone rat insulin promoter RIP-B7-1 C57BL/6 mice. Rajagopalan et al. [53] showed that spontaneous diabetes occurred in RIP-B7-1 transgenic mice expressing transgenic HLA-DR3 or HLA–DQ8 molecules and the incidence of the disease was comparable between the two (approximately 30% in either sex up to 50 weeks of age). However, Kudva et al. [54] found that in NOD mice lacking endogenous class II molecules, transgenic expression of HLA-DR3 and HLA-DQ8 associated with predisposition to T1DM alone was not sufficient to induce spontaneous diabetes. It should be noted that the induction of immunodominant, protective $CD8^+$ T cell responses to *T. gondii* infection requires proteolysis by the endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) in the endoplasmic reticulum [55]. Although a key function for ERAAP is shaping many precursor peptides to the appropriate length for presentation by MHC class I molecules, this pathomechamism may also partly participate in the immune processes reported in mice by Kudva et al. [54]. This reasoning is supported by the finding that *T. gondii* infection caused downregulation of MHC class II molecules and inability to upregulate class I molecules in murine macrophages [56-58].

### *T. gondii*

*T. gondii* tachyzoite division is composed of single G1, S, mitosis /cytokinesis phases with infectious daughters formed following each nuclear cycle [59,60]. Consequently, replicating parasites, which can divide 5 to 6 times in a single host cell, are continuously infective when mechanically liberated from host cells [59]. Gaji et al. [59] showed that tachyzoites preferentially egress and invade in the G1 phase of the parasite cell cycle, thus demonstrating functional coordination between the cell cycle and intercellular transmission. The parasite rapidly alters the expression of many mRNAs soon after cell invasion to intracellular replication [59].

Mack et al. [61] demonstrated a cause and effect relationship between human MHC genes and resistance to *T. gondii* infection and associated inflammatory processes. They found that in Caucasians, the DQ3 gene frequency was significantly higher in infected infants with hydrocephalus (0.783) than infected infants without hydrocephalus (0.444) or published controls (0.487). Consistent with the observed association between DQ3 and hydrocephalus in human infants, was the finding in the murine model that the DQ3 (DQ8; DQB1*0302) gene protected less than DQ1 (DQ6; DQB1*0601) [61].

Dubey et al. [62] studied pathogenesis of *T. gondii* oocysts in HLA transgenic mice infected with different doses of the parasite strains of different genotypes derived from several countries. It was found that the decreasing order of infectivity and pathogenicity was the following: mice C57BL/6 background IFN-γ gene knock out, HLA-A*1101, HLA-A*0201, HLA-B*0702, Swiss Webster, C57/black, and BALB/c.

| Percent *T. gondii* positive | Age (yrs) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 18-20 | 21-25 | 26-30 | 31-35 | 36-40 | 41-45 | 46-50 | 51-55 | 56-60 | 61-65 | 66-70 | 71-75 |
| 100 | | | | | | | | | | | | 100 |
| 80 | | | | | | | | | 80 | | 80 | |
| 60 | | | | | | | 58 | 58 | | 60 | | |
| 40 | | 35 | 32 | 40 | 39 | 38 | | | | | | |
| 20 | 19 | | | | | | | | | | | |
| 0 | | | | | | | | | | | | |

In the control individuals 45 yrs old or younger recruited from the same geographical region as the psychiatric patients admitted to the hospital, serofrequency of *T. gondii* infection ranged between 20 and 40% without any systematic age effect, whereas in the individuals older than 45 yrs serofrequency systematically increased with age from about 40% to almost 100% [45]

**Table 5:** Percentage of *T. gondii* positive individuals among 214 nonpsychiatrically affected controls depending on age analyzed during a large epidemiologic study of 869 psychiatric patients [45,46].

Mice fed as few as 1 oocyst of type I and several atypical strains died of acute toxoplasmosis within 21 days post inoculation, while some *T. gondii* type II, and III strains were less virulent [62]. In North America, *T. gondii* serotype II and NE-II caused congenital toxoplasmosis, and prematurity and severe disease at birth was related with the parasite NE-II serotype [63]. This serotype was also associated with rural residence, lower socioeconomic status and Hispanic ethnicity ($p<0.01$-$0.001$) [63].

In summary, literature data indicate that there are considerable similarities of genetic contribution between chronic latent *T. gondii* infection, parasite strain affecting infectivity and pathogenicity, its developmental stage, dose, route of infection, innate immune state of the host, and local environmental factors, and development of diabetes mellitus.

## Disturbances of Innate Immunity in Patients with Diabetes Important for Triggering Development of the Disease

Han et al. [64] showed an overall depressed immunity in long-term patients with T1DM, and markedly increased gene expression for IFN-γ, IL-4 and IL-10 mRNA levels in new-onset patients compared to at-risk and long-term types of T1DM patients.

Clinical investigations performed in diabetic patients and experimental studies in diabetic rats and mice showed defects of neutrophil chemotactic, phagocytic and microbicidal activities [65]. Also, there were many other abnormalities, including decreased microvascular response to inflammatory mediators, reduced protein leakage and edema formation, decreased mast cell degranulation, impairment of neutrophil adhesion to the endothelium and migration to the site of inflammation, production of ROS, reduced release of cytokines and prostaglandins by neutrophils, increased leukocyte apoptosis, and reduction in lymph node retention capacity [66]. Endothelial dysfunction appeared to precede the development of overt hyperglycemia in the patients with T2DM [66]. Metabolic routes by which hyperglycemia is linked to neutrophil dysfunction included the advanced protein glycosylation reaction, the polyol pathway, oxygen free radical formation, the NO-cyclic guanosine-3'-5' monophosphate pathway, and the glycolytic and glutaminolytic pathways [65].

Diabetes causes marked changes in function and metabolism of neutrophils, e.g. glutamine oxidation and glutaminase activity are markedly decreased in neutrophils from diabetic rats [67] and glutamine plays an important role in protein (as an amino acid source), lipid (by NADPH production) and nucleotide synthesis (by purine and pyrimidine production), and in NADPH oxidase activity [68]. It must be emphasized that the tachyzoite stage of *T. gondii*, responsible for an acute infection, rapidly metabolizes glucose via glycolysis [69,70]. Blume et al. [70] demonstrated however that glucose is nonessential for *T. gondii* tachyzoites because host-derived glucose and its transporter in the parasite are dispensable by glutaminolysis. Thus, eventually increased requirements for glutamine and competition for this amino acid between *T. gondii* and neutrophils (and probably other cells) may result in diminished sources of glutamine and development of disturbances in maintaining regular metabolic and immune processes in many host cells. Moreover, this amino acid raises the *in vitro* bacterial killing activity and the rate of ROS generation by neutrophils [71,72], and delays spontaneous apoptosis of these cells [73].

Defective phagocytosis and decreased activity of intracellular killing of bacteria and Candida [74-76] (probably including also *T. gondii*) in neutrophils, are examples of the impaired mechanisms responsible for the increased susceptibility to infections in the patients with advanced stages of DM.

Rodacki et al. [77] found that the onset period of T1DM is marked by a slight reduction in blood NK cells, but in some patients the cells were unusually activated, as estimated by increased IFN-γ expression). NK cells patients with in long-standing T1DM had a markedly lower expression of p30/p46 NK-activating receptor molecules compared with control individuals, and this abnormality may be rather consequence than cause of the disease [77]. T1DM follows an immunologically mediated destruction of the pancreatic cells and autoreactive T-cells play a pivotal role in this process. NK cells are also potentially involved in this process, given their ability to kill target cells and interact with antigen-presenting cells and T-cells [78-80]. Indeed, NK cells can lyse islet cells *in vitro* [81,82]. Because NK cells are a major source of IFN-γ, their pathophysiological impact on the disease is to modulate the aggressiveness of the immune attack and the rate of its progression from insulitis to overt diabetes [77].

It was reported that pancreatic β-cell destructive insulitis was associated with increased expression of several proinflammatory cytokines (IL-1, TNF-α, TNF-β, INF-α, INF-γ, IL-2, and IL-12), whereas non-destructive (benign) insulitis was linked with the expression of antiinflammatory cytokines (IL-4 and IL-10) and TGF-β [83] (Table 6). Proinflammatory cytokines such as IL-1, TNF-α, TNF-β and IFN-γ may be directly cytotoxic to pancreatic β-cells by inducing NO and ROS in these cells. These data are in agreement with the findings that systemic administration of a wide variety of cytokines demonstrated to prevent development of IDDM in NOD mice and/or BB rats depending on the dose and frequency of administration [83].

Lajoix et al. [84] demonstrated the presence of neuronal NO synthase (nNOS) in rat pancreatic islets and INS-1 cells. Electron microscopic study showed that nNOS was mainly localized in insulin secretory granules and to a lesser extent in the mirochondria and the nucleus. It appeared that β-cell nNOS exerted, like brain nNOS, two catalytic activities: a NO production and a NOS monooxidative reductase activity [84]. Kröncke et al. [85] found that NO is extremely

| | Proinflammatory cytokines | | | Type 1 cytokines | | | Type 2 cytokines | | | | Type 3 cytokine |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | IL-1 | TNF-α | IFN-α | IL-12 | IFN-γ | TNF-β | IL-2 | IL-4 | IL-6 | IL-10 | TGF-β |
| NOD mice | ++ | ++ | 0 | ++ | ++ | ++ | ++ | + | ++ | + | + |
| BB rats | ++ | ++ | ++ | ++ | ++ | ? | ++ | 0 | ? | + | + |
| Humans | 0 | nd | ++ | ? | ++ | ? | nd | 0 | 0 | ? | ? |

++: cytokine presence correlates with β-cell destructive insulitis; +: cytokine presence correlates with benign insulitis; 0: cytokine presence does not correlate with either destructive or benign insulitis; nd: not detected;

?: not reported; NOD: Non-Obese Diabetic mice; BB: Biobreeding rats; Humans: pancreas of humans with IDDM

**Table 6:** Correlations of cytokines expressed in islets with β-cell destructive or benign insulitis (according to Rabinovitch [83]; with own modification).

toxic for islet cells and even in the absence of other macrophage-generated potentially toxic products can rapidly and completely kill these cells.

Proinflammatory cytokines are increasingly thought to contribute to beta-cell dysfunction and death not only in T1DM, but also in the progression of T2DM. It was established that pancreatic beta cells, as well as neural cells, can be destroyed by several toxic agents and noxious stimuli, such as for example: 1) ROS ($H_2O_2$, $O_2^-$, $HO^-$) and NO, 2) cytokines (TNF, IL1-β, IFN-γ), 3) hyperglycemia and 4) islet amyloid polypeptide [86]. Cytokines can alter intracellular calcium levels by depleting calcium from the endoplasmic reticulum (ER) and by increasing calcium influx from the extracellular space [87]. Depleting ER calcium leads to protein misfolding and activation of the ER stress response. Disrupting intracellular calcium may also affect organelles, including the mitochondria and the nucleus, and as a chronic condition, cytokine-induced calcium disruption may lead to beta-cell death in both T1DM and T2DM [87]. Host cell mitochondria and ER have an intimate relationship with *T. gondii* due to their recruitment to and association with the parasitophorous vacuole membrane (Table 7) [88]. Moreover, discharge of adhesive proteins *T. gondii* apical storage organelles (micronemes) is stimulated by contact with host cells and this process is regulated by increases in intracellular calcium within the parasite [89]. In addition, gliding of the parasite is controlled by secretion of microneme proteins and factors that alter calcium fluctuation in the cytosol, while chelation of intracellular calcium blocked parasite motility [90].

Experimental studies indicated that disruption of endothelial insulin signaling through the activity of protein kinase C-β and NFκB reduces NO availability, and Tabit et al. [91] observed 1.7-fold higher basal eNOS phosphorylation at serine 1177 in patients with diabetes (p=0.007). Nitrotyrosine levels were higher in diabetic patients indicating endothelial oxidative stress, and protein kinase C-β expression was higher in those patients and was associated with lower flow-mediated dilation (r=-0.541, p=0.02) [91]. In *T. gondii,* calcium-dependent protein kinases (serine/threonine kinases) are key mediators of signaling [92], and a protein kinase C receptor 1 was identified in tachyzoites [93]. Proliferation of the parasite is dependent on its ability to invade host cells, which is mediated partly by calcium-dependent protein kinase 1 [94]. cAMP-dependent protein kinase plays an important role in the growth of tachyzoites [95], and a plant-like calcium-dependent protein kinase in *T. gondii* is required for optimal *in vitro* growth, regulates microneme secretion when parasites are intracellular and its egress from host cells [96].

Leem and Koh [97] suggested that impaired mitochondrial function and ER stress are closely associated with pancreatic β cell dysfunction and peripheral insulin resistance, and each of these factors contributes to the development of T2DM [98-103]. ROS generation is thought to act as local messengers between the ER and mitochondria [103] and many ROS sources and targets are localized to the ER and mitochondria [104]. NO signals the ER stress response via inhibition of mitochondrial respiration because in NO-generating cells the respiratory chain is disrupted [105] and NO can bind to cytochrome c oxidase and inhibit the enzyme, in competition with oxygen [106]. In addition, NO mediates cytokine-induced (IL-1β, TNF-α, and IFN-γ) inhibition of insulin secretion by human islets of Langerhans through generation of iron-nitrosyl complexes that inactivate enzymes, such as aconitase and ribonucleotide reductase [107-109], while on the other hand, IL-1β and TNF induce NO formation and accumulation of cyclic GMP in pancreatic β cells [110,111].

Weaver et al. [112] showed that stimulation of human donor islets with a cocktail of inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) significantly induced NADPH oxidase-1 (NOX-1) gene expression ($p<0.05$), and concomitantly induced loss of islet glucose stimulated insulin response ($p<0.05$), elevated expression of MCP-1 ($p<0.01$), increased cellular ROS production, and induced cell death [112]. Recently, the role of NOX in mitochondrial dysregulation in diabetes was reported [113], and NOX-1 participated in ROS-dependent cell death of $Caco_2$ cells [114].

T1DM results from the destruction of insulin-producing pancreatic beta cells by a beta cell-specific autoimmune process leading to absolute insulin deficiency. Beta cell autoantigens, macrophages, dendritic cells (DC), B lymphocytes, and T lymphocytes have been found to be involved in the pathogenesis of autoimmune diabetes [115-117]. Beta cell autoantigens are thought to be released by cellular turnover or damage, then processed, and finally presented to T helper cells by antigen-presenting cells. Macrophages and DC are the first cell types to infiltrate the pancreatic islets. Naive $CD4^+$ T cells can be activated by IL-12, a proinflammatory cytokine, released from macrophages and DC. The $CD4^+$ T cells secrete IFN-γ and IL-2, and IFN-γ activates other resting macrophages, which release IL-1β, TNF-α, and free radicals, all toxic to pancreatic beta cells if produced in excess [118]. Beta cell antigen-specific $CD8^+$ T cells activated by IL-2 produced by the activated $T_H1$ $CD4^+$ T cells differentiate into cytotoxic T cells and are recruited into the pancreatic islets, finally leading to the destruction of beta cells [115-117]. In addition, beta cells can also be

| Sample treatment | Sample size (n) | SvPVM Mean (SE) | SvM Mean (SE) | SvER Mean (SE) | Percentage PVM-mitochondrial association | Percentage PVM-ER association |
|---|---|---|---|---|---|---|
| Untreated, 4 hrs | 31 | 3.20 (0.16) | 0.58 (0.07) | 1.78 (0.22) | 18 | 56 |
| Nocodazole, 4 hrs | 31 | 3.70 (0.17) | 0.33 (0.08) | 1.79 (0.15) | 9 | 47 |
| Untreated, 20 hrs | 30 | 2.70 (0.19) | 0.61 (0.06) | 0.80 (0.13) | 23 | 30 |
| Nocodazole, 20 hrs | 30 | 3.22 (0.19) | 0.81 (0.09) | 1.21 (0.20) | 25 | 38 |
| Pyrimethamine 20 hrs | 30 | 2.96 (0.14) | 0.79 (0.09) | 1.00 (0.14) | 27 | 34 |

ER: Endoplasmic Reticulum. PVM: Parasitophorous Vacuole Membrane. The extent of PVM-organelle association was measured at 4 hrs and 20 hrs post-infection. The specific parameters measured are the Surface to Volume ratios (Sv) of the PVM (SvPVM), PVM-associated mitochondria (SvM) and PVM-associated Endoplasmic Reticulum (SvER), all relative to the volume of the PV. The mean surface densities and the Standard Error (SE) for the sample size measured are presented. The extent of PVM-mitochondrial and PVM-ER association was represented as a percentage of their surface densities (SvM and SvER) relative to SvPVM. The values for the mean surface densities and the percentage association were rounded off to the level of significance indicated

**Table 7:** Morphometric analysis of *T. gondii* PVM-host organelle association (acc. to Sinai et al. [88]; with own modification).

damaged by granzymes and perforin released from $CD8^+$ cytotoxic T cells, and by cytokines and reactive oxygen/nitrogen species generated by activated macrophages accumulated in the islets. Thus, activated macrophages, $T_H1$ $CD4^+$ T cells, and beta cell-cytotoxic $CD8^+$ T cells act synergistically to destroy beta cells, resulting in the development of autoimmune T1DM [115-117].

Abscisic acid (ABA) is a membrane-permeant hormone rapidly produced and released from human islets stimulated with high glucose concentrations, which regulates several important physiologic functions related to stress [119]. ABA acts as an endogenous proinflammatory cytokine in human granulocytes because it activates many functions of these cells including phagocytosis, migration, production of ROS and NO [119,120]. Nanomolar ABA concentrations increase glucose-stimulated insulin secretion from human and murine pancreatic β cells, and the paracrine production of the hormone by activated granulocytes and monocytes suggests that ABA may also be involved in dysregulation of insulin release during inflammation of pancreatic islets [119,120].

ABA has been detected as a product of human granulocytes [120,121], pancreatic islet cells [119], and other cell types [122]. Nagamune et al. [121] demonstrated production of ABA also by *T. gondii*. ABA induced the release of intracellular calcium stores via the generation of cAMP ribose [121], controlled calcium signaling within the parasite, and calcium was responsible for several critical bioevents related to *T. gondii*, including its motility, secretion, cell invasion, and egress. It appeared that ABA-mediated calcium signaling controls the decision between lytic and chronic stage growth, a developmental switch that is crucial in pathogenicity and transmission of the parasite [121]. Parasite- or host cell-derived ABA represents a potential initiating agent of calcium mediated host cell autophagy [123]. The above-presented processes, with possible involvement of *T. gondii* infection, may be at least in part responsible for the increased β-cell apoptosis and deficit of β cells development found in pancreatic tissue from 124 autopsies in humans with T2DM and in mouse model of type 2 diabetes [124,125].

Pathologic or radiologic evidence of pancreatitis was noted with *T. gondii*, especially in HIV-infected patients or in other immunocompromised hosts. The majority of these individuals had no clinical symptoms of pancreatitis and infection was demonstrated histologically during postmortem examination [126].

## Immunomodulatory Effects of Latent Toxoplasmosis in Animals and Humans

### Animals

Kankova et al. [127] showed that mice in the early phase of latent *T. gondii* infection had transiently increased serum IL-12 levels and decreased generation of IL-10. There was also a decreased production of NO by stimulated macrophages, diminished generation of IL-2 and IL-4, and a markedly lower proliferative activity of splenocytes compared with controls in the early and also in the late phases of the infection, which suggested that immunosuppression processes play an important role during latent toxoplasmosis [127].

Tachyzoites of *T. gondii* stimulate production of IL-12 [128-131] and this proinflammatory cytokine activates NK cells and T cells to produce IFN-γ that is crucial for host resistance [132-136]. IFN-γ and TNF-α act synergistically to mediate killing of tachyzoites by macrophages and the combination of these two cytokines results in a greatly enhanced production of free oxygen radicals and NO, both of which can affect parasite killing [128,136-138], although NO and its metabolites appear to be the primary effectors. NO is produced as a result of iNOS activation, which is dependent on activation of NFκB [139]. Gomez-Marin [140] obtained evidence of NO generation not only in the host cells, but also in *T. gondii* [141,142], which has its own cNOS producing 2-6 μmol of nitrites that could be essential in intracellular signaling. The NO defensive mechanism, where levels of nitrites can reach 120 μmol or more, is probably toxic for human and mice tissues [140].

*T. gondii* induces overproduction of IFN-γ and other proinflammatory cytokines which may contribute to host tissue injury and death [143,144]. IFN-γ-induced antitoxoplasmatic activity is mediated by NO and indeed, induction of iNOS was demonstrated during the parasite replication in murine macrophages [145]. IFN-γ combined with TNF-α activated macrophages to produce increased quantities of RNI that are involved in the control of parasite replication [138] (Table 8).

Seabra et al. [194] found that NO was produced by monocyte-derived macrophages only if cultured in the presence of macrophage-colony-stimulating factor. Monocyte-derived or peritoneal macrophages infected with *T. gondii* presented lower iNOS expression, had a marked reduction in NO production, and only viable parasites caused partial inhibition of this process [194].

Lüder et al. [195] showed that infection of primary bone marrow-derived macrophages or monocyte/macrophage RAW264.7 cells with a mouse-avirulent *T. gondii* strain markedly decreased NO production that had been induced by activation with either IFN-γ or bacterial lipopolysaccharide (LPS), or IFN-γ plus LPS. The down-regulation of NO production by the parasite enabled its considerable replication in macrophages activated with IFN-γ or LPS alone. iNOS transcripts induced by IFN-γ alone or in combination with LPS were also dose-dependently down-regulated after infection of RAW264.7 cells with *T. gondii* [195].

Infection of mice with *T. gondii* elicits a dominant $T_H1$ cytokine response involving IFN-γ, IL-12, IL-1β, and TNF-α. TNF-α induction has a serious impact on the parasite-induced pathology at early stages of infection. $T_H2$-associated cytokines, such as IL-4 and IL-10, appear relatively late after infection, and may limit immune pathology [196]. To resolve acute infection, IFN-γ induces indoleamine 2,3 dioxygenase (IDO) release, resulting in tryptophan degradation and kynurenic acid accumulation [197]. IDO activated T cells and blocked their conversion into $T_H17$-like T cells [198]. Tryptophan depletion is thought to be responsible for suppression of the growth of the acute stage tachyzoites. Kynurenic acid accumulation in the brain could potentially alter dopamine metabolism due to its NMDA antagonistic property [199-201] (Figures 1-3).

In mice, *T. gondii* infection caused a significantly increased formation of RNI probably due to elevated serum NO concentrations [143], and a significantly higher serum kynurenine/tryptophan ratio compared with control animals ($p<0.05$). The authors suggested that increased free radical toxicity may cause elevation in tissue malondialdehyde (MDA) levels arising from lipid peroxidation in *T. gondii*-infected mice, while unchanged serum MDA concentrations might indicate the increased oxidative stress due to the parasite infection restricted to intracellular area [143].

Wang et al. [123] presented evidence that *T. gondii* induces host cell autophagy in both HeLa cells and primary fibroblasts by a mechanism dependent on calcium, and that it exploits the nutritive function of host

| Cytokines | Sources | Functions |
|---|---|---|
| IL-12 | Dendritic cells [147-149] | Promotes T cell proliferation and differentiation [134,150] |
| | Neutrophils [151-153] | Promotes NK cell responses [129,154] |
| | Inflammatory monocytes [153,155] | Promotes IFN- production [122,149] |
| IFN-γ | NK cells [137,145,154] | Promotes iNOS expression [158] |
| | $CD4^+$ T cells [159] | Promotes p47 GTP-ase-mediated killing of *T. gondii* [160,161] |
| | $CD8^+$ T cells [159] | Promotes tryptophan degradation [135,162-164] |
| TNF-α | Neutrophils [151,165] | Promotes macrophage activation [166] |
| | Dendritic cells [165] | Promotes control of parasite in non-hematopoietic cells [167] |
| | Macrophages [168] | |
| | Microglia [169] | Promotes iNOS expression [138,170-172] |
| | T cells [173] | |
| IL-6 | Monocytes [174] | Necessary for optimal neutrophil responses [175] |
| | Astroglia [176] | Necessary for optimal IFN-γ responses [175] |
| | Stromal cells [177] | |
| | Retinal pigment epithelial cells [178] | |
| LT-α | Lymphocytes [179] | Necessary for normal secondary lymphoid architecture [180] |
| | | Necessary for optimal antibody and IFN-responses early during infection [138] |
| | | Necessary for optimal expression of iNOS [170] |
| IL-10 | NK cells [181] | Inhibits $CD4^+$ T cell-mediated pathology [182] |
| | Macrophages [183] | |
| | $CD4^+$ T cells [184] | |
| | $CD8^+$ T cells [183] | |
| IL-27 | Antigen-presenting cells [185] | Inhibits IL-17 production [186] |
| | | Inhibits IL-2 production [187] |
| | | Promotes IL-10 production [188] |
| | | Promotes PD-L1 expression [189] |
| CD40L (surface protein) | Expressed on T cells [190] | Promotes $T_H1$ responses in humans [191] |
| | | Promotes iNOS expression [192] |
| | | Promotes xenophagic killing of *T. gondii* [193] |

Numbers in parentheses denote reference nos

**Table 8:** Cytokines necessary for survival during *T. gondii* infection (according to Dupont et al. [146]; with own modification).

autophagy to enhance its proliferation. Autophagy is a lysosomal self-digestion process essential for cellular homeostasis, differentiation and survival, which protects organisms against a wide range of pathologies, including infection, neurodegeneration, ageing. (Nb. autophagy is considered a basis for the health promoting effects of vitamin D because of at least two functions, the induction of cancer cell death and the clearance of *Mycobacterium tuberculosis* in macrophages [202]). Autophagy-dependent *T. gondii* growth correlates with autophagy-dependent consumption of host cell mass that is dependent on parasite consumption. In macrophages infected with the parasite, fusion of the parasitophorous vacuole with lysosomes can be induced in an autophagy-dependent manner when host cell anti-parasitic function is activated via CD40 [203]. Parasite-induced autophagy is dependent on calcium sigaling and on abscisic acid. Autophagy as a component of host defense may be upregulated by proinflammatory factors, such as LPS [204] and IFN-γ [205]. The parasite is able to actively sequester host cell lysosome-derived vesicles, thereby potentially gaining access to their content [206]. *T. gondii* reside in host cell vacuoles which resist typical phagosome-lysosome fusion because phagosome acidification necessary for microbicidal effect is blocked by intracellular parasite [207]. Extracellular *Toxoplasma* is highly susceptible to acidic pH conditions, indicating that the acidification block is important for intracellular survival of the parasite [207]. Thus, it seems that hypoglycemia-induced neuronal death, increased superoxide production and microglia activation reported in patients with diabetes, and is not a simple result of glucose deprivation, but instead the end result of a multifactorial process [208]. These changes may at least in part reflect a defense reaction of the host against chronic latent *T. gondii* infection through increasing systemic and local acidification of the tissues.

## Humans

Flegr and Striz [22] found that in 128 analyzed male patients the prevalence of *T. gondii* infection was 10.9% which contrasted with 23.7% in 312 female patients and 20-30% in general population reported in Prague (Czech Republic). The male patients with latent *T. gondii* infection had significantly decreased leukocyte, NK-cell and monocyte counts while the *T. gondii*-positive women had increased these values, as compared with controls. The B-cell counts were markedly reduced in both men and women with toxoplasmosis [22]. Karaman et al. [209] found a significantly higher serum NO levels in patients with latent toxoplasmosis as compared with seronegative controls, and Dzitko et al. [210] demonstrated markedly increased serum prolactin (a strong immunomodulator) levels in women with latent *T. gondii* infection. Prolactin has been show to enhance production of IFN-γ, IL-12, and IL-10, but not of TNF-α, in a stimulus specific manner [211]. *In vitro* preincubation of tachyzoites with recombinant human prolactin resulted in a significant reduction (up to 36.15%) in replication abilities of the parasite, and the inhibition of replication was caused by a limited capacity of the parasites to penetrate host's cells as demonstrated by the reduced number of infected cells [212]. More detailed effects of chronic *T. gondii* infection concerning modulation of human innate immunity and metabolism were presented in other works [213-216]. Tables 9 and 10 summarized host cell-mediated evasion strategy against infection with the parasite.

In the sera of 37 IgG-seropositive patients with *T. gondii* infection, Karaman et al. [209] demonstrated significantly increased malondialadehyde (MDA) and NO concentrations, and a decrease in glutathione activity as compared with healthy controls. It was also found a markedly higher MDA levels ($p<0.001$) paralleled with significantly decreased concentrations of glutathione peroxidase ($p<0.0188$) and tocopherol fractions (alpha, gamma and lambda) ($p<0.001$) in *T. gondii* seropositive than in seronegative blood donors [248,249]. These significant alterations in redox status between the two groups of blood donors indicate that chronic *T. gondii* infection is associated with oxidative stress because MDA is arising from the lipid peroxidation and is an indicator of oxidative stress, glutathione defends cells against oxidative damage by ROS and peroxidase, and tocopherol is an antioxidant [249,250]. The increased NO concentrations can be associated with the stimulation of the cell-mediated immune system in these individuals reflecting a defense of the host against the infection with the parasite. This may be supported by the finding that NO is a major effector molecule of macrophage cytotoxicity against *T. gondii*,

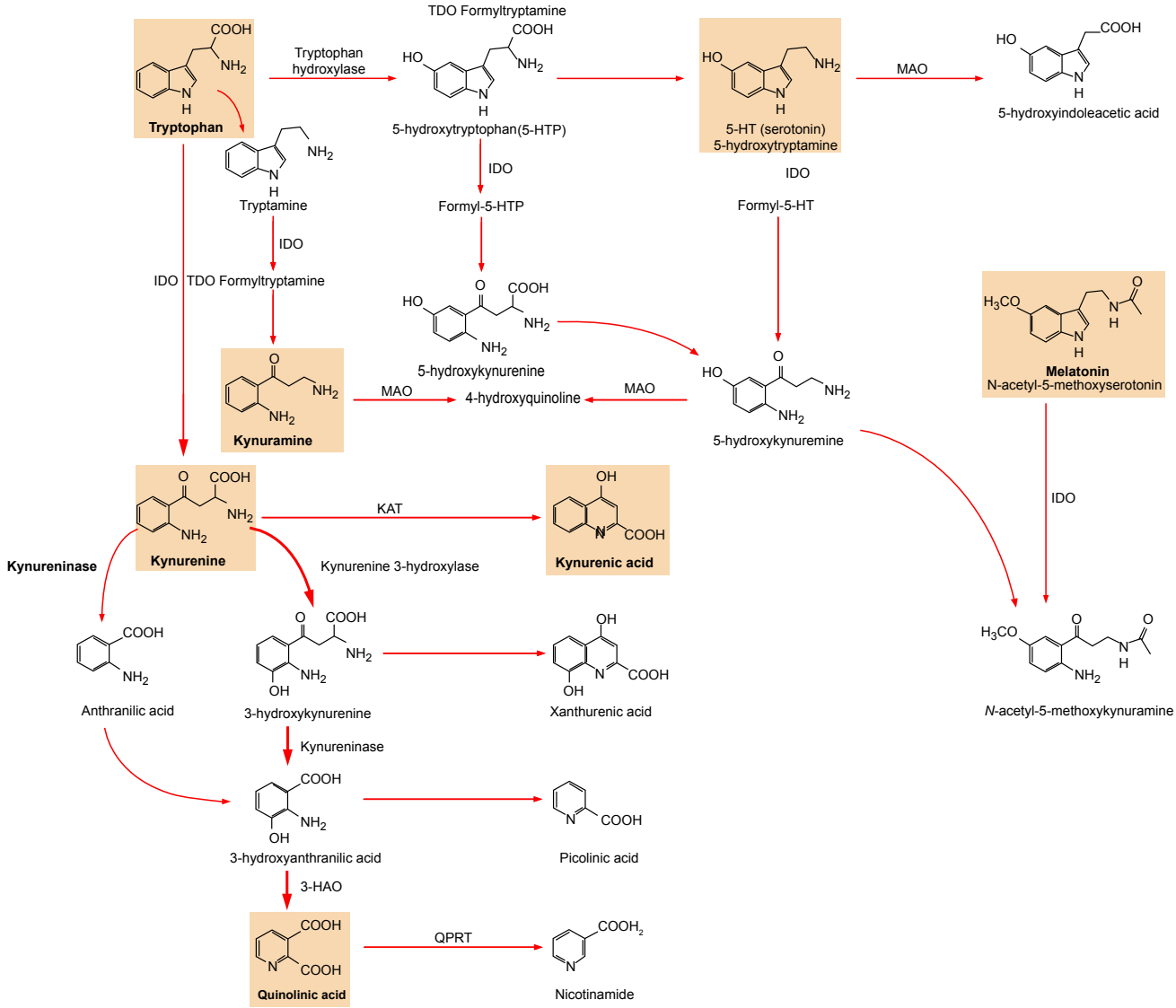


**Figure 1:** Various pathways of the essential amino acid tryptophan metabolism. About 99% of the dietary tryptophan is metabolized along the kynurenine pathway (red arrows). Alternative pathways are the conversion of tryptophan to 5-hydroxytryptamine (5-HT) and then to melatonin, or to tryptamine and then to the kynuramines (or kynurenamines). N1-acetyl-5-methoxykynuramine is a metabolite deriving from melatonin by mechanisms involving free radicals, exhibits potent antioxidant properties exceeding those of its direct precursor N1-acetyl-N2-formyl-5-methoxykynuramine, a biogenic amine generated through either an enzymatic or a chemical reaction (free radicals) pathway. 3-HAO, 3-hydroxyanthranilate oxidase; IDO, indoleamine 2,3-dioxygenase; KAT, Kynurenine aminotransferase; MAO, monoamine oxidase; QPRT, quinolinic-acid phosphoribosyl transferase; TDO, tryptophan 2,3-dioxygenase [200].

and its production by macrophages is catalyzed by a cytokine-inducible form of the NO synthase positively controlled by TNF-α, IFN-γ and IL-2 [178]. Other cell types, such as endothelial cells and hepatocytes, display a similar capacity for NO generation in response to cytokine stimulation [251].

Baltaci and Mogulkoc [252] reported that in rats *T. gondii* infection caused also a significant increase of plasma leptin concentrations ($p<0.01$), with no change in body weight of the animals. In the obese individuals, including diabetics, this biomarker exerts a proinflammatory activity, and its structure is similar to that of IL-2, an important proinflammatory cytokine (Table 11).

## Maternal Microchimerism (Mmc) in the Circulation and Tissues of Mothers' Immune-Competent Children with T1DM. Pancreatic Islet β Cell Microchimerism may Play an Important Role in Development of T1DM because of Possible Intracellular Transmission of *T. gondii* Infection as a Trojan Horse to Mother's Progeny

Pregnant women infected with *T. gondii* for the first time can transmit the infection to their fetuses across the placenta. The risk of congenital infection is lowest (10-25%) when acute maternal toxoplasmosis occurs during the first trimester and highest (60-90%) when it occurs during the third trimester [257]. In primarily infected pregnant woman invasion of the placenta by tachyzoites that then multiply within placenta cells, may cross the placenta, and enter the fetal gastrointestinal tract with amniotic fluid, invade circulation and/or fetal tissue [258]. Passage of erythrocytes between mother and

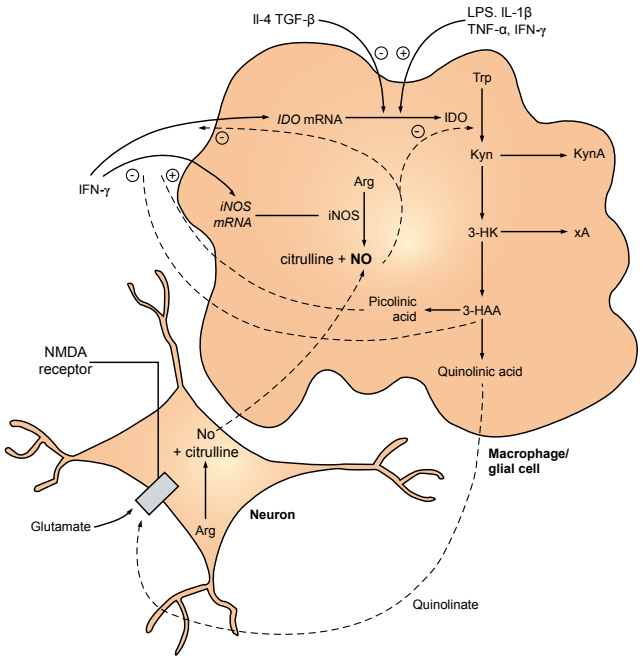


**Figure 2:** Interrelationships between indoleamine 2,3-dioxygenase (IDO) and nitric oxide synthase (NOS) in macrophages or glial cells, and the potential interactions with neurons by means of N-methyl-D-aspartate (NMDA)-receptor-induced nitric-oxide (NO) formation. Arg, arginine; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; IFN-γ, interferon-γ; IL, interleukin; Kyn, kynurenine; KynA, kynurenic acid; LPS, lipopolysaccharide; mRNA, messenger RNA; iNOS, inducible nitric-oxide synthase; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Trp, tryptophan; xA, xanthurenic acid. The broken lines represent possible reactions [200].

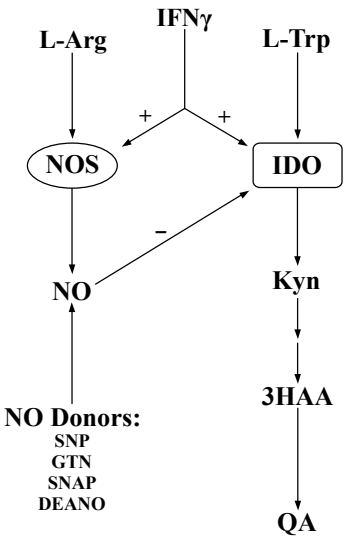


**Figure 3:** Possible model for NO-mediated regulation of IDO in IFN-γ-primed mononuclear phagocytes. NOS, nitric-oxide synthase; IDO, indoleamine 2,3-dioxygensae, L-Arg, L-arginine; L-Trp, L-tryptophan; IFN-γ, interferon-; NO, nitric oxide; Kyn, kynurenine; 3-HAA, 3-hydroxyanthranilic acid; QA, quinolinic acid; SNP, sodium nitroprusside; GTN, glyceryl trinitrate; SNAP, S-nitroso-N-acetylpenicillamine; DEANO, diethylaminodinitric oxide. SNP, DEANO, and SNAP release NO extracellularly, while GTN is thought to release NO intracellularly [201].

fetus is a well-documented phenomenon and it was reported that maternal erythrocytes have been found in 10-80% of all newborns [259]. Also, materno-fetal passage of leukocytes and platelets has been demonstrated [260].

Investigations of MMc in subsets of whole blood from 31 healthy adult women showed that 39% (12/31) of probands had MMc in at least one cellular subset [261]. MMc was demonstrated in T lymphocytes in 25% and B lymphocytes in 14%, while monocyte/macrophages had MMc in 16% and NK cells in 28%, thus demonstrating that MMc cells are detectable in the peripherial blood of many healthy individuals [261,262].

## Diabetics

Zhou et al. [263] demonstrated that two independent pathways of maternal cell transmission to offspring, i.e. transplacental passage during pregnancy and breast milk-feeding after birth.

Bidirectional trafficking of maternal and fetal cells occurs during almost all pregnancies resulting in the persistence of low levels of these cells in the mother and/or her offspring for several decades after delivery [264-267]. The cell types exchanged between mother and fetus include leukocytes and T cells [268-271] in addition to progenitors of different line-ages [267,272], such as hematopoietic [267] or mesenchymal stem cells [273] and/or endothelial progenitors [274]. It was suggested that maternal T and potentially B cells transferred during pregnancy or lactation might play a role in the development of T1DM [275].

Roy et al. [275] believe that fetal microchimeric cells did not seem to influence the level of islet inflammation in mothers despite their anti-beta cell specificity. They showed that fetal lymphoid progenitor cells enter the maternal thymus and develop into double positive and single positive thymocyte [270]. A possible role of microchimerism in the pathogenesis of some autoimmune diseases has been suggested because microchimeric cells may differentiate into many lineages in various tissues finally inducing diverse pathophysiologic processes during the host lifetime [276] (Table 4). For example, proliferation of these cells in maternal tissue environment may be at least in part responsible for development of autoimmune thyroid diseases because of a significant prevalence of anti-IgG *T. gondii* antibodies present in those individuals [25,277,278], as well as papillary thyroid cancer induction [279]. Fetal microchimerism may take place during pregnancy starting from the 4$^{th}$ to 6$^{th}$ week of gestation [280]. This traffic of cells is primarily composed by immune cells, such as T and B-lymphocytes, monocytes, and NK cells, including hematopoietic stem cells CD34$^{+}$ and CD34$^{+}$/38$^{+}$ committed to early B and T-cells with the capacity for multilineage differentiation [281]. The number of fetal progenitor cells circulating in the blood of pregnant women, has been estimated to be 0-2/mL, but in normal second-trimester pregnancies may vary from 1 to 6 cells per ml of maternal venous blood [280]. At 36$^{th}$ week of gestation, 100% of pregnant women have fetal cells in their circulation and after delivery and 30-50% of healthy women have detectable fetal cells in their blood from 4 weeks to decades after delivery [279,282] (Table 12). Interestingly, in mice maternal background strain and strain differences between the mother and father significantly affected fetal-maternal trafficking (both the number of fetal cells and the relative distribution of cell types in maternal organs) more than maternal immune competence [284].

Chimeric maternal cells (e.g. hematopoietic cells) have been found in human fetal [285] (including a second trimester fetus [286]), newborn [287] and infant tissues [288]. It must be emphasized that T1DM has been mostly related to maternal cell microchimerism

| Evasion strategy | Consequence(s) | Molecular mechanism(s) | Parasite effect | References |
|---|---|---|---|---|
| Induction of IL-10 | Decreased $T_H1$ response;<br>Deactivation of macrophages | <br>Independent of $PGE_2$ | Indirect<br>Indirect | [218,219] |
| Induction of TGF-β | Reduced TNF-α production by macrophages;<br>reduced IFN-γ production by NK cells | Antagonizes IL-12 | Indirect | [130,220,221] |
| IFN-γ/-β upregulation | Reduced IFN-γ levels and splenocyte proliferation | | Indirect | [222] |
| Inhibition of TNF- α and IL-12 production | Deactivation of macrophages; inhibition of $T_H1$ responses | Reduced phosphorylation of p65/ RelA; defective nuclear import of NF-κB; IL-10-independent STAT3 phosphorylation | Direct | [15,223-226] |
| Decreased IL-12 production by DCs | Inhibition of $T_H1$ responses | $LXA_4$-mediated downregulation of CCR5 | Indirect | [227,228] |
| Blockade of MHC class II upregulation | Defective antigen presentation to $CD4^+$ T cells | Reduced activity of CIITA and IRF-1 promoters | Direct | [56,229] |
| Inhibition of NO production | Defective antiparasitic activity | Inhibition of iNOS transcription | Direct | [194,195] |
| Inhibition of NO production in microglia | Reduced antiparasitic activity | Secretion of $PGE_2$,<br>**IL-10 and TGF-β** | Indirect | [230,231] |
| Inhibition of p47 GTPases | | Reduced transcription | | [232] |
| Significant suppression of IL-2, IFN-γ (but not IL-10). Markedly lower levels of IgG1, IgG2a, IgG2b, IgG3, IgA, IgM | Suppressed cytokine and immunoglobulin secretions by murine splenic lymphocytes | | **Indirect** | [21] |

CIITA: Master Regulator of Major Histocompatibility Complex Class II Transcription; CCR5: CC Chemokine Receptor; DCs: Dendritic Cells; iNOS: Inducible Nitric Oxide Synthase; IRF-1: Interferon Regulatory Factor-1; $LXA_4$ Lipoxin $A_4$; MHC: Major Histocompatibility Complex Molecules; $PGE_2$: Prostaglandin $E_2$; TGF-β: Transforming Growth Factor-β. Proliferation of *T . gondii* in inflammatory macrophages was associated with diminished ROS production in host cells [233]. In young children with congenital toxoplasmosis specific T cell response to the parasite antigens was impaired and such hyporesponsiveness has been restored during childhood. The acquisition of functional T cell response was disease-unrelated and indistinguishable
in terms of strength, epitope specificity, and cytokine profile from the corresponding responses in immunocompetent adults with asymptomatic acquired *T. gondii* infection [234].
In pregnant mice, *T. gondii* infection caused a decrease of $CD4^+CD25^+$-regulatory T cells [235]. It must be noted that peripheral blood leukocytes (PBL) from healthy children older than 36 mths responded to several stimuli at levels comparable to those of PBL from adults, but surprisingly, cord blood leukocytes appeared to be more efficient in antigen-presenting function than PBL from children younger than 13 months [236].

**Table 9:** Partial downregulation of cell-mediated immune responses after infection with *T. gondii* (Lang, Gro & Lüder [217]; with own modification).

| Evasion strategy | Consequence(s) | Molecular mechanism(s) | Parasite effect | References |
|---|---|---|---|---|
| Apoptosis of $CD4^+$ cells | T-cell unresponsiveness | Cell death by neglect | Indirect | [237] |
| Apoptosis of leukocytes[a] | Unrestricted parasite replication and host death | Upregulation of Fas and FasL;<br>TNF-dependent mechanisms | Indirect | [238-240] |
| Inhibition of apoptosis in parasite-positive cells | Blockade of host cell suicide; avoidance of CTL- and NK-mediated cytotoxicity | Inhibition of cytochrome c-release; upregulation of anti-apoptotic molecules<br>Interference with caspase activation; degradation of PARP (?) | **Direct** | [241-245]<br>[246] |

CTL: Cytotoxic T lymphocyte; Fas: Receptor; FasL: Fas Ligand (a cell surface molecule belonging to TNF family and death factor, which binds to its receptor Fas, thus inducing apoptosis of Fas-bearing cells); NK: Natural Killer cells; PARP, Poly(ADP-Ribose) Polymerase. [a]*T. gondii* delayed neutrophil apoptosis by inducing granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor secretion by the parasite-infected human fibroblasts. Although neutrophils are unable to kill *T. gondii*, this can retard their division time from the usual 6-8 hrs cycle to a 24 hrs cycle and this enhanced neutrophil survival may contribute to the robust proinflammatory response elicited in the pathogen-infected host cells [247].

**Table 10:** Suppression of immune responses to *T. gondii* by parasite-triggered modulation of host cell apoptosis (acc. to Lang, Gro β & Lüder [231]; with own modification).

[289]. Nelson et al. [30] reported markedly higher levels of maternal microchimeric cells in the peripheral blood of 94 patients with T1DM, ranging from 3 to 28 years of age, than 54 unaffected siblings as well as 24 unrelated healthy individuals studied.

Recently, patients with juvenile diabetes were found to have increased levels of maternal cell microchimerism when compared to their unaffected siblings. Nelson et al. [30] reported that maternal microchimerism (MMc) levels, expressed as the genome equivalent per 100,000 proband cells, were markedly higher in T1DM patients' circulation than unaffected siblings and healthy individuals. In the patients with the T1DM-associated DQB1*0302-DRB1*04 haplotype, MMc was found more often when the haplotype was paternally (70%) rather than maternally transmitted (14%). Female islet β cells (presumed maternal) formed 0.39-0.96% of the total, whereas female hematopoietic cells were very rare [30]. There are also strong suggestions of a prominent role of the maternal perinatal environment in the predisposition to juvenile diabetes among the offspring [290,291] (Tables 12 and 13). Fetal DNA in maternal organs also has been detected in animals with streptozocin-induced diabetes [292].

In humans, fetal cells have been detected for decades in the maternal blood and tissues affected by both autoimmune and non-autoimmune diseases, and these cells had morphological features of both hematopoietic and non-hematopietic tissue lineages cells [293-295]. In mice, 30 days after STZ-induced diabetes fetal cells were

detected in the maternal bone marrow, pancreas, liver and kidney (Table 13). Histological examination showed differentiated fetal cells within the pancreatic acinar cells, hepatocytes and tubular epithelial cells, and their morphological appearance was undistinguishable from their maternal counterparts and constant over the long term after delivery [293].

In pancreas samples obtained from 6 males with T1DM Vanzyl et al. [296] found that the frequency of MMc was significantly higher (range 0.32-0.80%, mean 0.58%) than in 4 male controls (0.24-0.50%, mean 0.38%; p=0.05). Interestingly, clusters of 2-3 MMc were occasionally found in the pancreases, particularly T1DM pancreases, suggesting replication of these cells [296]. In all pancreas samples analyzed MMc were identified within islets that appear to be insulin positive but non-insulin staining MMc were also observed in the exocrine tissue [296]. In a separate analysis of pancreatic autopsy material from 4 males with T1DM they demonstrated that female cells in the pancreases of these male individuals were often positive for insulin. It was suggested that maternal cells could contribute to endocrine function in offspring [30,297]. It appeared that in all pancreases analyzed, female (presumed maternal) cells have been identified and immunochemistry showed insulin-producing XX beta cells in males. VanZyl and Gillespie [262] suggested that some cells that transfer from mother to child must therefore be adult stem cells with the capacity to differentiate into islet beta cells. Because these cells are 50% genetically distinct from the host, it could be a trigger for autoimmune attack, but so far there is no evidence to support this hypothesis [262]. On the other hand, this idea may however be in agreement with the fact that *T. gondii* is transmitted as a Trojan horse in many hematopietic cells, and therefore these cells could be the focus of lymphocytic attack and selectively destroyed in T1DM pancreas. This reasoning may be consistent with the findings that islets in T1DM pancreas are often present in a lobular pattern, with islets completely intact on one lobe and totally destroyed on an adjacent lobe [259,260].

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

Results are mean ± 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 [255]. 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 [256].

**Table 11:** Serum proinflammatory cytokines and leptin concentrations in obese children at prepubertal age compared with healthy children of the same age (according to Aygun et al. [253] and Kapiotis et al. [254]; with own modification).

| **Extravillous cytotrophoblasts** |
|---|
| Nucleated erythroblasts |
| Platelets |
| Mesenchymal stem cells |
| CD34+ hematopoietic stem/progenitor cells |
| CD34+ and CD38+ lymphoid progenitors |
| CD19+ and IgM+ B lymphocyte precursor cells |
| **CD8+ T cells** |
| CD4+,CD25high and FOXP3+ regulatory T-cells |
| CD45+ leukocytes |
| CD3+ and CD14+ mononuclear cells |
| CD56+ and CD16+ natural killer cells |

Maternal cells [263] and soluble maternal HLA are also transferred in breast milk [283]

**Table 12:** Different types of cells involved in fetal-maternal trafficking (according to Klonisch and Drouin [32]; with own modification).

| Group of animals | Days of pregnancy | Pancreas | Liver | Kidney |
|---|---|---|---|---|
| Controls | 6 | ND | ND | ND |
| | 13 | ND | ND | ND |
| | 19 | ND | ND | ND |
| Diabetics | 6 | 64.2 ± 6.8 | 9.7 ± 0.8 | 13.5 ± 3.2 |
| | 13 | 45.4 ± 5.1 | 13.3 ± 1.2 | 18.8 ± 3.5 |
| | 19 | 49.8 ± 7.5 | 10.7 ± 1.4 | 10.1 ± 2.9 |

Data are expressed as mean ± SE. Results represent the amount of fetal DNA per 1 x $10^5$ maternal (total) genome equivalents

**Table 13:** Fetal DNA in maternal organs at day 30 after streptozotocin-induced diabetes (200 mg/kg for 2 days) in mice (according to Sunami et al. [292]; with own modification).

## *T. gondii* infection

*T. gondii* can actively infect any nucleated cell type, including cells from the immune system. Persson et al. [298] observed that a large number of NK cells have been infected by the parasite early after intraperitoneal inoculation of *T. gondii* into C57BL/6 mice. It appeared that one mechanism of NK cell infection involved NK-cell-mediated targeting of infected dendritic cells (DC), and infected NK cells were not efficiently targeted by other NK cells. It was suggested that rapid transfer of the parasite from infected DC to effector NK cells may contribute to sequestration of *T. gondii* and shielding from immune recognition shortly after infection [298]. It must be noted that NK cells do not posses intracellular killing pathway, such as for example NO, therefore NK cell infection may provide a niche in which the parasites proliferate and promote its persistence in a less hostile environment [298]. Table 14 presented *in vitro* differences in division rates of intracellular *T. gondii* tachyzoites in various types of cells [300-309].

The risk of infection with the parasite is 0.1% to 1% of all pregnancies [314]. All eukaryotic cells may function as systemic parasite transporters. Maternal microchimerism was found to be more common than fetal microchimerism (40% vs. 15%, p=0.05) [315]. In the mouse model, the effect of maternofetal transmission of *T. gondii* after oral infection was measured by the mortality rate in the mother, the fetus and the neonate [316]. When the infection preceded the mating, the percentage of neonates who died ranged from to 52% to 74%. In contrast, when the mating preceded the infection, these percentages were much more elevated ranging from 74% to 96% [316]. Weight and Carding [10] showed that *T. gondii* upregulated intercellular junction-associated proteins, such as intercellular adhesion molecule 1 (ICAM-1), in MDCK II and BeWo cells [317]. The parasite influenced the cellular distribution of occludin to transmigrate the intestinal epithelium. ICAM-1 is expressed on leukocytes and endothelial cells and binding to its ligand (leukocyte function-associated antigen-1) activates leukocyte transmigration via actin-cytoskeletal rearrangements [318,319]. Migration is most common in type I strains and tachyzoites that move between epithelial cells, via the paracellular pathway, and do so without affecting the integrity of the monolayer [258,317]. It must be also added that NO mediates IFN-γ-induced hyperpermeability, dilates tight junctions and depletes ATP in cultured human intestinal epithelial monolayers [320,321].

| Cell type | Parasite division rate | | Mechanism | References |
|---|---|---|---|---|
| | Unprimed | IFN-γ primed | | |
| Hemopoietic | | | | |
| **Lymphocyte** | S | | | [285] |
| Neutrophil | S | | | [285-287] |
| Adherent monocyte | S | | | [287-290] |
| Nonadherent monocyte | R | R | ROS; not TS | [285,291] |
| Dendritic cell | R | | | [285] |
| Alveolar macrophage | R | S | Partly TS | [155] |
| Peritoneal macrophage | R | S | | [292] |
| Monocyte-derived macrophage | R | S | ROS; not RNI | [155,156,157,288,290,292,293] |
| Nonhematopietic | | | | |
| **Neuron** | S | | | [294] |
| Foreskin fibroblast | R | S | TS | [154,295] |
| Umbilical vein endothelial cell | R | S | TS or ROS; not RNI | [290,296] |
| Retinal pigment epithelal cell | R | S | TS | [297] |
| Fetal astrocyte | R | S | RNI | [294,298] |
| Fetal microglial cell | R | R | | [299] |

R: Rapid; S: Slow; RNI: Reactive Nitrogen Intermediates; ROS: Reactive Oxygen Species; TS: Tryptophan Starvation

**Table 14:** Division rate of intracellular *T. gondii* tachyzoites in primary human cells *in vitro* (according to Channon et al. [299]; with own modification).

Studies in animals performed by Chen et al. [322] showed that multipotent mesenchymal stromal cells were transferred to rat maternal venous blood. They trafficked via VEGF-A and integrin-dependent pathways across the placenta, engrafted in various fetal organs, and persisted in offspring for at least 12 weeks. Silveira et al. [323] found that *T. gondii* may circulate in the peripheral blood of recently and chronically infected immunocompetent individuals with or without ocular lesions. In the patients with active ocular lesions and positive anti-toxoplasma IgG but negative IgM, propagation of the infection may still occur through the blood [323].

*T. gondii* can be also transmitted via breast milk [324,325], and achlorhydria observed in human newborn infants during the first 10 days of life [326] may favor infectivity with various forms of the parasite, such as oocysts, tachyzoites (invasive forms), and/or tissue cysts filled with bradyzoites [327]. It was demonstrated that breastfeeding seems to influence the development of diabetes in non-obese diabetic (NOD) neonates [328], because NOD progeny from NOD mothers deficient in T and B cells failed to develop diabetes [329]. Given the absence of activity of autoantibodies in this process [330,331], it seems that maternal T and predominantly B cells transferred during gestation or lactation might play a role in development of autoimmune diabetes. Roy et al. [275] found that in mice maternal T cells reactive against the endocrine pancreas of their progeny markedly increased islet infiltration. Specific maternal microchimeric T cells targeting fetal antigens in beta cells may also constitute evidence of their predisposing role in development of autoimmune T1DM. It must be emphasized that the maternal environment during gestation and a genetic predisposition to diabetes also play an important role in future development of T1DM in the offspring [290,332].

## Possible Link between a Specific Role of T Cell Membrane Metalloproteinases (MMPS) in Development of Diabetes and *T. Gondii* Infection-Associated Positive Modulation of Macrophages Migration by Increasing Expression of Matrix Metalloproteinases and Decreasing CD44 Receptor at Cell Surface

### Diabetes

MMPs, a family of secreted zinc proteases capable of degrading collagen and other matrix components, may participate in a wide variety of pathophysiological responses, including inflammatory pocesses, embryonic development, and cell apoptosis [333,334]. MMP-2 appeared to be critical in rat-pancreatic islet development and is activated between embryological days 17 and 19, and TGF-β is responsible for islet morphoghenesis by regulating MMP-2 expression [335], On the other hand, tissue inhibitor of metalloproteinase-1 (TIMP-1) prevents cytokine-induced dysfunction and cytotoxicity in pancreatic islets and β-cells, and in addition to inhibiting MMP-2 and MMP-9 activity may also inhibit cytokine-mediated apoptosis in various cell lines. TIMP-1 mediated these effects by inhibiting cytokine-induced activation of NK-κB [333]. Several studies demonstrated that pancreatic islets contain detectable amounts of MMPs and TIMPs [333]. In the children and adolescents with T1DM, Florys et al. [336] found serum MMP-2 as well as TIMP-1 and TIMP-2 levels significantly higher than in controls ($p<0.01$, $p<0.02$, and $p<0.001$, respectively), and a strong positive correlation was noted between MMP-2 and TIMP-2 ($r=0.8$, $p<0.0001$).

Ridnour et al. [337] showed a dose-dependent, biphasic regulatory effect of NO on the activity of MMPs (MMP-1, -9, and 13) secreted from murine macrophages. Low exogenous NO perturbed MMP/tissue TIMP-1 levels by enhancing MMP activity and suppressing the endogenous inhibitor TIMP-1. Exposure of purified latent MMP-9 to exogenous NO demonstrated a concentration-dependent activation and inactivation of the enzyme, which occurred at higher NO flux. They suggested that NO regulation of MMP-9 secreted from macrophages might occur by RNI-mediated protein modification [337].

Zhou et al. [338] demonstrated that MMPs contribute to pancreatic islet fibrosis and insulin insufficiency in Zucker diabetic fatty rats. In both male and female rats, the authors found marked increases the mRNAs encoding proteases and extracellular matrix components that are associated with fibrosis and tissue remodelling. The mRNAs for MMP-2 (>10-fold increase in activity), -12, -14 were sharply increased with the onset of islet dysfunction and development of diabetes [338].

The pathogenesis of T1DM begins with the activation of autoimmune T killer cells and is followed by their homing into the pancreatic islets where they directly contact and subsequently destroy insulin-producing β cells [339]. Autoreactive IS $CD8^+$ T killer cells transmigrated from bloodstream through pancreatic microvessels endothelial cell barrier and into the islets of Langerhans, are specific for islet-derived insulin antigen [339,340]. The cell surface-associated signalling and adhesion CD44 receptor and other adhesion receptors including selectins, cadherins, immunoglobulin superfamily cell adhesion molecules, such as VCA, ICAM-1, and -2) and integrins, which are expressed in both T cells and endothelial cells, contribute to the adhesion of T cells to the endothelium [341]. The high affinity interactions of T cell CD44 with its abundant endothelial ligand, hyaluronian, are essential for firm adhesion and the subsequent transmigration events [342,343]. T cell membrane type-1 MMP ($MT_1$-MMP) regulates the functionality of CD44 (a marker of activated T cells) and thus control the rate at which T cells home into the pancreatic islets, finally affecting the severity of the disease [339]. CD44 is heavily glycosylated and its glycosylation negatively regulates oligomerization, the movement of CD44 across the plasma membrane, and recognition of hyaluronian [339,344].

## *T. gondii* infection

The parasite is able to infect macrophages and dendritic cells for dispersal throughout the body. Seipel et al. [345] found that *T. gondii* infection positively modulated the macrophages migratory molecular complex by increasing MMPs, CD44 and alpha(v)beta(3) integrin. Migration in Matrigel™ of infected macrophages was augmented after 48 hrs of infection, and inhibition of MMPs abolished this process. *T. gondii* infection also induced a decrease of CD44 receptor at cell surface and increased secretion of active MMP-9. Infected macrophages showed increased expression of $MT_1$-MMP and ADAM10 (a disintegrin and metalloproteinase membrane10) MMPs [345]. Expression and function of several MMPs in experimental animals and humans during *T. gondii* infection were presented in table 15 [346-350].

*T. gondii* tachyzoites store toxolysin 4 (TLN4), an extensively processed putative metalloproteinase, in the micronemes and secrete

| Model | Mmp[a]-MMP[b]/TIMP[b] expression pattern[c] | Functional data/associations | Comments | References |
|---|---|---|---|---|
| **1. *In vitro*** | | | | |
| ***T. gondii*-infected THP-1 cells** | Mmp-2,-9, Timp-2↓, Mt1-mmp↑<br>proMMP-2, proMMP-9, TIMP-2↓<br>proMT1-MMP↑, actMT1-MMP↑ | | MT1-MMP activation by *T. gondii* infection probably explains parasite dissemination and access to immune-priviledged sites. | [347] |
| *T. gondii*-infected (macrophage-like) RAW 264.7 cells | ActMMP-9↑ in infected supernatants<br>MT1-MMP↑ in *T. gondii*-infected cells<br>ADAM-10↑ in *T. gondii*-infected cells | **MMP inhibitor I**<br>→ Abolished invasiveness of *T. gondii*-infected macrophages over 3D ECM<br>MMPs might facilitate the access of infected leukocytes to immune-privileged sites | Increasing levels of MT1-MMP → shedding of CD44, a docking molecule for MMP-9 | [345] |
| **2. *In vivo*** | | | | |
| Ileum tissue from *T. gondii*-infected mice | Mmp-2,-9/MMP-2,-9↑ in small intestine<br>ActMMP-2↑ in small intestine<br>IL-23p19KO; mice<br>Mmp-2,-9/MMP-2,-9↓<br>No actMMP-2<br>→ Significantly reduced intestinal pathology | MMP-2KO mice (compared to MMP-9KO and WT mice)<br>→ Protected against the development of intestinal immunopathology and early death; MMP-2 mediates immunopathology in *T. gondii*-infected ileitis.<br>Treatment with gelatinase inhibitors (doxycycline and MMPI RO28-2653)<br>→ Ameliorated intestinal pathology<br>Treatment with gelatinase inhibitors protects mice against *T- gondii*-induced immunopathology | Selective blockage of gelatinoses may be a safe and effective strategy in prevention and treatment of intestinal inflammation | [348] |
| Brain tissue from *T. gondii*-infected mice | Mmp-8, -10 and TIMP-1↑ in brain<br>MMP-8↑ in brain infiltrating $CD4^+/CD8^+$ T cells<br>MMP-10↑ in brain infiltrating $CD4^+$ T cells<br>TIMP-1↑ in CNS-resident astrocytes and in brain infiltrating $CD4^+/CD8^+$ T cells | TIMP-1 deficient mice<br>→ Little morphological changes in tissue structure<br>→ ↑ $CD4^+$ T cells in brain<br>→ Reduced parasite burden in brain<br>TIMP-1 is associated with inhibition of pathogen clearance without development of adverse pathology | MMP-8 and –10 production by brain-infiltrating T cells implies a role for MMPs in brain tissue penetration; TIMP-1 is associated with inhibition of pathogen clearance | [349] |
| **3. Clinical studies** | | | | |
| Serum from pregnant women with or without *T. gondii* infection vs. healthy non-pregnant women | MMP-12 - > associated with ↑ elastin degradation products; Pregnant women with toxoplasmosis > healthy pregnant women > healthy non-pregnant women | | Interaction between MMP-12 and elastin in the serum of infected pregnant women suggests MMP-12 mediates damage to elastin and contributes to *T. gondii*-associated pathology during pregnancy | 350] |

↑↓: Increased or decreased levels; ADAM: A Disintegrin And Metalloprotease; actMMP: activated MMP; CNS: Central Nervous System; ECM: Extracellular Matrix; KO: Knockout; MMP: Matrix Metalloproteinase; MT-MMP: Membrane-Type Matrix Metalloproteinase; TIMP: Tissue Inhibitor Of Metallopproteinases; [a]Mmp/Timp refers to mRNAs; [b]MMP/TIMP refers to proteins. WT: Wild Type.

**Table 15:** Expression and function of MMPs/TIMPs/ADAMs in toxoplasmosis (*T. gondii* infection) (acc. to Geurts et al. [346]; with own modification).

it in response to elevated calcium, suggesting a possible role in cell invasion [351]. TLN4 is initially synthesized as a large precursor (~260 kDa) that is extensively processed into multiple proteolytic fragments within the parasite secretory system, and at least some of these fragments remain associated in a large molecular complex [351].

Niehaus et al. [352] found that T. *gondii* glycosylphosphatidylinositols (GPIs) induced MMP-9 in human macrophage-like THP-1 cells via TLR2/4-dependent mechanism and the degradation of human extracellular galectin-3 (a substrate for MMPs-2 and -9, and a lectin specific for β-galactosides, which binds to both the glycan and lipid moieties of the parasite GPIs). It must be added that the parasite activated TLR2/4 receptors and induced a NF-κB-dependent production of TNF-α in macrophages [353,354].

## Disturbed Carbohydrate Metabolism in Diabetes Mellitus. *T. gondii* Infection also Significantly Affects Glycolysis, Gluconeogensis and Tricarboxylic Acid (TCA) Cycle

### Animals

Glycolysis, gluconeogenesis and the TCA cycle are central pathways of the carbohydrate metabolism which need to be tightly regulated depending on the cellular demand for energy, reducing power and precursors for biosynthesis pathways. Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism [1].

Palsamy and Subramanian [355] found altered activities of the key enzymes of carbohydrate metabolism, such as hexokinase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphatase, fructose-1,6-biphosphatase, glucose-6-phosphate dehydrogenase, glycogen synthase and glycogen phosphorylase in liver and kidney tissues of streptozocin-nicotinamide-induced diabetic rats. During diabetic conditions, the glycogen levels, glycogen synthase activity and responsiveness to insulin signaling were diminished and glycogen phosphorylase activity was markedly increased [356] (Table 16). Glucose-6-phosphatase, a key enzyme in the homeostatic regulation of blood glucose level is critical in providing glucose mainly to the liver and kidney during diabetes, prolonged fasting or starvation [357]. It catalyzes the dephosphorylation of glucose-6-phosphate to free glucose as the terminal step in gluconeogenesis and glycogenolysis. This reaction occurs in the lumen of the endoplasmic reticulum and the enzyme complex is composed of a glucose-6-phosphate transporter that transport glucose-6-phosphate from the cytoplasm into the lumen of the endoplasmic reticulum and a glucose-6-phosphatase catalytic subunit that hydrolyzes the glucose-6-phosphate to glucose and phosphate [358]. Glucose-6-phosphate dehydrogenase catalyzes the first and rate-limited step of the hexose monophosphate shunt and produces NADPH needed for the maintenance of reduced glutathione and reductive biosynthesis [359]. NADPH is essential for both the production of ROS, such as superoxide anions and NO and the elimination of these radicals via glutathione peroxidase and catalase in hepatic as well as extrahepatic tissues [360]. It has been shown that modest changes in glucose-6-phosphate dehydrogenase activity itself have significant effects on cell growth and cell death in a variety of cell types [361,362].

### Diabetic patients

Suhail and Rizvi [363] analyzed activities of the key glycolytic enzymes of red blood cells, i.e. hexokinase (HK), phosphofruktokinase (PFK) and pyruvate kinase (PK) and found that they were significantly ($p<0.01$) increased in patients with T1DM. It was suggested that the increased activity of the enzymes might be due to a greater proportion of young erythrocytes in diabetic individuals because of a shortened red cell life span as compared to healthy persons [363]. However, Sitzmann [364] measured the activity of the same three enzymes in 119 children suffering from juvenile T1DM and reported that the values were mildly diminished both in the group with good state of metabolic control and in those with bad control compared with the respective reference values [364]. This discrepancy may be at least in part explained by the exposure time of these patients to *T. gondii* infection, its intensity, cell/tissue invasiveness of the parasite, its strain, etc. This suggestion may be supported by marked modulations of activities of enzymes participating in the metabolic pathways during glycolysis, gluconeogenesis and TCA-cycle found in *T. gondii* tachyzoites and bradyzoites [365-367] (Tables 17-19). However, it appeared that the level of mRNA upregulation of some enzymes important for carbohydrate metabolism was only moderate, therefore other mechanisms, such as an increase in gluconeogenesis enzyme activity or uptake of glucose-6-phosphate from the host cell by the hexose transporter [377] may also take place [365]. *T. gondii* utilizes host sugars for energy and to generate

| Groups | Glycogen | Glycogen synthase | Glycogen phosphorylase |
|---|---|---|---|
| Controls | 60.83 ± 1.35 | 842.17 ± 10.91 | 658.33 ± 11.46 |
| Diabetic rats | 19.50 ± 2.35[a] | 525.67  14.39[a] | 897.50 ± 28.26[a] |

Units are expressed as: mg/g wet tissue for glycogen, μmoles of UDP formed/h/mg protein for glycogen synthase and μmoles Pi liberated/h/mg protein for glycogen phosphorylase. Values are given as mean ± SEM, for groups of 6 rats in each. [a]Values statistically significant at $p<0.05$

**Table 16:** Level of glycogen content and activities of glycogen synthase and glycogen phosphorylase in liver tissues of streptozotocin-nicotinamide-induced diabetic rats (according to Palsamy and Subramanian [355]; with own modification).

| Protein name | Gene name | Change in expression | Microarray experiment |
|---|---|---|---|
| Aldolase[a] A and B | ALDOA,ALDOB | ↑ | |
| ATP synthase[a] beta subunit | ATP5D | ↑ | |
| Cytochrome c oxidase subunit Vib | COX6B | ↓ | ↑ |
| Dimethyl arginine dimethyl aminohydrolase | DDAH1 | ↓ | ↓ |
| Enolase[a] 1 | ENO1 | M | ↑ |
| Glyceraldehyde 3 phosphate dehydrogenase[a] | GAPDH | ↑ | ↑ |
| Phosphoglycerate kinase[a] 1 | PGK1 | M | ↑ |
| Protein disulfide isomerase | P4HB | | ↑ |
| Pyruvate kinase M2 isozyme | PKM2 | M | |
| Thioredoxin domain 5 isoform 2 | TXNDC5 | ↑ | |
| Triose phosphate isomerase | TPI1 | M | ↑ |

Host cell proteins were designated as being downregulated in expression (↓),upregulated (↑),or modulated (M). [a]These host cell proteins also changed expression in the brains of patients with mild cognitive impairement,early AD,or AD [369]. It must be noted that *T. gondii* tachyzoites are thought to rely upon both glycolysis and the tricarboxylic acid cycle,while bradyzoites are largely dependent upon glycolysis [364,371]. Although tachyzoites utilize both glycolysis and oxidative phosphorylation to obtain energy,glycolysis seems to be the predominant pathway for ATP synthesis in the bradyzoite [372,373]. Moreover,ENO2 and lactate dehydrogenase1 are only found in tachyzoites,while ENO1 and lactate dehydrogenase 2 are exclusively expressed in bradyzoites [374,375]. Silencing of tachyzoite ENO2 altered nuclear targeting of bradyzoite ENO1 in *T. gondii* [376,377].

**Table 17:** Selected modifications in the proteomes of human foreskin fibroblasts infected with *T. gondii*: proteins implicated in glycolysis Nelson et al. [368]; with own modification).

| Enzyme | Gene accession numbers from ToxoDataBase[a] |
|---|---|
| Glucosephosphate-Mutase I (GPM1) | 76.m00002 |
| Glucosephosphate-Mutase II (GPM2) | 641.m00009 |
| Fructose-Biphosphatase I (FBP1) | 20.m03907 |
| Fructose-Biphosphatase II (FBP2) | 50.m00005 |
| PEP-Carboxykinase I (PEP-CK1) | 80.m00002 |
| PEP-Carboxykinase II (PEP-CK2) | 80.m02252 |
| Pyruvate-Carboxylase (Pyc) | 76.m01567 |

In contrast to most of the other *T. gondii* gluconeogenesis genes,namely fructose-biphosphatase,glucosophosphate-mutase and PEP-carboxykinase, which are encoded twice in the genome,pyruvate-carboxylase is only encoded once and the enzyme is localized inside the single mitochondrion,while the remaining reactions of gluconeogenesis are typically cytosolic [365]. [a](www.toxodb.org/toxo/home.jsp)

**Table 18:** Gene prediction for irreversible steps in gluconeogenesis in *T. gondii* (according to Fleige et al. [365]; with own modification).

| Enzyme | Gene accession numbers from ToxoDataBase[a] | Localization |
|---|---|---|
| Citrate Synthase I (CS1) | 59.m03414 | Mitochondrion |
| Citrate Synthase II (CS2) | 20.m03767 | |
| Citrate Synthase III (CS3) | 42.m03311 | |
| Aconitase (ACN) | 42.m03524 | Mitochondrion |
| Isocitrate-Dehydrogenase I (IDH1) | 583.moo674 | Mitochondrion |
| Isocitrate-Dehydrogenase II (IDH2) | 57.m00028 | |
| -Ketoglutarate-Dehydrogenase E1 (OGDH E1) | 49.m03397 | |
| Dihydrolipoly-Transacetylase (OGDH E2) | 38.m00017 | |
| Dihydrolipoly-Transacetylase (OGDH E3) | 20.m03954 | |
| Succinyl-coa-synthetase alpha (Scsa) | 80.m00087 | Mitochondrion |
| Succinyl-coa-synthetase (ATP) (Scsb) | 583.m00592 | Mitochondrion |
| Fumarase (FUM) | 57.m01846 | |
| Malate-Dehydrogenase (MDH) | 641.m00168 | Mitochondrion |
| FAD Malate-Dehydrogenase (MDH-FAD) | 80.m00006 | Mitochondrion |
| Branched-Chain α-Ketoglutarate-Dehydrogenase E1 (BCOGDH-E1) | 49.m00028 | |

It seems that tachyzoites, but not bradyzoites, posses a functional tricarboxylic acid cycle because in cell homogenates of tachyzoites, activities of Succinate Dehydrogenase (SDH) and NADP+-dependent isocitrate dehydrogenase were detected, while no SDH activity could be found in bradyzoites [365,372] [a](www.toxodb.org/toxo/home.jsp)

**Table 19:** Gene prediction for putative tricarboxylic acid cycle-associated enzymes in *T. gondii* and their localization (according to Fleige et al. [365]; with own modification).

glycoconjugate that are important for its survival and virulence. The parasite glucose transporter is proficient in transporting mannose, galactose and fructose besides glucose, and serves as a major hexose transporter at its plasma membrane [70].

## *T. gondii* infection

The parasite posses a complete glycolytic pathway [366], as well as all enzymes for the TCA cycle and mitochondrial electron transports chain [365-368]. Tachyzoites are thought to rely upon both glycolysis and the TCA cycle, unlike the bradyzoites, which are thought to be largely dependent upon glycolysis [370]. Several enzymes of the glycolytic pathway have been shown to be modulated also during differentiation [370,378], with some showing stage-specific isoforms, such as enolase and lactate dehydrogenase (LDH) [379]. LDH1 and LDH2 genes and their products have been implicated in the control of a metabolic flux during parasite differentiation, and LDH knockdown parasites exhibited variable growth in either the tachyzoite or the bradyzoite stage, as compared with the parental parasites [380].

Denton et al. [372] found that both developmental stages of *T. gondii* (i.e. tachyzoites and bradyzoites) contained high activities of phosphofructokinase (specific for pyrophosphate rather than ATP), pyruvate kinase and lactate dehydrogenase (LDH), suggesting that energy metabolism in both forms of the parasite may center around a high glycolytic flux linked to lactate production. The significantly higher activity of the latter two enzymes in bradyzoites suggested that lactate production is particularly important in this developmental form. It should be noted that LDH is a terminal glycolytic enzyme that plays an indispensable role in the interconversion of pyruvate to lactate to yield energy under anaerobic conditions [378] and the reaction occurs in both cytosolic and mitochondrial compartments [357]. Both parasite forms contained low activities of NAPD(+)-linked isocitrate dehydrogenase. The results were consistent with the bradyzoites lacking a functional TCA cycle and respiratory chain [372]. Moreover, *T. gondii* uses PP(i) in place of ATP as an energy donor in at least two reactions: the glycolytic PP(i)-dependent phosphofructokinase and V-H(+)-PPase [vacuolar H(+)-translocating PPase (pyrophosphatase)] [381]. Pace et al. [381] showed that overexpression of cytosolic pyrophosphatase (TgPPase) in extracellular tachyzoites led to a 6-fold decrease in the cytosolic concentration of PP(i) relative to wild-type strain RH tachyzoites. This reduction of PP(i) was associated with a higher glycolytic flux in the overexpressing mutants. In addition to elevated glycolytic flux, TgPPase-overexpressing tachyzoites also possessed higher ATP concentrations relative to wild-type RH parasites. The authors believed that PP(i) had a significant regulatory role in glycolysis and, potentially, other downstream processes that regulate growth and cell division [381].

Glycolysis is a major source for *T. gondii* motility [366,373]. In the presence of glucose, a carbon source for both glycolysis and oxidative phosphorylation, a robust movement in a majority of the tachyzoites was observed, while omission of glucose resulted in a drastic decrease in the fraction of mobile parasites [71.6 ± 7.4% vs. 2.7 ± 2.9% (SD)]. Host cell egress and invasion induce marked relocations of glycolytic enzymes in *T. gondii* tachyzoites [366,373]. In the parasite, Coppin et al. [373] identified several genes and proteins associated with amylopectin

synthesis or degradation and glucose metabolism, including different forms of certain glycolytic enzymes, which are stage-specifically expressed during tachyzoite-bradyzoite interconversion. This interconversion is central to the pathogenic process and sometimes is associated with recrudescence of infection observed especially in the patients with AIDS. Biochemical analysis revealed that the glycolytic metabolite lactate is an inhibitory component of resistant for conversion cells, and upregulation of glycolysis in permissive cells through the addition of glucose or by overexpression of the host cell kinase, Akt (an intracellular ligand), was sufficient to convert cells from a permissive to a resistant phenotype [382]. In chronic infection with the parasite, the bradyzoites are located within tissue cysts that are enclosed by the wall containing specific lectin binding sugars, while the bradyzoites have accumulated large amounts of amylopectin, the storage polysaccharide of glucose [373].

Pomel et al. [367] demonstrated that the glycolytic enzymes of *T. gondii* tachyzoites undergoes a striking translocation from the parasites' cytoplasm to their pellicles upon the parasite egress from host cells. Specifically, the glycolytic enzymes are relocated to the cytoplasmic face of the inner membrane complex as well as to the space between the plasma membrane and inner membrane complex. The glycolytic enzymes remain pellicle-associated during extended incubations of parasites in the extracellular millieu and do not revert to a cytoplasmic location until well after parasite have completed invasion of new host cells [367]. Translocation of glycolytic enzymes to and from the parasite pellicles occurred in response to changes in extracellular [$K^+$] experienced during egress and invasion, a signal that requires changes of [$Ca^{2+}$] concentration in the parasite during egress [367].

Nelson et al. [368] found a considerable up-regulation of the glycolytic pathway in *T. gondii*-infected cell because six of the ten enzymes involved in glycolysis showed evident modification, with either an increase or modulation (aldolase A and B, glyceraldehyde 3 phosphate dehydrogenase, phosphoglycerate kinase, enolase, and pyruvate kinase), and one which showed a decrease in expression (triose phosphate isomerase) [368,383]. Increased glycolysis results in the production of both ATP and pyruvate, which enters most biosynthetic processes in the cell and may considerably affect oxidative phosphorylation (modulation of ATP synthase beta subunit, cytochrome c oxidase subunit Vib, and inorganic pyrophosphatase) (Tables 17 and 20). It should be noted that Kimata and Tanabe [384] found a markedly reduced invasion rate of *T. gondii* observed in ATP-depleted chick embryo erythrocytes, and the rate was restored in ATP-restored cells. This indicated that *T. gondii* invasion was dependent on the ATP level of the erythrocytes). It was also demonstrated that during *T. gondii* infection, six proteins involved in carbohydrate metabolism were modulated in the infected cell (aldose reductase, aldehyde dehydrogenase 1A3, aldehyde dehydrogenase X, hexoaminidase B, phosphoenoylpyruvate carboxykinase, and 6-phosphogluconolactonase) [368]. Infection with *T. gondii* resulted also in dysregulation of the host cell cycle by promoting the G1-to-S transition in infected human foreskin fibroblasts [385].

Fleige et al. [365] showed that in *T. gondii* all analyzed TCA cycle enzymes were localized in the mitochondrion, including both isoforms of malate dehydrogenase (Table 19). The TCA cycle metabolizes acetyl-CoA into $CO_2$, thereby generating ATP and reducing power, which typically enters the respiratory chain. The authors suggested that tachyzoites, but not bradyzoites, posses a functional TCA cycle [365]. Moreover, data on the parasite carbohydrate metabolism by localizing enzymes for glycogenesis and amylopectin synthesis were provided (Table 21). It was found that reactions of gluconeogenesis are mainly cystosolic, including PEP-carboxykinase-I and both isoforms of fructose bisphosphatase and glucosephosphate-mutase, while pyruvate-carboxylase is localized in the single mitochondria [366] (Table 18). *In vitro*, bradyzoites displayed a 2-fold upregulation of fructose-biphosphate I and glucosephosphate-mutase I (5-fold) and II (also 5-fold) compared with tachyzoites. This moderate upregulation of gluconeogenesis genes was likely contributing to satisfying the increased demand of glucose-6-phosphate for amylopectin synthesis, which take place during bradyzoite development [365] (Table 21). However, since the level of mRNA upregulation is only moderate, other mechanisms, such as an increase in gluconeogenesis enzyme activity or uptake of glucose-6-phosphate from the host cell by the hexose transporter [377] might also take place [365]. All these findings strongly suggest that chronic *T. gondii* infection plays an important role in carbohydrate metabolism disturbances characteristic for T1DM and T2DM.

| Protein name | Gene name | Change in expression | Microarray experiment |
|---|---|---|---|
| 6-Phosphogluconolactonase | PGLS | ↓ | ↓ |
| Acetyl coenzyme A acyltransferase | ACAT1 | M | ↑ |
| Adenylate kinase 2 isoform a | AK2 | ↑ | ↑ |
| Adenylate dehydrogenase 1A3 | ALDH1A3 | ↑ | ↓ |
| Aldehyde dehydrogenase X | ALDH1B1 | ↓ | ↓ |
| Aldose reductase | AKR1B1 | ↑ | ↑ |
| Carbonyl reductase[a] | CBR1 | ↓ | |
| Cathepsin B | CTSD | ↓ | ↓ |
| Coproporphyrinogen oxidase | CPOX | ↑ | |
| Glutathione synthetase | GSS | ↑ | |
| Glutathione-S-transferase[a] chain A | GSTA2 | ↓ | ↓ |
| Phosphoenolpyruvate carboxykinase | pck1 | ↓ | |
| Protein-L-isoaspartate *O*-methyltransferase | PCMT1 | ↑ | |
| Pyridoxine 5'-phosphate oxidase | PNPO | ↓ | |
| Pyrophosphatase (inorganic) | PPA1 | ↓ | |

Host cell proteins were designated as being down regulated in expression (↓), upregulated (↑), or modulated (M). Modulated proteins had expression altered across several isoforms on the same gel using the Amersham difference gel electrophoresis, and this probably indicated a posttranslational modification event [354]. [a]The host cell proteins, which also changed expression in the brains of patients with mild cognitive impairement, early AD, or AD [369]

**Table 20:** Selected modifications in the proteomes of human foreskin fibroblasts infected with *T. gondii:* proteins implicated in metabolism (Nelson et al. [368]; with own modification).

| Enzymes | Bradyzoite | Tachyzoite |
|---|---|---|
| Actin | ↑ | ↑ |
| UDP-glucose phosphorylase | ↑ | ↑ |
| Starch (glycogen) synthase | ↑ | ↑ |
| Branching enzyme 1 | ↑↑ | ↑ |
| Branching enzyme 2 | ↑ | ↑↑ |
| Isoamylase | ↑ | ↑ |
| D-enzyme (α-1,4-glucanotransferase) | ↑ | ↑ |
| α-glucan | | 0 |
| α-amylase | | 0 |
| α-glucosidase | ↑ | 0 |
| Glycogenin | ↑ | |
| R1 protein (α-glucan water dikinase) | ↑ | 0 |
| Debranching enzyme | ↑ | ↑ |

↑↑: Markedly increased gene expression; ↑: Increased expression; ↑: Weak expression; 0: No gene present; T. gondii enzymes were identified at the genome Web site: htttp://www.toxodb.org. The parasite genome encodes two fructose 1,6-biphosphatase isoenzymes, a single pyruvate-carboxylase, and two PEP-carboxykinases. The conversion from glucose-6-phosphate into glucose-1-phosphate,which forms the link between amylopectin metabolism and gluconeogenesis, is catalysed by two isoforms of glucosephosphate-mutase [365]. The following soluble tachyzoite antigenic proteins have been identified: a putative protein disulfide isomerase,Hsp60,Hsp70,a pyruvate kinase, a putative glutamate dehydrogenase, a coronin, a protein kinase C receptor 1, a malate dehydrogenase, a major surface antigen 1, an uridine phosphorylase, and a peroxiredoxin [93]

**Table 21:** Bradyzoite and tachyzoite stage-specifically expressed genes coding the enzymes involved in *T. gondii* amylopectin metabolism (Coppin et al. [386]; with own modification).

## N-Linked Glycosylation of Proteins by *T. Gondii* may Participate in the Increased Generation of $HbA_{1c}$ Characteristic for Diabetes Mellitus

### Diabetes

In 1968, Rahbar [387] first discovered the association of increased $HbA_{1c}$ with diabetes mellitus [388]. $HbA_{1c}$ results from glucose condensing nonenzymatically with the N-terminal valine residue of the hemoglobin beta chain [389], and the HbA to $HbA_{1c}$ conversion takes place during the entire life span of the red blood cells, so the $HbA_{1c}$ concentration is higher in old red cells than in new red cells [389]. It must be noted that a significantly higher incidence of major congenital anomalies occurred in the offspring of women who had elevated glycosylated hemoglobin levels in the early part of their pregnancy [390]. This finding may be consistent with the severity of anomalies observed in newborn infants with congenital *T. gondii* infection.

Gould et al. [391] found that erythrocyte 2,3-diphosphoglycerate, a catalyst of glycation, was elevated in high compared with low glycators ($5.61 \pm 0.26$ vs. $4.81 \pm 0.24$ mmol/l, $p<0.001$), and mean centile glycated hemoglobin was positively correlated with intra-erythrocyte pH ($r=0.55$, $p<0.05$).

Merino-Torres et al. [392] found that hemoglobin glycosylation index (HGI) is not related with blood glucose. In addition, the percentage of self-monitored blood glucose was the same for high glycosylators (HGI<0) as for low glycosylators (HGI>0). Moreover, Lachin et al. [393] showed that glycation index was not an independent risk factor for microvascular complications, and effect of this bioparameter on the risk was wholly explained by the associated level of A1c. Therefore, it was suggested that HGI should not be used to estimate risk of complications or guide therapy [393]. These findings may be consistent with an important role of *T. gondii* infection being a potential risk factor in development of diabetes.

### *T. gondii* infection

The post-translational modification of proteins by the addition of N or O-linked oligosaccharides is common in most eukaryotic cells. They are added onto proteins either during their transport into the endoplasmic reticulum (in the case of O-linked oligosaccharides), or during their transport through the Golgi complex (in the case of the O-linked oligosaccharides) [394]. Protein glycosylation plays a critical role in the interaction of various pathogens with their host cells and organisms [395], and the correct folding of proteins and their export from the endoplasmic reticulum [396], as well as the correct intracellular targeting of proteins [397]. Luk et al. [394] showed that N-glycosylation is a common post-translational modification of proteins in *T. gondii* essential for the survival of the parasite and its viability, but the structure of these glycans differs substantially from that usually encountered in animals, plants and other unicellular organisms. The parasite synthesizes a large number of proteins with N and O-linked glycans that are found throughout the secretory pathway of tachyzoites. *T. gondii* tachyzoites contain at least 11 major and multiple minor N-glycosylated proteins, but so far only two have been reported to be glycosylated. The presence of N-linked glycans on GAP50, the membrane anchor for the myosin XIV motor complex in the parasite and gp23, which is a GPI-anchored *Toxoplasma* surface protein , have been demonstrated [384]. It was found that tachyzoite surface glycoprotein gp23 has N-linked glycans in the hybrid-type glycans composed of at least N-acetylgalactosamine, N-acetylglucosamine and mannose. T. gondii microsomes have the ability of to synthesize in vitro a glycosylated lipid-bound high mannose structure that is assumed to be identical with the common precursor for N-glycosylation in eukaryotes [398].

Garenaux et al. [399] demonstrated that *T. gondii* independently transfers endogenous truncated as well as host-derived N-glycans onto its own proteins, and suggested that the parasite scavenges N-glycosylation intermediates from the host cells to compensate for the rapid evolution of its biosynthetic pathway, which is primarily devoted to modification of proteins with glycosylphosphatidylinositol rather than N-glycans. In a similar way, *T. gondii* has been shown to mobilize selected host lipids to fulfill its high metabolic requirements during proliferation [400-402]. These exchanges could be facilitated by the close association of the parasitophorous vacuole (PV) membrane with the host endoplasmic reticulum (and mitochondria), where the early steps of N-glycan biosynthesis take place [399]. It must be emphasized that the PV membrane surrounding intracellular *T. gondii* functions as a molecular sieve allowing exchange of molecules up to 1300-1900 Da between the host cell cytoplasm and the parasitophorous vacuolar space [403], i.e. protein antigens consisting of about 9-12 amino acids with a mean molecular weight of approximately 160 Da.

Finally, Fauquenoy et al. [404] demonstrated that N-glycans are required for efficient binding of *T. gondii* to gliding partners, because the parasite motility and host cell entry was severely impaired in the unglycosylated GAP50 mutants. In addition, N-glycosylation was found to be a prerequisite for GAP50 transport from the endoplasmic reticulum to the Golgi apparatus and for its subsequent delivery into the inner complex membrane. Thus, it seems that *T. gondii* uses N-glycosylation intermediates from the host cell for its own metabolic processes and such metabolic intervention may interfere with the rate of $HbA_{1c}$ generation in diabetics.

## *T. Gondii* Infection may be atleast in Part Responsible for Development of Glucose-6-Dehydrogenase (G6PD) Deficiency, and Probably Participate in the Enhancing Diabetic Ketoacidosis Severity

G6PD deficiency, the most common enzyme deficiency and an X-linked inherited disorder, is affecting over 400 million people world-wide, and causes several diseases, including neonatal hyperbilirubinemia, with acute and chronic hemolysis, although persons with this condition may be asymptomatic.

Tabbara et al. [405] performed serology testing for the presence of *T. gondii* and also analyzed frequency of glucose-6-phosphate dehydrogenase (G6PD) deficiency in 91 blood donor male volunteers aged 17 to 52 years. They found that 53 (58%) individuals were G6P dehydrogenase deficient. In addition, it appeared that 31 (58.5%) G6PD deficient subjects had positive titers for *T. gondii* as compared to 9 (24%) G6PD normal persons ($p<0.002$). It was suggested that G6PD deficiency increased the risk for the parasite infection by 2.5 fold probably due to decreased killing effect of phagocytic cells [405,406]. In addition, Gupta et al. [407] demonstrated that the parasite uses its secretory apparatus to modify lipids in the PVM and host cell membranes, because secreted *T. gondii* soluble phosphatidylserine decarboxylase reduced externalized phosphatidylserine on host cells enabling evasion of phagocytosis. The presence of increased serum anti-*T. gondii* IgM and IgG titers and G6PD deficiency were also reported in a 5-years-old male child [408].

Carette et al. [409] described two patients, one with ketosis-prone type 2 diabetes and the other with T1DM and ketoacidosis, who developed hemolysis during acute decompensation of the disease states. It appeared that both these patients had G6PD deficiency. It was suggested that this abnormality might also be facilitated by the erythrocyte depletion of glutathione, an important antioxidant, which is observed in the patients with diabetes [409,410].

Sobngwi et al. [411] demonstrated that G6PD deficiency alone is not a causative factor of ketosis-prone diabetes because they found a high (20.3%) prevalence of G6PD deficiency also in individuals without G6PD gene mutation, which may suggest a novel pathomechanism predisposing to ketosis-prone diabetes. In addition, in an adolescent with G6PD and T1DM it was found that disorders of hemolysis reduce the exposure time of hemoglobin to glucose, resulting in a falsely low hemoglobin A1c level discordant with blood glucose measurements [412]. Xu et al. [413] found that chronic hyperglycemia caused inhibition of G6PD activity via decreased expression and increased phosphorylation of G6PD, which therefore increased oxidative stress. In cultured cells high glucose concentrations caused activation of protein kinase A (PKA) and subsequent phosphorylation and inhibition of G6PD activity and hence decreased NADPH generation [414]. One may suggest that in the patients with ketosis-prone diabetes, a concomitant infection with *T. gondii* exerted similar effects on PKA activity and phosphorylation of the enzyme (Table 22), which could potentiate harmful effects of high glucose levels, thus finally aggravating clinical course of the disease. This reasoning may be supported by the findings that mononuclear leukocytes from obese patients with T2DM also have reduced activity of 6GPD, hexokinase, and 6-phosphofructokinase [419], and an increased prevalence of proliferative retinopathy was found in the patients deficient in G6PD [420]. In addition, moderate upregulation of gluconeogenesis genes was likely to contribute to the increased demand of glucose-6-phosphate for amylopectin synthesis (this reflects process of gluconeogenesis in contrast to glycolysis, i.e. degradation of this storage material), which takes place during bradyzoite development [365] (Table 21). However, since the level of mRNA upregulation was only moderate, other mechanisms, such as an increase in gluconeogenesis enzyme activity or uptake of glucose-6-phosphate from the host cell by the hexose transporter [363] might also take place [361]. This may suggest that *T. gondii* infection of the red blood cells is able to filch this enzyme from the host cells for its own metabolic requirements, thus leading to the enhancement of G6PD erythrocyte deficiency and aggravation of diabetes state described above by Sobngwi et al. [411]. These reasoning is consistent with the finding of Usher-Smith et al. [421] that one of several factors associated with the presence of diabetic ketoacidosis at diagnosis of diabetes in children and young adults was a preceding infection (OR 3.41, CI 0.94 to 10.47).

## *T. Gondii* Infection may be Associated with Amyloid Deposition in the Pancreatic Islet Beta Cells. The Presence of Lactoferrin (Lf) in Amyloid Fibrils Characteristic for Patients with T2DM Probably Reflects Host Defense Reaction to the Parasite Latent Infection

### Patients with diabetes

Amyloidosis is a disorder of protein metabolism in which normally soluble autologous proteins are deposited in tissues as abnormal insoluble fibrils, causing structural and functional disruptions [422]. In mouse model of type 2 diabetes evidence was presented for role of islet amyloid formation rather than direct action of amyloid [125]. Amyloid fibril formation is the hallmark of T2DM and amyloid fibrils deposit in the extracellular space and generally co-localize with the glycosaminoglycans (GAGs) of the basement membrane, a specialized component of the extracellular matrix that mainly is built of collagen and GAGs. GAGs have been shown to accelerate formation of amyloid

| Protein (localization) | Phosphorylation status in infected cells | Functional category |
|---|---|---|
| Caldesmon | Unphosphorylated | Mitosis |
| Calreticulin | Unphosphorylated | Protein folding |
| Nucleobindin | Unphosphorylated | Protein fate |
| Protein disulphide isomerase | Unphosphorylated | Structural |
| Thyroid hormone binding protein | Unphosphorylated | Signal transduction/cellular communication |
| Chaperonin HSP60 (mitochondria) | Phosphorylated | Energy metabolism |
| Lamin A protein (mitochondria) | Phosphorylated | Unknown |
| Vimentin (mitochondria) | Phosphorylated | Structural |

*T. gondii* kinase activity is involved in phosphorylation of host IκBα and this unusual mechanism can be utilized in manipulating the NF-κB pathway [415]. Moreover, a novel parasite kinase activity at the *T. gondii* parasitophorus vacuole membrane is also capable of phosphorylating host IκB [416]. There is biochemical evidence for the presence of an oxidative phosphorylation and functional respiratory chain in the mitochondrion of tachyzoites [417]. Recently, cAMP dependent protein kinase important for the tachyzoite growth was identified in the parasite [95], and protein phosphorylation is a key event in the process of *T. gondii*-host cell interaction [418]. Changes in the proteomes of human foreskin fibroblasts following infection with *T. gondii* included, e.g. protein kinase C (delta binding protein) (modulated),protein kinase NYD-Sp9 (↑, up-regulated in expression), protein serine/threonine kinase (↓,down-regulated),glutathione synthetase (↑), glutathione-S-transferase chain A (↓), as well as several enzymes involved in glycolysis (Table 17) [368].

**Table 22:** Proteins undergoing a change in phosphorylation state following *T. gondii* infection (Nelson et al. [368]; with own modification).

fibrils *in vitro* for a number of protein systems [423]. Monsellier et al. [423] found that the GAGs acceleration effect was mainly governed by three parameters that account for about 75% of the observed experimental variability: the GAG sulfation state, the solute molarity, and the ratio of protein and GAG molar concentrations. GAGs are long unbranched polysaccharides that often occur as O- or N-linked chains of proteoglycans, with the exception of hyaluronic acid existing in a free form. Naturally occurring GAGs include heparin, heparin sulfate, dermatan sulfate, keratan sulfate, chondroitin sulfate and hyaluronic acid [423]. GAGs have been found intimately associated with all types of amyloid deposits *in vivo* so far analyzed, which may suggest that they play a pivotal role in amyloidogenesis [424-426] because they display an ability to promote fibrillogenesis *in vitro* for a number of protein or peptide systems [427-430]. For example, heparan sulfate and HS proteoglycans accumulation with amyloid β-peptide deposits was found in AD and Tg2576 mice [431]. In addition, for ordered protein to fibrillate, its unique and rigid structure has to be destabilized and partially unfolded, while on the other hand, fibrillogenesis of a natively unfolded protein involves the formation of partially folded conformation, i.e. partial folding rather than unfolding [432]. Moreover, the AD-associated amyloid β-protein exerted potent antimicrobial activity against eight common and clinically relevant microorganisms [433]. Lf, a component of amyloid fibrils in pancreatic islet β-cells, was found to have strong anti-toxoplasmatic activity [434]. In addition, long-term lactation exerted a protective effect on the development of T2DM in women with recent gestational diabetes mellitus [435], at least in part probably because the breast-milk contains increased amounts of Lf [434].

Amyloid formation and aberrant protein aggregations play a role in a range of human diseases including T2DM, Alzheimer's diseases and Parkinson's disease [436-438] (Table 23). Human islet amyloid polypeptide (IAPP, or amylin) is the major protein component of the pancreatic islet amyloid associated with T2DM [124,440-443] and IAPP induced toxicity is believed to contribute to the loss of β-cell mass associated with the late stages of T2DM. This amorphous proteinaceous material originally described as hyaline has tinctorial properties of amyloid [444]. Islet amyloid polypeptide is a 37 aminoacid, beta-cell peptide with is co-stored and co-released with insulin [445,446].

Amyloid deposition in pancreatic islet is one of the most common pathological features of T2DM found in at least one islet at post-mortem in more than 90% of diabetic patients, but also demonstrated in about 15% of elderly (>60 years old) non-diabetic individuals [445,447,448]. In human diabetes, islet amyloid can affect less than 1% and up to 80% of islet mass indicating that islet amyloidosis largely results from diabetes-related pathologies. Interestingly, in the aged rats vitamin D administration mitigated age-related cognitive decline through the modulation of proinflammatory state, increased beta amyloid clearance and decreased amyloid burden [449].

Islet amyloid polypeptide (amylin) is secreted from pancreatic beta-cells, converted to amyloid, and its immunoreactivity was localized to beta-cell lysosomes. Pharmacological doses of islet amyloid polypeptide were found to inhibit insulin secretion as well as insulin action on peripheral tissues (insulin resistance) [450].

## *T. gondii* infection

Tachyzoite forms of the parasite grow within an intracellular vacuole surrounded by host's cell mitochondria and endoplasmic reticulum [88] suggesting that the rapid replication and propagation of the tachyzoites may imply essential metabolites supplied by these organelles of the infected cells [451]. Bradyzoites transform the vacuolar membrane to an envelope called a cyst-wall which loses the ability to bind to mitochondria and endoplasmic reticulum of the infected cells [452]. Tables 24 and 25 are presented with number of organelle and inclusion bodies in different forms of the protozoan, which contain various substances, including amylopectin, probably important for generation of the amyloid present in diabetic islet cells. Guerardel et al. [454] found that *T. gondii* synthesizes amylopectin following changes in the environmental conditions and this storage polysaccharide differs from glycogen and starch in terms of glucan chain length (Table 21). The authors demonstrated that the origin of the host cell can affect the physiology and some key metabolism of the parasite. Using HepG2 cells they discovered that the culture medium of growing intracellular tachyzoites turned yellow rapidly, compared to that of tachyzoites

| Diseases | Protein that forms toxic oligomers | Cell lost |
|---|---|---|
| T2DM | islet amyloid polypeptide | β-cells |
| Alzheimer's disease[a] | β-amyloid protein | cortical neurons |
| Parkinson's disease[a] | synuclein | dopaminergic neurons |
| Prion encephalopathy/ transmissible spongiform encephalopathies | prion | cortical neurons |
| Amyotrophic lateral sclerosis | mutant superoxide dysmutase | motor neurons |
| Polyglutamine/Huntington's disease | Huntington's polyglutamine | pyramidal neurons |

The islet amyloid is derived from islet amyloid polypeptide (IAPP,amylin),a protein co-expressed and co-secreted with insulin by pancreatic β-cells. [a]It must be noted that anti-IgG *T. gondii* antibodies were found to be significantly increased in both Alzheimer's and Parkinson's diseases as compared with controls ($p<0.001$)

**Table 23:** The common molecular basis of islet amyloidogenic proteins-related T2DM and neurodegenerative diseases (according to Haataja et al. [439]; with own modification).

| Stage | Mean no. (range) of: | | | | |
|---|---|---|---|---|---|
| | Rhoptries | Micronemes | Dense granules | Amylopectin | Lipid |
| Sporozoite | 5.9 (2-11) | 55 (40-78) | 9.4 (5-15) | 7.8 (3-13) | 1.25 (1-3) |
| Tachyzoite | 6.7 (2-11) | 25 (19-38) | 9.1 (5-17) | 2.4 (1-6) | 0.6 (0-2) |
| Bradyzoite | 5.5 (2-8) | 75.5 (36-112) | 2.7 (1-5) | 21.8 (7-38) | 0 |

Sporozoites were freshly excysted from 34-day-old oocysts. Tachyzoites were obtained from the peritoneum of an IFN-γ knockout mouse 8 days after inoculation of tissue cysts. Bradyzoites were from cysts in the brains of mice at 8 months after inoculation of oocysts. It must be noted that tachyzoites and bradyzoites could both be present in the same parasitophorus vacuole indicating that stage conversion from tachyzoite to bradyzoite is asynchronous. Numbers represent means that were obtained by counting all organelles or inclusion bodies in 20 longitudinal sections of each type of zoite; ranges are given in parentheses

**Table 24:** Relative numbers of organelle and inclusion bodies in sporozoites, tachyzoites, and bradyzoites of the VEG strain of T. gondii (according to Dubey et al. [453]; with own modifications).

| Stage | Mean size (nm) (range) in: | | |
|---|---|---|---|
| | Dense granules | Amylopectin | Lipid |
| Sporozoite | 208 (175-250) | 356 (200-460) | 388 (200-550) |
| Tachyzoite | 244 (133-334) | **201** (103-333) | 224 (150-400) |
| Bradyzoite | 181 (167-**201**) | 358 (192-603) | 0 |

For the source of these infectious stages, (Table 23). It must be noted that tachyzoites and bradyzoites could both be present in the same parasitophorus vacuole indicating that stage conversion from tachyzoite to bradyzoite is asynchronous

**Table 25:** Relative sizes of inclusion bodies in sporozoites, tachyzoites, and bradyzoites of the VEG strain of *T. gondii* (according to Dubey et al. [453],with own modifications).

growing in non-transformed human foreskin fibroblast cells. This lead to the acidification of the culture medium from pH 7-7.5 to pH 6-6.5 and this change in the pH probably was a consequence of glycolysis responsible for the major energetic metabolism driving ATP synthesis in the parasite, and the elevation of lactic acid level [454]. It is known that the culture medium acidification causes an accumulation of large amounts of amylopectin (10-20 mg of amylopectin per $2\times10^{10}$ parasites). Nutrient starvation occuring during this process may also be involved in amylopectin biosynthesis and accumulation. In another intracellular apicomplexan, *Eimeria spp*., depletion of amylopectin by incubation of the parasite at 37°C or 41°C lead to the impairment of these functions [455,456]. In addition, Ryley et al. [457] incubated Eimeria tenella sporozoites anaerobically, and correlated the decrease in amylopectin stores of the parasite with production of lactic acid plus lesser amounts of $CO_2$ and glycerol.

Sawesi et al. [458] found that in mice *T. gondii* infection resulted in highly increased extracellular levels of glycosaminoglycans, including hyaluronan and chondroitin sulfate A, suggesting a role of these substances in the general defense mechanism of the host cells. This suggestion is consistent with the finding that cell surface heparan sulfate (HS) and glycans, which contain sialic acid, have been shown to act as potential receptors for the parasite [459-461]. Loss of HS chains or sialic acid from cellular glycoconjugates resulted in marked reduction of *T. gondii* infection *in vitro* [460]. Inhibition of glycosaminoglycan-mediated amyloid formation by islet amyloid polypeptide and pro-IAPP processing intermediates reported by Meng and Raleigh [436], as well as the presence of Lf in amyloid fibrils and deposits in the cornea, seminal vesicles, and brain [462], are in agreement with the above-presented findings.

Nilsson and Dobson [462] found a highly amyloidogenic region of Lf (sequence NAGDVAFV) that forms amyloid fibrils at pH 7.4 when incubated at 37°C. Although full-length Lf does not by itself form amyloid fibrils, the protein binds to the peptide fibrils and the binding constitutes a selective interaction with the NAGDVAFV fibrils. The Lf appears to coat the peptide fibril surface to form mixed peptide/protein fibrils, but there was no formation of Lf-only fibrils. It was suggested that such process could be generally important during formation of amyloid fibrils *in vivo* because the identification of both full-length protein and protein fragments was common in *ex vivo* amyloid deposits [462].

Ando et al. [463] reported that in three patients with corneal amyloidosis electrophoresis of amyloid fibrils revealed Lf with and without sugar chains, and N-terminal sequence analysis demonstrated full-length Lf and a truncated tripeptide of N-terminal amino acids, Gly-Arg-Arg. Carboxymethylated wild-type Lf formed amyloid fibrils *in vitro*. Lf gene analysis in the 3 patients revealed a Glu561Asp mutation, and a compound heterozygote of Ala11Thr and Glu561Asp mutations in 1 patient. Heterozygotic Glu561Asp mutation was found in 44.8% of healthy Japanese volunteers, suggesting that the mutation may not be an essential mutation for amyloid formation (p=0.104), and that Lf was this precursor protein [463].

Lf is a cationic iron-binding glycoprotein belonging to the transferrin family, which accumulates in the amyloid deposits in the brain in neurodegenerative diseases, such as AD and Pick's disease. Iwamaru et al. [464] showed that bovine Lf inhibited amyloidogenic isoform of cellular prion protein (PrP(Sc)) accumulation in scrapie-infected cells in a time- and dose-dependent manner. Lf mediated the cell surface retention of normal cellular prion protein by diminishing its internalization and was capable of interacting with it as well as with PrP(Sc). In addition, Lf partially inhibited the formation of protease-resistant prion protein. Lf present in the breast milk also protected against *T. gondii* infection [420], and current evidence indicate that a short duration of breastfeeding may constitute a risk factor for development of T1DM later in life [465].

## Gastrointestinal Sensory-Motor Dysfunction Reported in Patients with Diabetes Mellitus may be at least in Part Caused by *T. Gondii* Infection

### Diabetic patients

Gastrointestinal disturbances are common in diabetic patients and the entire gastrointestinal tract (GT) may be involved [466-469]. In patients with diabetic autonomic neuropathy (DAN), a number of abnormal conditions have been reported in different segments of the GT, such as esophagus (dysmotility), stomach (dysmotility, delayed emptying) and small and large intestine (dysmotility, delayed transit, bacterial overgrowth and diarrhea) [470] (Table 26). At present, it is believed that DAN is the major factor in the pathogenesis of these GT abnormalities and prolonged hyperglicemia play an important role in development of DAN through the glycation of the enteric nervous system [466,484-486]. Morphological and biomechanical changes as well as alterations of the enteric nervous system in experimental animals and patients with diabetes mellitus are presented in tables 27 and 28.

### Diabetic rats

In animals, streptozotocin-induced model of diabetes was associated with development of serious pathophysiological abnormalities, including morphological changes in the gastrointestinal tract and quantitative alterations of the myenteric plexus neurons

| GT segment | Motility disorders | References |
|---|---|---|
| Esophagus | Increased amplitude and number of peristaltic contractions | [471-474] |
| | Increased number of spontaneous and non-propagated contractions | [475] |
| | Decreased amplitude of lower esophageal sphincter pressure | [471] |
| | Multi-peaked contractions | [476, 477] |
| Stomach | Decreased antral IMMC | [478] |
| | Decreased postprandial antral activity and the number of antral contractions | [479] |
| | Pyloric dysmotility | [480] |
| Small intestine | Decreased or increased frequency and amplitude of the antropyloroduodenal contractions | [481] |
| | Increased duration of MMC cycle | [482] |
| | Early recurrence of the MMC and clusters of contractile activity | [483] |

IMMC: Inter-digestive Migrating Motor Complex; MMC: Migrating Motor Complex

**Table 26:** Disorders of GT motility in patients with DM (according to Zhao et al. [466]; with own modification).

and other cells. For example, seven days after diabetes induction by streptozotocin, a significant reduction in the circumference and area of the colon (Table 29), size of the myenteric neurons and their nuclei (Table 30), and a decrease in the overall neuronal population as compared with controls, were demonstrated [504]. These changes were associated at least in part with marked body weight loss by the animals with diabetes [504]. Also, the number of myenteric plexus neurons per calculated area on the small and large stomach curvatures in diabetic rats examined after two months were found to be significantly decreased in diabetics compared with controls rats (Table 31). In addition, in the ileum, the relative percentage of the NADH-diaphorase positive myenteric neurons with the smaller area of cell body size in the ileum of diabetic rats increased and these with larger area decreased, in contrast to that found in control animals (Table 32). Izbeki et al. [496] also found that in the jejunum, ileum and colon of rats with streptozotocin-induced diabetes, both the total and the nitrergic neuronal cell number decreased significantly, while in the duodenum only the number of nitrergic neurons decreased. De Mello et al. [495] also demonstrated a significant reduction in density of the duodenal myenteric neurons stained with HuC/HuD compared with controls (18.6 and 19.77%, respectively, $p<0.001$). The density of nNOS neurons was also lower than that of controls (8.62 and 7.30%, respectively), but the difference did not attain statistical significance, which may suggest that the nitrergic neurons are less sensitive to acute diabetes induced by streptozotocin [495]. On the other hand, the density of NADHd-positive neurons in these groups was markedly higher than in control animals indicating that acute diabetes increases the activity of respiratory chain enzymes on these neurons, enhancing their staining [495] (Table 33).

## *T. gondii* infection

Immunochemistry studies performed by Haroon et al. [506] showed that in infected neurons of chronically infected BALB/c mice upon oral infection with *T. gondii* cysts, not only parasitic cysts but also the host cell cytoplasm and some axons stained positive for the parasite antigen suggesting that parasitic proteins might directly interfere with neuronal function. It appeared that both bradyzoites and tachyzoites functionally silence infected neurons because the activity-dependent uptake of the potassium analogue thallium was reduced in cysts harboring neurons, and the percentage of nonfunctional neurons

| Morphological changes | Biomechanical changes |
|---|---|
| Increased intestinal weight, length, weight per unit length | Decreased opening angle and residual strain in duodenum |
| Increased surface area of mucosa | Increased opening angle and residual strain in jejunum and ileum |
| Increased number of goblet cells per villus | Increased circumferential stiffness of the intestinal wall |
| Increased smooth muscle mass | Increased longitudinal stiffness of the intestinal wall |
| Increased different layer thickness | Decreased stress relaxation of small intestine |
| Increased proliferating cell nuclear antigen | |
| Decreased volume of interstitial cells of Cajal | |

**Table 27:** Morphological and biomechanical changes of small intestine in diabetic patients (according to Zhao et al. [466]; with own modification)

| GT segment | Type of abnormality | Species | References |
|---|---|---|---|
| Esophagus,stomach,intestine | Loss of ICCs | Human,mouse,rat | [488] |
| Esophagus,stomach,intestine | Diabetic gastroenteropathy | Human,mouse,rat | [489] |
| Stomach | Gastroparesis, oxidative stress | Mouse | [490] |
| Stomach | Gastroparesis, regional injury of ICCs | Rat | [491] |
| Stomach | Gastroparesis | Human | [492] |
| Stomach | Gastroparesis | Human | [493] |
| Stomach,intestine | Oxidative stress | Human,mouse,rat | [494] |
| Duodenum | Loss of enteric neurons | Rat | [495] |
| Duodenum,jejunum,ileum,colon | Region-specific nitrergic neuronal loss, gastrointestinal motility disorders | Rat | [496] |
| Duodenum,cecum | Loss of enteric neurons | Rat | [497] |
| Jejunum | Decreased NO responsiveness and nNOS protein expression | Rat | [498] |
| Ileum | Loss of enteric neurons | Rat | [499] |
| Small intestine | Loss of enteric neurons, gastrointestinal motility disorders | Human,mouse,rat | [500] |
| Colon | Loss of enteric neurons, gastrointestinal motility disorders, increased oxidative stress | Human | [501] |
| Colon | Reduction in GFAP and neurotrophins | Rat | [502] |

GT: Gastrointestinal Tract; ICCs, Interstitial Cells of Cajal; nNOS, Neuronal NO Synthase; GFAP, Glial Fibrillary Acidic Protein

**Table 28**: Alterations of the enteric nervous system found in experimental and clinical diabetes mellitus (according to Bagyanszki and Bodi,[487]; with own modification).

| Parameters | Controls (n=8) | Diabetics (n=8) |
|---|---|---|
| Length (cm) | 16.33 ± 1.57 | 14.49 ± 0.61 |
| Weight (g) | 2.90 ± 0.42 | 2.51 ± 0.11 |
| Circumference (cm) | 2.60 ± 0.15 | 2.05 ± 0.10[a] |
| Area ($cm^2$) | 42.58 ± 5.04 | 29.91 ± 2.27[a] |

[a]Values significantly different compared with controls ($P < 0.05$)

**Table 29:** Length, weight, circumference, and area of the colon in streptozotocin-induced diabetic rats (according to Furlan et al. [503]; with own modification).

| Parameters | Controls (n=8) | Diabetics (n=8) |
|---|---|---|
| Total profile of the cell body (μm²) | 219.20 ± 4.99 | 193.60 ± 4.32[b] |
| Nucleus profile (μm²) | 81.88 ± 1.57 | 76.77 ± 1.31[a] |
| % of the cell profile occupied by the nucleus | 39.89 ± 0.46 | 42.28 ± 0.42[b] |

*Values significantly different compared with controls ([a]p< 0.05; [b]p< 0.001)

**Table 30**: Areas of cell body and nucleus profiles of the proximal colon myenteric neurons in streptozotocin-induced diabetic rats (according to Furlan et al. [503]; with own modification).

| Rat No. | Small curvature | | Large curvature | |
|---|---|---|---|---|
| | Controls | Diabetics | Controls | Diabetics |
| 1 | 7743 | 4827 | 1331 | 962 |
| 2 | 6495 | 5544 | 1661 | 1172 |
| 3 | 8127 | 5172 | 2269 | 1770 |
| 4 | 9050 | 5018 | 1524 | 1032 |
| 5 | 7854 | 5784 | 1535 | 1458 |
| Mean | 7854 | 5269 | 1664 | 1279 |
| SD | 917 | 390 | 358 | 334 |
| P | | 0.007319 | | 0.008835 |

**Table 31:** Number of neurons per 11.6 mm² areas of the small and large stomach curvatures in streptozotocin-induced diabetic rats (according to Fregonesi et al. [504]; with own modification).

| Area of cell body size (μm²) | Absolute (F) and Controls F | relative (%) % | frequency of Diabetics F | neurons/group % |
|---|---|---|---|---|
| <100 | 14 | 2.8 | 24 | 4.8 |
| 100-200 | 186 | 37.2 | 311 | 62.2 |
| 201-300 | 212 | 42.4 | 144 | 28.8 |
| 301-400 | 74 | 14.8 | 21 | 4.2 |
| 401-500 | 11 | 2.2 | - | - |
| > 501 | 3 | 0.6 | - | - |
| Total | 500 | 100 | 500 | 100 |

**Table 32:** Changes of absolute (F) and relative (%) frequency of NADH-diaphorase positive myenteric neurons classified according to the area of their cellular body profiles in the ileum of diabetic rats (n=5 per group) 15 weeks after induction of the disease with streptozotocin (according to Alves et al. [505]; with own modification).

| Technique | Group of animals | Density of neurons |
|---|---|---|
| HU | Controls (n=5) | 1472.80 ± 179.14 |
| | Diabetics (n=5) | 1198.80 ± 237.24[a] |
| | Insulin-treated rats (n=5) | 1181.60 ± 179.53[a] |
| NADH | Controls (n=5) | 631.80 ± 5.18 |
| | Diabetics (n=4) | 820.0 ± 8.13[a] |
| | Insulin-treated rats (n=4) | 987.5 ± 19.69[a] |
| NOS | Controls (n=5) | 454.80 ± 59.41 |
| | Diabetics (n=5) | 415.60 ± 109.30 |
| | Insulin-treated rats (n=5) | 421.60 ± 48.22 |

Results are expressed as mean ± SD; [a]p<0.001. HuC/HuD,anti-human neuronal protein HuC/HuD identifies all neuronal cell bodies in the ganglion; NADHd, diaphorase positive

**Table 33:** Density of neurons (neurons/11.07 mm²) reactive to the HuC/HuD and nNOS immunohistochemical techniques and NADHd histochemistry of the myenteric plexus of the duodenum in adult rats with experimental acute diabetes (according to de Mello et al. [495]; with own modification).

increased over time in these animals. *In vitro*, live cell calcium ($Ca^{2+}$) imaging investigations revealed that tachyzoites actively manipulated $Ca^{2+}$ signaling upon glutamate stimulation leading either to hyper- or hyporesponsive neurons, and depleted $Ca^{2+}$ stores in the endoplasmic reticulum [506].

In rats, after oral infection with *T. gondii* oocysts, Sant'Ana et al. [507] found a marked reduction in the number of goblet cells producing neutral mucins (PAS+) and sulphomucins (AB pH 1.0) as compared with control animals, and these changes reflected production of a more fluid mucous (Table 34). The number of vasoactive intestinal peptide (VIP-IR) submucosal neurons as well as the area of the VIP-IR neuronal cell bodies also decreased significantly compared with control rats (667 ± 6.98 vs. 856 ± 14.89 per 1.74 mm² of the jejunum, p<0.05, and 317.29 ± 9.28 vs. 404.24 ± 11.10 μm², p<0.05, respectively). All these abnormalities indicated that oral *T. gondii* infection caused alterations in the chemical composition of the intestinal mucous and reduction in the number of submucosal neurons associated with atrophy of the remaining neurons in this cell subpopulation [507]. In this context, it is suggested that the morphological changes reported in duodenal atresia (a well known neonatal intestinal disease), such as the neuronal cells decreased in number and size, the circular musculature moderately-to-severely hypertrophic, and the interstitial cells of Cajal decreased even around the myenteric plexus [508], were due to chronic latent *T. gondii* infection. Also inflammatory lesions involving esophagus, stomach and duodenum frequently observed in neonates may be caused by the infection with the protozoan acquired prenatally [509-511]. Recently an association between *T. gondii* infection and development of abdominal hernia has been proposed [512]. More detailed gastrointestinal disturbances caused by chronic infection with the parasite in experimental animals and patients with several neurodegenerative diseases have been described elsewhere [434].

## Possible Links between Elevated Plasma Levels of TGF-β1 in both T1DM and T2DM, and *T. gondii* Infection. Dual Role: TGF-β Inhibits Inflammation and Autoimmunity, and Increases Intracellular Parasite Replication

### Diabetes

TGF-β is a multi-functional cytokine with anti-inflammatory activities, such as inhibition of proliferation, maturation and/or activation of macrophages, lymphocytes and NK cells [513-516]. Smart et al [517] reported that TGF-β signaling is crucial for establishing and maintaining defining features of mature pancreatic β cells. Several authors demonstrated significantly increased plasma TGF-β1 levels in patients with NIDDM (7.9 ± 1.0 ng/ml vs. 3.1 ± 0.4 ng/ml, P<0.001; correlation with glycosylated hemoglobin ($r^2$ = 0.42, P<0.001), women with T2DM, and women with prior history of gestational diabetes mellitus as compared with control individuals [518,519]. Elevated TGF-β1 concentrations were associated with retinopathy and neuropathy. It was interpreted as an anti-inflammatory response as these patients were known to have sublinical inflammation presumably triggered by hyperglycemia [519], although there might be also a concomitant *T. gondii* infection. Smoker diabetic patients showed also much higher plasma and urinary TGF-β1 levels than non-smoker diabetic individuals (12.6 ± 4.9 vs. 7.7 ± 4.7 ng/ml, P < 0.001; 27.5 ± 16.0

| Animals | IELs | Goblet cells | | |
|---|---|---|---|---|
| | HE | PAS+ | AB 1.0+ | AB 2.5+ |
| Controls | 7.80 ± 1.65 | 21.30 ± 3.29 | 9.71 ± 1.22 | 7.10 ± 1.12 |
| Infected rats | 8.90 ± 1.42 | 18.60 ± 2.18[a] | 5.70 ± 1.79[a] | 6.20 ± 1.73 |

Values are means ± SD; [a]Results significantly different compared with controls (P <0.05); AB 1+ or 2.5+, Alcian blue pH 1 or 2.5; HE: Hematoxylin/Eosin; IELs: Intraepithelial Lymphocytes; PAS: Periodic Acid Shiff

**Table 34:** Changes in proportion of IELs and goblet cells/100 epithelial cells in the jejunum mucous tunica of rats 36 days post oral infection with 500 sporulated genotype 2 *T. gondii* ME-49 strain oocysts (according to Sant'Ana et al. [507]; with own modification).

vs. 15.3 ± 6.3 ng/mg urinary creatinine, P = 0.01; and 15.3 ± 6.3 vs. 8.1 ± 4.4 ng/mg urinary creatinine, P<0.02, respectively) compared with control subjects [520]. These are important findings because TGF-β system mediated also diabetic renal hypertrophy and fibrosis build-up due to the extracellular matrix production [521,522], and TGF-β1 was found to induce vaginal tissue fibrosis in animal model [523], while the beta cell hypertrophy, beta cell damage and fibrosis, with reduction in insulin secretion, is characteristic for patients with T2DM [7]. In this context, a significant increase of plasma TGF-β1 levels caused by latent *T. gondii* infection may play a key role in development of pancreatic islet beta cell abnormalities found in T2DM.

Tonkin & Haskins [524] demonstrated that regulatory T cells (Tregs) transfer causes a reduction in the number of effector $T_H1$ T cells and macrophages, and also inhibits effector T cell cytokine and chemokine production. Transfection of effector T cells with a dominant negative TGF-β receptor showed that *in vivo* suppression of diabetes by TGF-β-induced Tregs is TGF-β-dependent [524]. Anti-islet autoimmunity can be inhibited by transfer of „natural" $CD4^+CD25^+$ Tregs [525-528], or by induced Tregs which upregulate the Treg transcription factor Foxp3 after activation of CD4 T cells in the presence of TGF-β [524,529,530]. However, in pregnant mice, the infection of *T. gondii* caused the decrease of $CD4^+CD25^+$-regulatory T cells [235].

TGF-β plays a critical role in the suppression of lymphocyte proliferation and differentiation therefore preventing hazardous autoimmune responses, and its immunosuppressive effects are mediated through the inhibition of TNF-α and IL-1 [531] and blocking the induction of adhesion molecules like ICAM-1 and VCAM-1 [519,532,533]. Filisetti & Candolfi [534] reported that TGF-β is well known for its immunosuppressive action on leukocyte cell lines. This cytokine was found to be an antagonist of TNFα, TNF-β, IFN-γ and IL-2 [130,221]. The antiinflammatory action of TGF-β control development of immunopathological processes related to $T_H1$ immune response in the brain [535] and the intestines [536]. However, TGF-β was reported to increase *in vitro* replication of *T. gondii* in retinal cells, suggesting that this cytokine may be involved also in immunopathological phenomena [537]. Elevated expression of TGF-β in vitreous, retina and retinal pigment epithelium has been correlated closely with retinal fibrosis and choroidal neovascularization [513]. Thus, the development of pancreatic islet β cells fibrosis characteristic for the patients with T2DM may be associated with the increased levels of this cytokine due to *T. gondii* infection because TGF-β belongs to biomediators favouring growth of the parasite [538].

## *T. gondii* infection

Normal pregnancy is characterized by a preferentially $T_H2$ immune response, with the production of antiinflammatory cytokines, such as IL-4, IL-5, IL-10 and TGF-β by both maternal and fetal cells [539-543]. Host protection to *T. gondii* infection involves $T_H1$ type immune response of inflammatory cells, lymphocytes and macrophages with enhanced production of IFN-γ, TNF-α, and IL-1β [534, 544]. Activated macrophages by IFN-γ inhibit parasite replication through a number of potent microbicidal mechanisms such as oxidative [545] and non-oxidative [546] mechanisms as well as the induction by IFN-γ of IDO that degrades tryptophan, which is required for the parasite replication [547]. Barbosa et al. [539] showed that in contrast with HeLa cells, treatments with IL-10 or TGF-β1 induced a considerable augmentation in both *T. gondii* intracellular replication and invasion into BeWo cells. BeWo trophoblasts were unable to control replication of the parasite even in the presence of exogenous IFN-γ [548]. In addition, treatment with IFN-γ alone or associated with IL-10 or TGF-β1 increased the same parameters in BeWo cells, whereas the opposite effect was observed in HeLa cells. When endogenous IL-10 or TGF-β was blocked, both BeWo and HeLa cells were able to control the parasite infection only in the presence of IFN-γ. It was suggested that the higher susceptibility of BeWo cells to *T. gondii* may be due to immunomodulation mechanisms, suggesting the role of trophoblast cells in maintaining a placental microenvironment favourable to pregnancy may facilitate the infection into the placental tissues [539].

Hunter et al. [130] demonstrated that TGF-β antagonizes the ability of IL-12 to stimulate production of IFN- by splenocytes from SCID mice, and suggested a role for TGF-β in regulation of T cell-independent resistance to *T. gondii.* Malipiero et al. [549] found that TGF-β is a potent deactivator of polymorphonuclear leukocytes (PMN) and macrophages since it suppresses the production of ROS, RNI and IL-1. TGF-β impairs expression of L-selectin on PMN and L-selectin is known to be essential for PMN recruitment in bacterial meningitis. On peripheral monocytes TGF-β is chemotactic, enhances phagocytosis, activates the production of cytokines – IL-1, TNF-β, and leads to increased expression of several integrin receptors. On tissue macrophages including microglia, the cytokine was found to inhibit phagocytosis and the production TNF-α, IL-1, IL-6, ROS, and to induce increased expression of IL-1 receptor antagonist [531,549-552].

Activation of macrophages plays an important role in the host resistance against intracellular pathogens. Langermans et al. [221] found that the IFN-γ-induced toxoplasmastatic activity of macrophages was inhibited by TGF- (mean fold increase = 6.3), which was also found for the IFN-γ-induced production of TNF-α, RNI and $PGE_2$ by macrophages. It was found that $PGE_2$, which has macrophage deactivating properties, was not involved in the inhibition of macrophage activation by TGF-β. It appeared that inhibition of TNF- production was a key factor in the TGF-β-induced suppression of macrophage activation with respect to toxoplasmastatic activity and RNI production [221].

Bogdan & Nathan [553] found that TGF-β can induce resting human monocytes to produce TNF, IL-1, and IL-6. It was found that IL-10 was about 25-fold more potent suppressor of LPS-induced TNF production by mouse macrophages than was TGF-β. TGF-β suppressed TNF release on a translational level. TGF-β, IL-4, and IL-10 have been shown to have strong macrophage-deactivating effects.

Seabra et al. [554]. Activated macrophages control growth by NO production. However, *T. gondii* active invasion inhibits NO production, allowing parasite persistence. The mechanism used by *T. gondii* to inhibit NO production persisting in activated macrophages depends on phosphatidylserine exposure. TGF-β1 led to iNOS degradation, actin filament (F-actin) depolymerization, and lack of NF-κB in the nucleus [554].

Nagineni et al. [513] reported that in human retinal pigment epithelial cultures TGF-β enhanced parasite replication. Soluble extracts of *T. gondii* stimulated secretion of both TGF-β1 and TGF-β2 significantly. T. gondii infection completely inhibited secretion of the active form of TGF-β2**.** Finally, Malipiero et al. [555] found that endogenous TGF-β suppresses host defense against pathogen infection also in the central nervous system. Thus, it seems that the increased levels of plasma TGF-β reported in the patients with T1DM and T2DM exert both beneficial and harmful effects because although this cytokine is an important regulator of pancreatic islet development

and has antiinflammatory and immunosuppresive activities, at the same time increases *T. gondii* replication in the host cells with further development of various pathophysiological irregularities.

## Beneficial Effect of Thermal Therapy on Glycemic Control in the Patients with T2DM may be Associated with Stage Conversion of *T. gondii* Tachyzoites to Bradyzoites and Increased Generation of NO by Endothelial NOS

Recently, it was reported that regular thermal therapy might promote insulin sensitivity while boosting expression of eNOS [556], and that control of glycemia was improved in the patients with T2DM receiving regular hot tub treatment [557]. Moreover, it was demonstrated that vascular endothelial constitutive isoform of NO synthase (eNOS) has been induced in cultured endothelial cells and cardiomyocytes exposed to mild heat (42°C) [558,559]. Endothelial eNOS mRNA expression and NO production also augmented about 40% in hamsters given daily hot treatments (15 min of infrared sauna) that increased core temperature by about 1°C [560,561]. In the patients with coronary risk factors who underwent daily sauna treatment for two weeks (15 min of 60°C infrared sauna followed by 30 min covered with blankets) endothelium-dependent vasodilation was found to increase markedly [562]. Recently, it was also found that in mice thermal induction and overexpression of hsp72 might counter high fat diet-induced insulin resistance [563]. Several studies [564,565] showed that a physiologically relevant hyperthermia (39°C) selectively induced constitutive hs-hsp70 (hsc70) in H9c2 cardiac myoblasts and conferred oxidative protection. Hsc70-enriched cells exhibited a marked resistance to oxidative challenge, including exposure to hydrogen peroxide, hydroxyl radical, and hypoxia/reoxygenation [565]. These are important findings because, for example, the seropositivity rate for anti-*T. gondii* IgG antibodies among patients with chronic heart failure was significantly higher than in healthy volunteers (68% vs. 36%, ($P < 0.05$), respectively) [566], and infection with the protozoan is associated with oxidative stress [209].

An increase in heat shock proteins 60, 70 and 90 (Hsp90), formation in *T. gondii* was demonstrated in bradyzoites on conditions which induce stage conversion, including increased temperature [567-569], and Hsp60 contributed to protection against the parasite infection [570]. Moreover, HSPs have been found to play a key role in the induction of a cellular immune response, including activation of NK cells by HSP70, which comprise 5-20% of peripheral blood mononuclear cells and are important in the control of bacteria, parasites and viruses [571]. Febrile temperatures (41°C) resulted in a synergistic increase in Hsp90 and Hsp70 synthesis induction [572,573], and a temperature-controlled shift from oligomeric complexes to smaller species with increasing temperature was found for small HSPs with low molecular mass of about 12-43 kDa [574] (Table 35).

Thus, the beneficial effects of thermal therapy reported in patients with T2DM may be associated with the changes in the stage of *T. gondii* during latent chronic pancreatic toxoplasmosis because bradyzoites to tachyzoites interconversion can be due to a variety of factors, including temperature-dependent HSPs induction (Table 36). This reasoning may be supported by the following findings: a) in mice a mutagenized strain tachyzoites of *T. gondii* showed variable growth at temperatures between 34 and 39°C, and inability to grow at 40°C, which correlated with a loss of virulence [583]; b) the reports on the parasite-induced changes in human behavior, including decreased psychomotor performance in individuals with latent asymptomatic toxoplasmosis [584]; and c) the fact that *T. gondii* may circulate also in the peripheral blood of immunocompetent persons with acute and chronic toxoplasmosis [323]. Virulence of the parasite is associated with distinct dendritic cell responses and reduced numbers of activated $CD8^+$ T cells [585]. It seems, therefore, that marked changes in the innate immune state associated with an improvement of balance between various proinflammatory and antiinflammatory cytokines and other inflammation mediators caused by fever probably resulted in *T. gondii* tachyzoite (representing a subacute persistent stage of cerebral toxoplasmosis) bradyzoite (representing a chronic stage) interconversion and/or apoptosis in tachyzoites, finally beneficially affecting glycemic control of some patients with T2DM.

| RH tachyzoites, ME49 strain of *T. gondii*. ESP molecular weight (kDa)[a] | Temp. (°C) 4 | 25 | 37 | 42 |
|---|---|---|---|---|
| 110 | | | + | + |
| 97 | | | + | + |
| 86 | | | + (Tg386) | + |
| 80 | | | + (Tg485) | + |
| 70 | + | + | + | |
| 60 | | | + | + |
| 54 | + | + | + | |
| 42 | | | + (Tg786) | + |
| 40 | | | + | + |
| 36 | + | + | + (Tg378) | |
| 30 | + | + | + | |
| 28 | + | + | + (Tg556) | |
| 26 | + | + | + | |
| 22 | + | + | + | |
| 19 | + | + | + | |

[a]The RH strain of *T. gondii* was maintained by peritoneal passages in Balb/c mice. For positive reference serum, mouse was infected with ME49 strain of the parasite for 8 weeks until the animal had brain cysts postmortem. The molecular mass of 15 ESP was estimated at 37°C. Among them,110, 97, 86, 80, 60, 42 and 40 kDa proteins were released temperature-dependently, while those of 70,54,36,30,28,26,22,and 19 kDa were released temperature-independently as low as 4°C. Five ESP of 86, 80, 42, 36 and 28 kDa reacted with monoclonal antibodies: Tg378 and Tg556 clones were detected 36 kDa and 28kDa proteins, respectively, in dense granules with involvement into parasitophorus vacuole, and these ESP were released regardless of temperature and time. Tg386 clone labeled presumably micronemal structure in tachyzoites, Tg485 clone labeled surface membrane protein; while Tg786 clone labeled probably rhoptry in the apical portion. ESP by Tg786 clone was released continuously with increment, whereas those by Tg378 and Tg556 clones were ceased to release after 3 and 4 hrs changes, respectively

**Table 35:** Profile of Excretory/Secretory Proteins (ESP) released from purified tachyzoites of *T. gondii* incubated for 1 hr at different temperatures and then analyzed by monoclonal antibodies (Son and Nam [575]; with own modification).

| Tachyzoite to bradyzoite conversion | Bradyzoite to tachyzoite conversion |
|---|---|
| High pH[a] | **Lack of NO** |
| Low pH[a] | Lack of IFN-γ |
| Heat shock[b] | Lack of TNF-α |
| Mitochondrial inhibition[c] | Lack of IL-12 |
| Presence of NO[c] | **Lack of T cells** |
| Elevation of both cAMP and cGMP[d] | Induction of a variety of HSPs, including HSP70, is associated with bradyzoite transition[e] |

[a]One cannot exclude that the differences in therapeutic efficacy between valproic acid and sodium valproate used in several neuropsychiatric diseases were related to acidic or alkaline target local tissue conditions induced by these two pharmaceutical forms of a drug (low doses vs. high doses, respectively) [577,578]. [b]Physiologically relevant circumstances that could play a role in stage conversion in vivo include heat shock through a fever [576]. [c]NO overproduction in ASD individuals [579] is an inhibitor of mitochondrial function [106]. [d]Stress-induced elevation of cAMP could play a role in bradyzoite induction because addition of cAMP or cGMP to tachyzoites can stimulate stage conversion [580]. PLK, a *T. gondii* ME49 clonal strain able to differentiate *in vitro*, exhibited a rise in cAMP in response to bradyzoite inducing conditions, but elevation of cAMP under the same conditions was not evident in RH, a strain that does not differentiate well [580]. It must be emphasized that inducers of oxidative stress (nb. a state characteristic for autistic patients) also have been demonstrated to cause parasite encystment *in vitro* [581,582]

**Table 36:** Factors associated with tachyzoite and bradyzoite interconversion (according to Lyons et al. [576]; with own modification).

## Vitamin D Deficiency is Linked with Development of Diabetes Mellitus. Protective Role of Vitamin D may be Partly Due to its Immunomodulatory and Antitoxoplasmatic Activities

### Diabetes

Sørensen et al. [586] reported a trend toward a higher risk of T1DM with the lower serum levels of vitamin D during pregnancy (the odds of the disease was more than 2-fold higher for the offspring of women with the lowest levels of 25-OH D compared with the offspring of those with levels above the upper quartile).

Evidence exists that patients with T1DM and T2DM have a higher incidence of hypovitaminosis D [587], and vitamin D deficiency has been associated with increased risk of T1DM. Vitamin D deficiency in early life accelerates T1DM in non-obese diabetic mice [588]. Children and adults need at least 1000 IU of vitamin D per day to prevent deficiency when there is inadequate sun exposure [589]. Interestingly, BCG vaccinated infants were almost six times (CI: 1.8-18.6) more likely to have sufficient plasma vitamin D concentrations than unvaccinated infants [590]. It appeared that *Mycobacterium tuberculosis* purified protein induced a significant increase of several immune factors in adolescents, including IFN-γ, TNF-α, IL-2, IL-6, IL-10, IL-17, GM-CSF, MIP1α, and IP-10 when compared to paired samples taken prior to BCG vaccination (P<0.0025) [591].

Moreover, intranasal vaccination with mycobacterial 65-kD heat shock protein (HSP) prevented development of insulitis and diabetes in non-obese diabetic mice [592], and DNA vaccine containing the mycobacterial hsp65 gene protected mice from streptozotocin-induced insulitis and diabetes [593]. These findings may be explained by the important role of HSPs acting as molecular chaperones in protection from and pathogenesis of infectious diseases [434,568,594]. Hisaeda & Himeno [595] showed that the expression of host-derived 65 kDa HSP was crucial in directing host immune system to achieve protective immunity against infection with *T. gondii*. A relationship was found between the biomolecule expression on/in host macrophages and development of immune defense against the parasite, regardless of differences in strains and forms of the protozoan (Tables 37 and 38).

Vitamin D treatment has been shown to improve, and even prevent, development and/or clinical course of T1DM in both humans and in animal models [596]. Pancreatic islet insulin-producing beta-cells as well as numerous cell types of the immune system express the vitamin D receptor and vitamin D-binding protein. Some organs have the capacity to metabolize 25-hydroxyvitamin D to its active form 1,25-dihydroxyvitamin D, which has a potent immunomodulatory activity that also enhances the production and secretion of several hormones, including insulin [589]. Pharmacologic doses of $1,25(OH)_2D$ prevented insulitis and T1DM in nonobese diabetic mice [597] and other models of T1DM, possibly by immune modulation, such as for example, increased monocyte differentiation to macrophages, thus increasing their cytotoxic activity, reduced the antigen-presenting activity of macrophages to lymphocytes, prevented dendritic cell maturation, decreased proliferation of activated lymphocytes, inhibited T lymphocyte-mediated immunoglobulin synthesis in lymphocyte B cells, delayed-type hypersensitivity reactions, and generation and activity of NK cells [598-603], as well as by direct effects on beta-cell function. It should be noted that vitamin D deficiency was found to be associated with retinopathy in children and adolescents with T1DM [604]. The prevalence of this clinical entity was higher as compared with the vitamin D sufficient patients (18 vs. 9%, P = 0.02; OR 2.12 [95% CI 1.03-4.33]), and was dependent of diabetes duration (1.13, 1.05-1.23), and $HbA_{1c}$ levels (1.24, 1.02-1.50) [604]. This is an

| Infected parasite | Host status[a] | Resistance to infection[b] | Expression of HSP65[c] |
|---|---|---|---|
| **Beverly strain** | | | |
| Bradyzoite | Non-immune | + | + |
| Bradyzoite | Immune with sonicated *T. gondii* | ++ | ++ |
| Tachyzoite After *in vivo* passage | Non-immune | - | - |
| RH strain | | | |
| Tachyzoite | Non-immune | - | - |
| Tachyzoite | Live-vaccinated with a low dose of the Beverly strain | ++ | ++ |

[a]BALB/c mice were used as hosts; [b]Symbols used here represent resistance to infection; +: resistant; ++: very resistant; -: susceptible; [c]Symbols used here represented levels of HSP65 expression as follows: +: strong; ++: very strong; -: none

**Table 37:** Relationship between HSP65 expression and resistance to infection with *T. gondii* (according to Hisaeda and Himeno [595]).

| Host[a] | Expression of HSP65[b] | Resistance to infection[c] |
|---|---|---|
| **CB17 scid/scid** | - | - |
| + Fetal thymus graft | + | + |
| + Fetal liver cell transfer | ++ | ++ |
| BALB/c nu/nu | ± | - |
| BALB/c +/+ | ++ | ++ |
| + Anti-TcR αβ | +++ | +++[d] |
| + Anti-TcR γδ | - | - |

[a]TcR: T-cell receptor; [b]Symbols represent as follows: -: none; ±: very weak; +: weak; ++: strong; +++: Very strong; [c]Infection with bradyzoites of the Beverly strain of T. gondii. Symbols represent as follows: -: Susceptible; +: weakly resistant; ++: resistant; +++: strongly resistant; "++" here (also expression level) is comparable with "+" in Table 34. [d]Especially at the early phase of infection. Scid, severe combined immunodeficiency; nu/nu, nude mice

**Table 38:** Requirements of T cells for HSP65 expression (according to Hisaeda and Himeno [595]; with own modification).

important finding because retinopathy and other abnormalities were frequent complications reported in individuals with ocular toxoplasmosis [605-607], and cataracts have been demonstrated also in both streptozotocin-induced diabetic rats [608] and T1DM pediatric population [609]. Tedesco et al. [607] demonstrated free parasites in the retinal vasculature, the presence of mononuclear inflammatory infiltrate and parasites in the vasculature of choroids in infected eyes. It was suggested that the increased levels of histamine found in the retina and choroid of diabetic rats may enhance permeability of local vascular bed and participate in development of diabetic ocular complications, including lens opacities [610].

T2DM involves impaired pancreatic β cell function, insulin resistance and inflammation [611]. In T2DM, several disturbances in concentrations of systemic inflammation mediators have been demonstrated, including proinflammatory cytokines and other factors: IL-2, IL-6, IL-12, IFN-γ, TNF- and TNF-β, C-reactive protein, and plasminogen activator inhibitor-1 [603,612,613]. Vitamin D supplementation can increase insulin sensitivity and decrease inflammation in the patients with T2DM [587]. Studies showed that hypovitaminosis D was associated with an enhanced inflammatory response manifested as significantly increased serum TNF-α, IL-6, and CRP levels in healthy [613-616] and obese persons [617, 618]. Moreover, in some clinical states associated with inflammation, vitamin D supplementation caused a marked decrease in serum levels of these proinflammatory factors [619, 620] and an increase in anti-inflammatory cytokine IL-10 concentration [621]. $1,25(OH)_2D_3$, vitamin $D_3$ analog, exerted direct action on purified mouse Langerhans cells reducing IL-10 production and enhancing the production of IL-6 and IL-12p40 upon activation by CD40 ligation [622]. In addition, $1,25(OH)_2D_3$ upregulated the production of IL-1β, CCL3, CCL4, and CCL5. The generation of $T_H2$-type chemokines, represented by CL17 and CCL22 was inhibited, whereas IFN-γ-triggered production of $T_H1$-type chemokines represented by CXCL9, CXCL10, and CXCL11, was increased [622]. It was reported that daily intake of 2000 UI vitamin D was associated with improved β cell function [623]. Moreover, results of a cross-sectional analysis showed that patients with serum 25OH D ≥ 80 nmol/l levels had reduced risk of developing T2DM when compared to those individuals who had ≤ 37 nmol/l [624].

Vitamin D is important for insulin synthesis and release because of the presence of both 1-α-hydroxylase and vitamin D receptor in pancreatic β cells [587,625]. Vitamin D is also involved in insulin sensitivity by controlling calcium flux through the membrane in both β cells and peripheral insulin-target tissues [611,626]. The opening of voltage-sensitive $Ca^{2+}$ channels allows $Ca^{2+}$ uptake by β-cell, thereby contributing to secretion of insulin [97]. Evidence exists that vitamin D has a potential antimicrobial activity and therefore may reduce the risk of various infections through multiple mechanisms [627]. Dendritic cells (DCs) are primary targets for the immunomodulatory activity of $1,25(OH)_2D_3$, as indicated by inhibited DCs differentiation and maturation, leading to down-regulated expression of MHC-II, costimulatory molecules and IL-12. Inhibition of this proinflammatory cytokine production by $1,25\text{-}(OH)_2D_3$ is associated with down-regulation of NF-κB protein levels in activated lymphocytes [628,629]. $1,25\text{-}(OH)_2D_3$ dose-dependently inhibited LPS-induced cytokines production in PBMC modulating intracellular calcium [630]. In addition, this active metabolite may protect against oxidative injuries caused by the NO burst because it dose-dependently inhibited iNOS messenger RNA expression of the LPS-stimulated RAW 264.7 cells and also significantly reduced the gaseous NO release and OONO- production [631]. Moreover, $1,25(OH)_2D_3$ enhances IL-10 production and promotes DCs apoptosis [632], as well as increases $PGE_2$ production by monocytes, a mechanism which partially accounts for the antiproliferative effect of $1,25\text{-}(OH)_2D_3$ on lymphocytes [633]. This metabolite has a direct effect on naive $CD4^+$ T cells to enhance development of $T_H2$ cells [634], increases regulatory T-cells and arrests autoimmune diabetes in NOD mice [635]. These actions emphasize the plethora of general benefits of vitamin D and its active metabolite in immunomodulating and antimicrobial mechanisms, thus favoring the host in courtailing present and imminent infections.

### *T. gondii*

In acute toxoplasmosis, $1,25(OH)_2D_3$ reduced survival rate of infected mice compared to untreated animals, and significantly decreased serum IFN-γ and IL-12p40 concentrations indicating inhibition of $T_H1$-type cytokines, as well as reduced $CD4^+$ T lymphocyte and splenocyte counts, thus enhancing host sensitivity to *T. gondii* infection [636]. Surprisingly, no increase in parasite load was observed in the organs, which suggested an inhibitory effect of $1,25(OH)_2D_3$ at a cellular level [636], like previously it was reported for *Plasmodium faciparum* [637]. Further studies showed that treatment with vitamin D dose-dependently inhibited both *in vivo* and *in vitro* growth of *T. gondii* intracellularly, possibly by limiting tachyzoite proliferation within the parasitophorous vacuole because of activity at the cellular level [627,638]. Ghaffarifar et al. [639] demonstrated that in RPMI 1640 cell culture vitamin $D_3$ (1000 IU) similarly like IFN-γ (100 IU) significantly decreased proliferation of *T. gondii* (RH stran) tachyzoites per infected peritoneal macrophage of BALB/c mice as compared with control animals (Table 39). It should be noted that the *in vitro* inhibiting effect of vitamin $D_3$ alone on tachyzoite proliferation, as well as the increase of NO generation by macrophages, were more distinct than the respective effects of IFN-γ [639]. This emphasizes the importance of NO activity against *T. gondii* tachyzoites in the infected cells (Table 40), despite the fact that *T. gondii* partially inhibits NO production of activated murine macrophage [194]. $1,25(OH)_2D_3$ also induced NO synthesis and suppressed growth of *Mycobacterium tuberculosis,* another intracellular microorganism, in a human macrophage-link cell line [640]. These findings are consistent with the beneficial effects of pretreatment with $1,25(OH)_2D_3$ (0.5 μg/kg for 2 days) on various tissue pathological changes caused by peritoneal administration of oocysts in mice and histologically examined after seven days post inoculation [638]. It appeared that $1,25\ (OH)_2D_3$ reduced tissue damage and parasite load in situ, and in particular the difference of the number of parasites per 1 mg of standardized tissue DNA was significant in the spleen (Table 41). Thus, vitamin D immunomodulatory and antitoxoplasmatic activities were probably at least in part responsible for inhibition of diabetes development in both patients with T1DM and T2DM.

## Beneficial Role of the Increased Indoleamine 2,3-Dioxygenase (IDO) Activity for both Diabetes and *T. gondii* Infection Prevention/Treatment

### Diabetes

IDO may exert an immunoregulatory function and has the capacity to affect the course of various infections, autoimmunity, cancer and transplantations [6]. Increasing evidence support suggestions that IDO may delay the onset and progression of autoimmune diseases, e.g. IDO expressing NK cells contributed and promoted acceptance of rat liver allograft [641]. Jalili et al. [642] demonstrated the long survival and viability of syngeneic islets exposed to IDO-expressing

| Experiment No. | Controls | Solvent[a] | Vit $D_3$ (1000 IU) | IFN-γ (100 IU) | Vit $D_3$ (1000 IU) plus IFN-γ (100 IU) |
|---|---|---|---|---|---|
| 1 | 3.01 ± 0.14 | 2.93 ± 0.16 | 2.49 ± 0.19[b] | 2.6 ± 0.2[b] | 2.37 ± 0.19[b] |
| 2 | 3.15 ± 0.12 | 3.03 ± 0.16 | 2.74 ± 0.16 | 2.5 ± 0.15[b] | 2.58 ± 0.13[b] |
| 3 | 3.05 ± 0.15 | 3.04 ± 0.14 | 2.82 ± 0.17 | 2.57 ± 0.16[b] | 2.69 ± 0.2[b] |
| 4 | 3.16 ± 0.14 | 3.0 ± 0.14 | 2.39 ± 0.19[b] | 2.59 ± 0.2[b] | 2.03 ± 0.19[b] |

Numbers of tachyzoites are given as a mean ± SD; [a]Ethanol 95; [b]Statistically significant differences compared with controls (P  0.05)

**Table 39:** Effect of vitamin $D_3$ and IFN-γ on proliferation of *T. gondii* (RH strain) tachyzoites per infected peritoneal macrophage of BALB/c mice after incubation for 96 hrs in RPMI1640 cells culture (according to Ghaffarifar et al. [639]; with own modification).

| Experiment No. | Controls | Solvent[a] | Vit $D_3$ (1000 IU) | IFN-γ (100 IU) | Vit $D_3$ (1000 IU) plus IFN-γ (100 IU) |
|---|---|---|---|---|---|
| 1 | 109 ± 8.02 | 108.2 ± 12.45 | 165 ± 11.30[b] | 146 ± 7.22[b] | 187.8 ± 9.82[b] |
| 2 | 108 ± 9.46 | 108.9 ± 6.93 | 121.2 ± 6.68 | 139.5 ± 5.76[b] | 136.2 ± 10.21[b] |
| 3 | 109.6 ± 7.35 | 108.2 ± 4.96 | 139 ± 7.01[b] | 146 ± 4.93[b] | 146.9 ± 9.62[b] |
| 4 | 109 ± 7.03 | 108.6 ± 4.26 | 166 ± 7.01[b] | 146.2 ± 5.60[b] | 191.5 ± 9.62[b] |

Values are given as mean ± SD. [a]Ethanol 95. [b]Statistically significant results compared with controls (P ≤ 0.05). NO production was estimated as a nitrite release from infected macrophages (μM/ml)

**Table 40:** Effect of vitamin $D_3$ and IFN-γ on NO production by peritoneal macrophages of BAL: B/c mice infected with *T. gondii* (RH strain) after incubation for 24 hrs in RPMI1640 cells culture (according to Ghaffarifar et al. [639]; with own modification).

| Tissue | Pathology | No treatment | Treatment with Vit $D_3$ |
|---|---|---|---|
| Lung | Alveolar macrophages | 1 | 0 |
| | Inflammatory foci | 2 | 1 |
| Liver | Inflammatory foci | 3 | 2 |
| | Hemorrhage | 2 | 0 |
| | Mitosis | 1 | 0 |
| Small intestine | Inflammatory infiltrates | 1 | 0 |
| | Necrotic mucosal cells | 2 | 1 |
| Brain | Presence of the parasite | 2 | 0 |
| Spleen | Granulocytes | 2 | 1 |

Histopathologic examination of the tissues was performed 7 days post inoculation. Numbers are based on severity of the lesions (0, no lesion, 1, mild, 2, slight, 3, moderate changes) and the total was divided the number of animals in the group. Also, *in vitro* studies with incubated intestinal epithelial cells showed a significant dose-dependent inhibition of intracellular *T. gondii* tachyzoites (RH strain, type I) proliferation at $10^{-7}$ mol/l of $1,25(OH)_2D_3$ concentration

**Table 41:** Effect of pretreatment with $1,25(OH)_2D_3$ (0.5 μg/kg/2 days) on tissue pathology caused by *T. gondii* avirulent ME49 strain infection with 20 cysts administered intraperitoneally in BALB/c mice (according to Rajapakse et al. [638]; with own modification).

fibroblasts within the composite grafts in a diabetic animal model. It was also found that transient up-regulation of IDO in dendritic cells by human chorionic gonadotropin down-regulated autoimmune diabetes [643]. Nb. it should be noted that IDO production by human dendritic cells results in the inhibition of T cell proliferation [644]. In addition, human chorionic gonadotrophin administration markedly inhibited T1DM onset in NOD female mice in an IDO-dependent fashion. Also, it was found that a defect in tryptophan catabolism impaired tolerance in NOD mice [643], and previously it was reported that IFN-γ blocks the growth of *T. gondii* in human fibroblasts by inducing the host cells to degrade tryptophan [161]. Moreover, functional IDO was induced when human islet were treated with IFN-γ [643], and otherwise it is known that IFN-γ is the key cytokine responsible for development of immune defense against *T. gondii* in all infected tissues and cells, including the central nervous system [161,231]. IDO is induced in the mouse brain in response to peripheral administration of LPS and superantigen [645], although LPS induction of IDO is mediated dominantly by an IFN-γ-independent mechanism [646]. These findings are important for diabetic patients with *T. gondii* infection because their metabolic-cytokine responses to a second immunological challenge might be excessive [647]. Recently, Fallarino et al. [648] provided promising evidence for treatment of T1DM using IDO expressing encapsulated Sertoli cells, and it appeared that IDO mediated TLR9-driven protection from experimental autoimmune diabetes induced in C57BL/6 mice by streptozotocin. In wild type animals, the disease was accompanied by up-regulation of IDO in pancreatic lymph nodes and would be greatly exacerbated by *in vivo* administration of an IDO inhibitor [648,649].

It should be emphasized that the increased IDO activity causes acceleration of tryptophan metabolism that results in enhanced generation of melatonin (Figure 1), a neuroimmunomodulator produced also by the pineal gland, retina, gut and immunocompetent cells including both bone marrow cells [650] and lymphocytes [651]. Melatonin is a free radical and peroxynitrite scavenger [652-654], and exerts anti-inflammatory effects, including inhibition of NF-κB activation [653,655,656], prevention of iNOS expression and direct inhibition of catalytic activity of NOS [657], decrease malondialdehyde production and increase glutathione peroxidase activity [658-660]. Also two melatonin metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine and N1-acetyl-5-methoxykynuramine, exerted potent anti-inflammatory and antioxidant effects [661,662]. The protective biological effects of melatonin on oxidative stress and inflammatory processes in both T1DM and T2DM, are important for reducing pancreatic β-cell damage caused by persistent autoimmune state and excessive production of proinflammatory cytokines during insulitis, eventually associated with chronic *T. gondii* infection. Interestingly, maternally administered melatonin differentially

regulated LPS-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver and brain [663].

On the other hand, melatonin was found to decrease insulin secretion specifically both *in vitro/in vivo* [664-666] and type 2 diabetic rats, as well as patients, exhibited decreased melatonin levels, whereas the levels in type 1 rats were increased [666]. Peschke et al. [666] suggested that catecholamines, which decrease insulin levels and stimulate melatonin synthesis, control insulin-melatonin interactions because the amines were increased in T1DM but were diminished in T2DM. This difference may be at least in part explained by the increased protective/defensive body requirements for this hormone in T2DM that resulted in its shortage in the host, as compared with T1DM, time of exposition to triggering factor(s) and underlying molecular and metabolic disturbances in these two clinical entities.

Finally, calcium ion is necessary for insulin exocytosis and -cell glycolysis, both processes important in signaling circulating glucose concentration [667]. Calcium also plays an essential role in *T. gondii* motility, enhanced invasion of the parasite to host cells and its increased intracellular replication [668,669]. Therefore, the competition for calcium may contribute to its shortage in islet β-cells and participate in the development of hypoinsulinemia.

### *T. gondii* infection

Increased levels of many tryptophan metabolites have been reported in several neurodegenerative disorders and were postulated to be secondary to induction of IDO and other enzymes of the L-tryptophan-kynurenine pathway. Inhibition of the increased IDO activity significantly exacerbated diseases scores and neuronal cell death in various central nervous system disorders [670]. Moreover, L-tryptophan degradation by IDO might have an important role in IFN-γ-induced antimicrobial effects [671]. Local accumulation of kynurenine metabolites, in particular, quinolinic acid, following IDO induction may represent a potentially detrimental event because quinolinic acid is a potent excitotoxin, and its overproduction has been linked to neuronal damage occurring in brain inflammation [672], initiation of lipid peroxidation [673], and development of disturbances in gluconeogenesis in the liver [674]. Spekker et al [675] proposed that IDO is responsible for the suppression of *Neospora caninum* growth, and other studies on *T. gondii* suggested that its growth could be contained when certain immune cells including dendritic cells were actively expressing IDO [163]. Moreover, L-tryptophan-L-kynurenine pathway metabolism accelerated by *T. gondii* infection was found to be abolished in IFN-γ-gene-deficient mice, and an antitoxoplasmatic mechanism of cross-regulation between iNOS and IDO that may vary among tissues, was demonstrated [671] (Figure 1). Thus, IDO and iNOS are involved in the immunomodulatory roles of IFN-γ, and evidence suggests that these functionally cross-regulated pathways [671] may play an important role in prediction and treatment of autoimmune diseases, particularly T1DM [6]. Dominant control of the regulatory T cells functional status and blocking their conversion into $T_H17$-like T cells in response to inflammatory stimuli may markedly contribute to these bioactions [191].

## Prevention of Hypoglycemia-Induced Neuronal Death by Minocycline may be Partly Associated with its Antimicrobial Activity against *T. gondii* Infection

### Hypoglycemia and minocycline

Recently, Won et al [208] demonstrated that minocycline treatment markedly reduced neuronal death induced by hypoglycemia and cognitive impairment associated with this clinical state was also significantly prevented. Hypoglycemia was reported even in the patients with T1DM and T2DM who were strictly monitoring their blood glucose levels [208,676,677], and may be associated with serious complications, such as deterioration of mental efficiency, focal neurological deficits, seizure, and neuronal death [678,679]. Interestingly, it was suggested that hypoglycemia-induced neuronal death is not only a result of markedly impaired glucose supply to the brain [680,681], but also other contributing factors are involved, including sustained stimulation of glutamate receptors, NADPH oxidase activation with enhanced ROS generation, and extracellular zinc release [682-684], which plays an important role in mediating positively gene expression and production of cytokines IL-2 and IFN-γ in the Th1 cell line and negatively TNF-α, IL-1, and IL-8 in the monocyte-macrophage cell line [685]. In experimentally induced toxoplasmosis, zinc added to diet stimulated cellular immunity, increased CD8 and total number of lymphocytes [686].

### *T. gondii* infection

Minocycline, a tetracycline broad-spectrum antibiotic, was developed as an antimicrobial drug for treatment of various infectious diseases, including *T. gondii* infection [687,688]. This antibiotic showed bacteriostatic effects limiting the growth of bacteria, inhibited production of proinflammatory cytokines, MMP-9 and activation of peripheral/central immunocompetent cells, including T cells, macrophages, and microglia [689]. Mice chronically infected with a low virulent strain Me49 of the parasite showed a significant reduction in the number of brain cysts after three weeks of treatment with 50 mg/kg per day of minocycline. Therapy of the infected animals with the chemotherapeutic (100 mg/kg/day for 12 days) increased their survival and cure rates [687]. Moreover, in acute and chronic neurological disease animal models, including Parkinson's [690], Huntington's [691] and Alzheimer's disease [692], as well as in human clinical trials minocycline had also neuroprotective, antiinflammatory and antiapoptotic properties [693-696]. These effects were thought to arise through the inhibition of microglial activation, iNOS, COX-2 expression and modulation of cytokine expression and release [208]. Moreover, minocycline was found to exert an inhibitory effect on TNF- and IFN-γ production by stimulated T cells [697]. In contrast to the effect on T cells, addition of minocycline to LPS-stimulated monocytes led to a dose-dependent increase in TNF-α and IL-6 production. These results indicated that minocycline exerted differential effects on the regulation of cytokine production by T cells and monocytes. Given the pleiotropic effects of minocycline, it was suggested that the immunostimulatory effect on monocytes might counteract its beneficial properties in the treatment of several forms of chronic inflammation [697]. Several authors found a possible relationship between *T. gondii* infection and etiology of Parkinson's disease [698], schizophrenia [699,700], Alzheimer's disease [701], and obsessive-compulsive disorder [702], and minocycline was found to exert beneficial effects in the treatment of schizophrenia [703,704], as well as in patients with toxoplasmic encephalitis [705]. Thus, it seems that preventive effect of minocycline in the hypoglycemia-induced neuronal death was at least in part associated with its antiparasitic activity.

## Prevention of Hypoglycemia-Induced Neuronal Death by Hypothermia may be Caused by Cold Stress-Related Modulation of Immune Responses Associated with Enhanced Activity Directed against *T. gondii* Infection

### Hypoglycemia and hypothermia

Shin et al. [706] demonstrated that hypothermia prevented neuronal

death induced by hypoglycemia. It was suggested that hypoglycemia-induced microglial activation resulted from the brain infiltration with peripheral and local inflammatory cells, enhanced release of several proinflammatory/neurotoxic substances (various cytokines, chemokines, NO, ROS) associated with morphological changes of microglial cells [707,708], and finally brain tissue inflammation [709,710]. The beneficial effect of cold stress on antibody production was found to be mediated via several nervous system-derived factors, such as glucocorticoids, catecholamines, and/or neuropeptides [711]. Shanks & Kusnecov [712] demonstrated stress-induced enhancement of B cell antibody responses to KLH (a keyhole limpet hemocyanin, a potent antigen in reaginic sensitization of mice and rats) in BALB/cByJ mice and B lymphocytes expressed the β2-adrenergic receptor, and binding of norepinephrine to this receptor was necessary to maintain optimal $T_H2$ cell-dependent antibody generation *in vivo* [713]. Moreover, norepinephrine appeared to be necessary for optimal IgG and IgM responses [713]. Experimental studies revealed adrenergic modulation of insulin sensitivity, i.e. drugs with β-adrenomimetic activity, such as epinephrine, novodrin, partusisten, α-adrenomimetic - phenylephrine, and one-hour immobilization stress enhanced insulin sensitivity in animals [714,715]. Interestingly, Keijzers et al. [716] suggested that caffeine might decrease insulin sensitivity in healthy humans possibly as a result of elevated plasma epinephrine levels. It seems, however, that the significant increases of epinephrine and norepinephrine ($P < 0.0005$ and $P < 0.02$, respectively) [716] reflected rather a defense reaction of the host, because Graham et al. [717] demonstrated that caffeine ingestion elevated insulin response in humans during an oral glucose tolerance test. The above-presented adrenergic modulation of insulin sensitivity in experimental animals is consistent with this reasoning.

In mice, chronic cold stress induced a regulatory phenotype in macrophages, characterized by diminished phagocytic ability, decreased TNF-α and IL-6 and increased IL-10 production [718]. Resting macrophages stimulated spleen cells to produce regulatory cytokines, and an immunosuppressive state that impaired cold stressed mice to control Trypanosoma cruzi proliferation [718]. These regulatory effects correlated with an increase in macrophage expression of 11β-hydroxysteroid dehydrogenase, an enzyme that converts inactive glucocorticosteroid into its active form [718]. Moreover, in experimental animals, exposure to cold significantly decreased insulin secretion induced by arginine, butyrate and tolbutamide, and the release of insulin by pancreatic β-cells appeared to be inhibited through stimulation of α-adrenergic receptors [719,720].

### Hypothermia and *T. gondii* infection

Several authors showed that cold stress profoundly modulated immune response and the outcome of infections because many mediators released during physical stress, such as glucocorticoids and catecholamines, influenced the growth and multiplication of pathogens, including *T. gondii* [711,721-724]. Cold stress can suppress [725,726] or enhance [727,728] immunity by inducing and/or enhancing the synthesis of specific proteins with relevant roles in transcription, translation, and recombination [729,730]. Aviles & Monroy [711] showed the increased serum specific anti-*T. gondii* IgG (but not IgM) levels in infected and in infected and stressed mice that underwent cold stress (the animals were kept in cold water, 1 ± 0.5°C for 5 min each day for 8 days) in the chronic phase of infection with the parasite. It appeared that cold stress modulated not only the physiologic processes of the host but also affected the pattern of antibody production against *T. gondii* antigens during longer parasite persistence in the circulation [711]. In mice with chronic phase of infection, cold water stress caused strong antibody response manifesting as the 5-kDa antigen on the surface of tachyzoites (not present in bradyzoite preparations), with significant diagnostic potential [731]. A similar protein (4-5kDa) was identified in human serum samples that reacted with IgM upon primary infection with the parasite [732]. Otherwise it is known that B cells (responsible for the production of antibodies) play an important role in resistance to persistent *T. gondii* infection, especially in the brain and lung [733].

Catecholamines act through adrenergic receptors and suppress the activation of $T_H1$ responses and stimulate $T_H2$ immune responses in antigen presenting cells and $T_H1$ cells [734-738]. Recent data however suggest that catecholamines play an important role in the induction of stress-induced (tailshock stress) proinflammatory cytokines and that β-adrenoceptors are critical for tissue (peripheral) IL-1β induction, while both α- and β-adrenoceptors contribute to the induction of plasma (systemic) cytokines [739]. Catecholamines also regulate host innate immune responses under stress situations. *In vitro* studies have shown that the treatment of human umbilical vein endothelial cells with α1-adrenergic receptor agonists inhibited multiplication of *T. gondii* [725], and norepinephrine influenced the growth and the production of virulence-associated factors in gram-negative bacteria [740,741]. Recently, it was found that cold water stress decreased parasite burden, expression of chemokines and their receptors in intestinal epithelial cells *in vitro* and *in vivo* [722], and down-regulated expression of TLR-2, -4, -9, and -11 in these cells [742]. Moreover, increased survival was found in mice infected orally with *T. gondii* and subjected to cold water stress as compared with controls [743]. In contrast, such stress had deleterious outcomes when mice were infected intraperitoneally [721]. These findings may support our suggestion that cold stress-induced host innate immune modulations during latent chronic *T. gondii* infection also may be partly responsible for the prevention of hypoglycemia-induced neuronal death by hypothermia.

## Impaired Vascular Endothelial Function and Abnormal Metabolic Fate of NO in the Patients with Diabetes Mellitus may be Due to the Preferential *T. Gondii* Infection of Endothelial Cells

Endothelial dysfunction with reduced bioavailability of NO characteristic for the patients with diabetes mellitus play an important role in the development of diabetic vasculopathy [744,745], but has been linked also to atherogenesis and non-diabetic glomerulosclerosis [746,747]. It is interesting that the effect of intensive glycemic control on levels of markers of inflammation in patients with T1DM in the diabetes control and complications trial appeared to be not good enough [748]. Thus, these discrepancies might be at least partly explained by the preferential *T. gondii* infection of the vascular endothelial cells.

### Diabetes mellitus

Hamed et al. [749] reported that the endothelial progenitor cells (EPCs) from diabetic patients generated more $O_2^-$, had higher NAD(P)H oxidase and superoxide dismutase activities, but lower NO bioavailability, as compared with healthy individuals. The function of EPCs, which are key cells in vascular repair, is impaired in diabetes mellitus, and NO and ROS can regulate their function. Plasma glucose and $HbA_{1c}$ levels in the diabetic patients were correlated negatively with the NO production from their EPCs [749]. NO is a biologically active unstable radical that is synthesized in vascular endothelial cells by eNOS,

and its bioavailability depends on the balance between its production and inactivation rates [750]. Exposure of EPCs to high glucose concentrations increases NAD(P)H oxidase activity which results in increased $O_2^-$ generation and reduced NO bioavailability because $O_2^-$ inactivates NO and uncouples eNOS [751]. It was demonstrated that NO bioavailability and the *in vivo* reendothelialization capacity of ECs from diabetic patients can be restored by inactivating NAD(P)H oxidase [752].

Milsom et al. [753] found preferential binding of endogenous and exogenous NO to glycosylated deoxy-hemoglobin and consequently an abnormal metabolic fate of NO in patients with T1DM. They showed that NO-hemoglobin binding was increased at a $HbA_{1c}$ concentrations greater than 8.5% compared with 5.9% ($P < 0.01$). In blood from diabetic patients, added NO was metabolized mainly to nitrosyl hemoglobin and plasma nitrosothiols, with a 2-fold increase in nitrosyl hemoglobin observed across all NO levels ($P < 0.05$), and these preferential increases correlated positively with $HbA_{1c}$ concentrations [753].

Cellek et al. [754] demonstrated NO-dependent morphological degeneration and functional nitrergic nerves impairment in diabetes mellitus, and reduced activity and protein amount of neuronal NOS in penile tissue of diabetic rats. Similar degenerative process of the peripheral nerves was reported in diabetic men [755]. Administration of L-NAME ($N^G$-nitro-L-arginine methyl ester), the inhibitor of NO synthase, appeared to be protective in this condition [754]. The atrophy of the nitrergic neurons in the enteric nervous system of the gastrointestinal tract has been documented also during chronic *T. gondii* infection in experimental animals and men, streptozotocin-induced diabetes, and in diabetic patients [434], because the parasite shows tropism to nerve cells [756]. These morphologic and functional abnormalities probably reflect a defense activity of the innate immune system of the host in response to the presence of the parasite, resulting in an increased level of NO produced by the iNOS [757,758]. NO is generated together with several proinflammatory and anti-inflammatory cytokines and chemokines during the invasion by *T. gondii,* which induces severe inflammatory process at the site of infection. However, excessive amounts of NO are cytotoxic not only for the parasite but also for the host cells [757], because this cytotoxic molecule inhibited the mitochondrial and nuclear enzymes [759]. On the other hand, however, inhibition of iNOS exacerbated chronic cerebral toxoplasmosis in *T. gondii*-susceptible C57BL/6 mice, although did not reactivate the latent disease in *T. gondii*-resistant BALB/c mice [760].

Glucose enters into the glycolytic pathway by phosphorylation to glucose-6-phosphate accomplished by hexokinase and glucokinase, with ATP required as phosphate donor. Glucose-6-phosphate is an important compound, being at the junction of glycolysis, gluconeogenesis, the hexose monophosphate shunt, glycogenesis, and glycogenolysis [761]. As in many reactions involving phosphorylation, magnesium must be present [761], and patients with T2DM frequently have hypomagnesemia, hypertriacylglycerolemia in association with enhanced $HbA_{1c}$ levels, retinopathy and neuropathy [762,763]. Glycolysis proceeds by the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, and glyceraldehyde-3-phosphate dehydrogenase is the enzyme responsible for the oxidation [761]. In many biological systems, nitrosation reactions transferring $NO^+$ from NO donor to a protein S group affect protein function and Mohr et al [764] demonstrated that NO-induced S-glutathionylation led to inactivation of glyceraldehyde-3-phosphate dehydrogenase, thus finally affecting metabolism of carbohydrates and lipid acids. In short, gluconeogenesis is the process whereby glucose is formed from noncarbohydrate metabolic substrates such as pyruvate, lactate, or amino acids, mainly alanine. This metabolic pathway occurs predominantly in the liver and kidney and is essential for the production of glucose during prolonged fasting when glucose stores have been depleted (Figures 4 and 5) [765,766].

## *T. gondii* infection

In congenital toxoplasmosis, infection of endothelial cells lining the umbilical cord and the placental blood vessels by the parasite is potentially the major transmission route to the fetus [767]. *T. gondii* invades and proliferates in HUVEC where it resides in parasitophorous vacuoles (PV), and at the time of PV formation, the cell surface anionic sites and fucose residues (human endothelial cells contain exposed fucose residues) are excluded, while HLA class I molecules are present only on a minority of the parasite-containing vacuoles. When the parasite invades the host cells, a PV sheltering are formed because the PV membrane contains proteins (ROP2) anchoring the mitochondria of the host cells to the external surface [768]. *T. gondii* cyst wall also has anionic sites and this negative charge is mainly produced by phospholipids [769]. The protozoan uses the mitochondria of the host cell to escape from the action of lysosome enzymes. Once within the PV, the parasite multiplies even in phagocytic cells until complete destruction of the host cell [768]. The formation of PV prevents it from

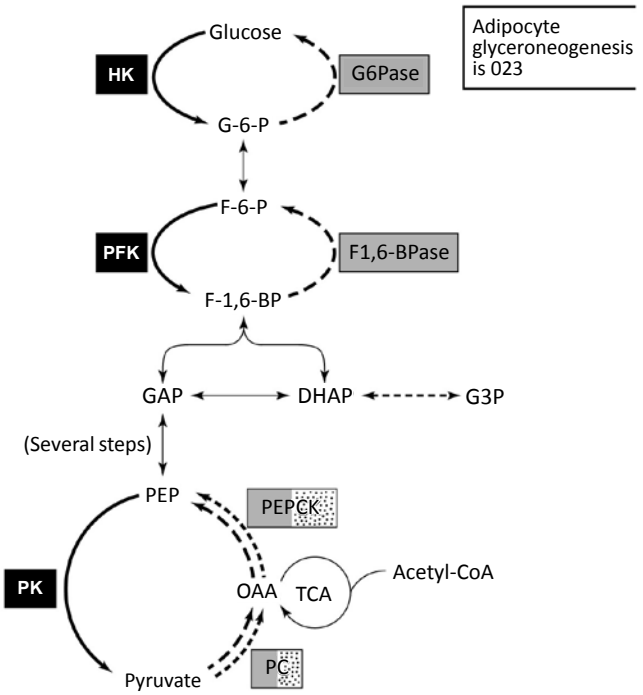


**Figure 4:** Glyceroneogenesis generates G3P (glycerol-3-phosphate) instead of glucose. Gluconeogenesis (broken arrows) and glyceroneogenesis (more frequently broken arrows) share several steps in common with glycolysis (solid arrows) because most of the reactions are readily reversible (two direction arrows). Three kinase reactions (HK, hexokinase; PFK, phosphofructokinase; and PK, pyruvate kinase) in glycolysis are not reversible and require separate enzymes for gluconeogenesis (PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; F1,6-Bpase, fructose-1,6-bisphosphatase; and G6Pase, glucose-6-phosphatase) and glyceroneogenesis (PC, PEPCK). PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; OAA, oxaloacetate; TCA, tricarboxylic acid cycle (Krebs cycle) [765].

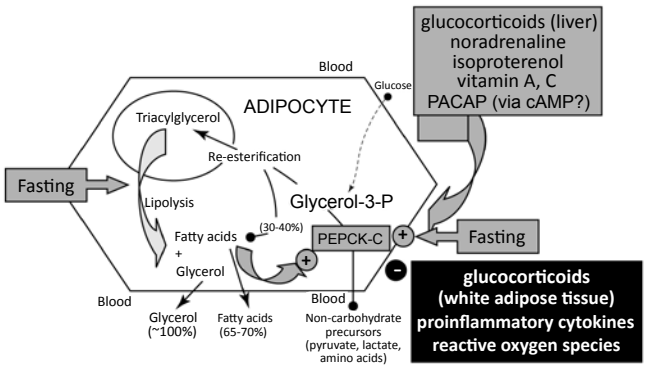


**Figure 5:** Simplified process of glyceroneogenesis in adipocytes and its regulation by PEPCK-C. Lipolysis releases glycerol and fatty acids from triglyceride stores in fat cells. Fasting increases PEPCK-C production and fatty acid release is restrained due to increased glyceroneogenesis and re-esterification in triacylglycerols, which requires glycerol-3-phosphate formation from non-carbohydrate precursors [766].

merging with the lysosome – a strategy of the protozoan developed to protect its growth within the host cells. During the intracellular life cycle of *T. gondii* there is no fusion of host cell lysosome with PV, however lysosome-phagosome fusion and parasite destruction occur when fixed or antibody-coated live parasites are internalized by macrophages [770].

Cortez et al. [771] found that HUVEC activated with INF-γ inhibited *T. gondii* infection and multiplication by 67.5% and 91.0%, respectively. After 4 hrs, 10.2% of INF-γ-activated HUVEC exhibited phagosome-lysosome fusion, and NAD(P)H oxidase present at the plasma membrane of activated HUVEC was internalized together with the parasite in 38% of the cells. This may suggest that NAD(P)H oxidase may participate in a mechanism by which INF-γ-activated HUVEC inhibit *T. gondii* multiplication [771]. This suggestion and the fact that diabetic patients had increased NAD(P)H oxidase activity [749] are consistent with the finding that two forms of lactate dehydrogenase LDH1 and LDH2 from *T. gondii* were inhibited competitively by gossypol and gossylic iminolactone, and this correlated with specific inhibition of tachyzoites growth in human foreskin fibroblast cultures [772]. Since the bradyzoite LDH2 was more sensitive *in vitro* to these compounds than the tachyzoite LDH1, it is likely that the growth of bradyzoites in cysts would be inhibited by gossypol and gossylic iminolactone as well [772]. Gossypol and derivatives are aldose reductase (polyol dehydrogenase) inhibitors [773], and gossypol, a natural product from cotton seed, is a non-selective competitive inhibitor of NADH binding to LDH with $K_i$ value 1.4 μM for LDH1 [774]. There was an association between the erythrocyte aldose reductase activity and the complications of diabetes mellitus [775,776]. It was reported that the patients with T1DM who had erythrocyte aldose reductase activity greater the mean ± 2 SD of the found in non-diabetic controls were four times more likely to have diabetic complication than non-diabetic individuals ($P < 0.0005$) [775]. In order to explain this relationship it should be noted that aldose reductase is a member a family of NADPH-dependent oxidoreductases, present in several human tissues that reduces glucose to sorbitol. In animal models there is evidence that the production of sorbitol is associated with the development of diabetic complications, including neuropathy, nephropathy, cataracts and retinopathy. In hyperglycemia, hexokinase is saturated and the fraction metabolized by aldose reductase increases which involves the sorbitol or polyol metabolic pathway resulting in accumulations of sorbitol and fructose [776]. Chandra et al. [777] showed that increasing NO availability inhibits aldose reductase, thus preventing sorbitol accumulation, whereas inhibiting NO synthesis promotes the activation of the rate-limiting enzyme. Inhibitors of aldose reductase have been developed to reduce the incidence or slow the progression of the major complications of diabetes mellitus. Flavonoids were found to possess aldose reductase inhibition and antioxidant activities *in vitro* as well as inhibition in the accumulation of sorbitol in the tissues of streptozotocin-induced diabetic rats [778].

Canedo-Solares et al. [779] demonstrated that invasion of both human microvascular endothelial cells (HMEC-1) and umbilical vein endothelial cells (HUVECs) by *T. gondii* RH and ME49 strains increased along with time. HMEC-1 cells were more susceptible to infection with the parasite than HUVECs, and ME49 parasites were faster than RH ones and this may be related to their ability to survive out of the cell and to the fact that *T. gondii* is more invasive during G1-S phase. In addition, both HMEC-1 and HUVECs showed higher number of parasitic vacuoles per cell when infected by ME49 tachyzoites than by RH protozoan, i.e. ~ 30 vs. 20 at 4 hrs, respectively [779]. These differences may also partly be explained by various division rates of intracellular *T. gondii* tachyzoites documented *in vitro* in many primary human cells, including endothelial cells (Table 14). Interestingly, compared to RH tachyzoites, ME49 tachyzoites induced a stronger upregulation of ICAM-1 in the brain vascular endothelial cells and an earlier and stronger IL-6 and MCP-1 secretion by these cells. *T. gondii* type I and II strains induced similar migration patterns of antigen-presenting cells but the infected antigen-presenting cells showed a more intensive migration compared to lymphocytes (4.63% vs. 0.6% of all cells) across the blood-brain barrier [780]. It was suggested that the parasite modulated gene expression of the brain vascular endothelial cells to promote its own migration through the blood-brain barrier as a Trojan horse [780]. Knight et al. [781] reported that the exposure of the rat retinal vascular endothelial cells to *T. gondii* infection after 2 hrs resulted in change of expression of approximately 6% of genes, including those involved in cell structure, protein and vesicle trafficking, cell-cycle regulation, transcriptional and translational machinery, and apoptosis. Infection of BUVECs with tachyzoites also enhanced transcription of several genes and induced adhesion of polymorphonuclear neutrophil cells (PMN) to both infected and noninfected BUVECs within one cell layer, suggesting parasite-induced paracrine activation of these cells and an effective role of PMN in development of the innate immunity to the parasites [782,783].

*T. gondii* is the common cause of posterior uveitis and a recurrent necrotizing retinochoroiditis worldwide, which may lead to a permanent loss of visual acuity. Tachyzoites spread throughout the body through the blood stream and lymphatics, but preferentially encyst in the eye and other parts of the CNS. Furtado et al. [784] found that human monocyte-derived dendritic cells infected with *T. gondii* tachyzoites transmigrated in larger numbers across stimulated human retinal endothelium than uninfected dendritic cells ($P \leq 0.0004$). Antibody blockade of ICAM-1, VCAM-1, and activated leukocyte cell adhesion molecule inhibited transmigration, while chemokines CCL21 or CXCL10 increased this process, which might be important for development of a novel therapeutic approach [784]. Zamora et al. [785] reported that *T. gondii* tachyzoites invade human retinal endothelial cells (HREC), which were more susceptible to infection with the parasite than other subpopulations human dermal endothelial cells. The enhanced susceptibility of human retinal vascular endothelium to infection by *T. gondii* may be related to preferential binding of

tachyzoites to the retinal vascular endothelial surface, relative ease of penetration into the cell, rate of replication within the cell and/or cell response to infection, as compared with aortic (55% more), umbilical vein (33%) and dermal (34%) endothelial cells [786]. It appeared that growth of the tachyzoites was approximately 2.8-fold higher in retinal endothelium than in foreskin fibroblasts [786]. Binding of the parasite to host cells is partially mediated by interaction with sulfated glycosaminoglycans (GAGs). Table 42 summarized the blocking effect of several soluble sulfated proteoglycans on attachment of *T. gondii* parasites to human host cells from a variety of lineages [787]. Heparin, heparin sulfate, chondroitin sulfate showed a negative charged sulfate groups on the molecules-mediated dose dependent inhibition in the average number of parasites attached to the substrate and gliding [787]. It was found that low concentrations of GAGs increased invasion of human fibroblasts while mutant CHO cells lacking in cell surface sulfated proteoglycans were less susceptible to infection [788].

Brunton et al. [789] found that pretreatment of rat retinal vascular endothelial cells infected with *T. gondii* RH strain tachyzoites, with IFN-γ, TNF or IL-1β resulted in a significant decrease in the parasite replication, and this inhibition appeared to be independent of NO production while L-tryptophan catabolism may have a role in this IFN-γ-mediated process. When the ovine umbilical vein endothelial cells were pretreated with IFN-γ, a high degree of inhibition of *T. gondii* replication was observed with the effect being dose-dependent, with a maximum IFN-γ activity of 625 U/ml (range 0.15-1250 U/ml) [790]. Studies of Däubener et al [163] confirmed that indoleamine 2,3-dioxygenase induction contributed to the antiparasitic effector mechanism inducible in human brain microvascular endothelial cells (HBMEC) by IFN-γ and TNF-α, because this enzyme was strongly induced in HBMEC, and its activity was enhanced by co-stimulation with TNF-α, while the addition of excess amounts of tryptophan to the HBMEC cultures resulted in a complete abrogation of the antitoxoplasmatic effect. The mechanism of inhibition of *T. gondii* in HUVEC activated by IFN-γ was found to be different from that present in mouse macrophages and human fibroblasts [310-313]. For example, Ji et al [791] demonstrated that exogenous NO triggered egress of *T. gondii* tachyzoites from infected peritoneal macrophages obtained from C57BL/6 mice, which then underwent necrosis. Moreover, addition of the calcium ionophore A23187 resulted in an increased release of merozoites from mature *T. gondii* meronts grown in cultured primary bovine umbilical vein endothelial cells (BUVECs) [792]. The extent and time course of the release was dependent on both, maturity of the meronts and concentration of the calcium ionophore because survival of parasitized host cells and the parasites themselves should be dependent on ion balances, especially on extra- and intracellular calcium concentrations [792].

Dimier & Bout [793] showed that when HUVEC were pretreated with IL-1β and TNF-α concentrations ranging from 1 to 100 U/ml, a dose-dependent inhibition of the intracellular parasite replication was observed. In addition, Benedetto et al. [724] demonstrated that pretreatment of HUVEC with an α-adrenergic resulted in a high degree of intracellular killing of *T. gondii* in these cells. Moreover, α-adrenergics activated HUVEC, and induced a marked dose-dependent toxoplasmastatic activity. Also a significant positive correlation was observed between the toxoplasmastatic activity and release of $NO_2^-$ during the activation phase before infection with the parasite, although this effect was not present during the infection phase.

## Diabetes Mellitus Comorbidities

### Possible association between diabetes, epilepsy and *T. gondii* infection

The prevalence of active epilepsy in European countries varies between 3.3 and 7.1 per 1000 for the age range 0 to 90 yrs, with a peak prevalence of 9 to 11.6 per 1000 in adults > 50 yrs of age, and 2.1 to 4.1 per 1000 in children and adolescence [794,795]. Ramakrishnan & Appleton [796] found that 6 of 285 children aged less than 16 yrs with T1DM had epilepsy giving a prevalence of 21/1000, which is approximately six times greater than the prevalence of epilepsy in general population of children in UK. Caletta et al. [797] found that among their cohort of 10 children suffering from both T1D and epilepsy, 5 was had generalized epilepsy and 5 was diagnosed with focal disease. Seizures caused by hypoglycemia in the patients with T1DM are quite frequent events with 18.2 to 62 per 100 patient years depending on age, type of treatment, and residual insulin secretion [798,799].

Schober et al. [794] found that children and adolescents with T1DM had an increased prevalence of epileptic seizures. There was also an association between epilepsy and diabetic ketoacidosis in these patients, and the risk of ketoacidosis was almost double in the patients with epilepsy compared with patients with diabetes alone. It must be noted that the frequency of severe hypoglycemia was lower in the patients treated with antiepileptics [800]. This may suggest that *T. gondii* infection was an important triggering agent because for example valproic acid, an epileptic drug, exerted strong antitoxoplasmatic effect (Table 43). It must be noted that uptake of the drug by bovine brain microvessel endothelial cells has been well documented [801A], and these cells represent frequent targets of the parasite in human host (Table 42). In addition, Glodek-Brzozowska et al. [802] analysed 784 children with T1DM and found that 8 of them (aged 7.5 to 18 yrs) had epilepsy, as a concomitant disease, with local or generalized abnormal changes in EEG. The patients had seizure types of different morphology, including petit mal, partial, myoclonic, or tonic-clonic

| Cell type | Cell line | $IC_{50}$ (μg/ml)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Heparin | CSC | CSA | Dextran sulfate[d] | Dextran | Fucoidin[e] |
| Endothelial | HUV-EC-C[b] | 1.8 ± 0.2 | 1.6 ± 0.4 | 2.9 ± 1.5 | 1.0 ± 0.4 | 9.7 ± 3.4 | 4.1 ± 1.8 |
| Epithelial | HEp-2[b] | 5.5 ± 1.6 | 4.5 ± 0.4 | 6.3 ± 2.3 | 5.7 ± 2.3 | 14.1 ± 2.1 | 19.5 ± 0.2 |
| Fibroblast | HFF | 4.8 ± 2.0 | 4.1 ± 1.4 | 7.3 ± 1.7 | 2.2 ± 0.4 | > 20 | > 20 |
| Glial or astrocyte | U373[b] | 3.0 ± 1.9 | 3.0 ± 0.8 | 1.1 ± 0.4 | 2.1 ± 0.4 | 7.4 ± 3.8 | 16.0 ± 3.7 |
| Macrophage | U937[b] | 2.0 ± 0.3 | 1.8 ± 0.8 | 2.6 ± 1.5 | 6.7 ± 2.6 | 7.0 ± 2.7 | 13.2 ± 4.9 |
| Melanocyte | G361[b] | 4.0 ± 0.3 | 2.0 ± 0.1 | 3.0 ± 1.9 | 4.6 ± 0.4 | 8.8 ± 4.7 | 0.3 ± 0.1 |
| Average[c] | | 3.5 ± 1.4 | 2.8 ± 1.1 | 3.9 ± 2.2 | 3.7 ± 2.1 | 11.2 ± 4.6 | 11.7 ± 6.2 |

[a]Mean ± SE, n = 2 or 3; [b]Obtained from the American Type Culture collection; [c]Average values (± SE) for all cell types tested. [d]A sulfated L-fucose oligosaccharide. [e]The synthetic polyanion. $IC_{50:}$ Inhibitory Concentration; CSC: Chondroitin sulfate C; CSA: Chondroitin Sulfate A; Several heparin binding proteins were identified in lysates of *T. gondii* based on their ability to agglutinate red blood cells in a heparin-sensitive manner [460].

**Table 42:** Inhibition by soluble glycosaminoglycans of *T. gondii* attachment to various mammalian cells (acc. Carruthers et al. [459]; with own modification).

seizures, and 7/8 patients received treatment with valproic acid as the main anticonvulsant. Poor control of diabetes was observed in 4 children during acute seizure attacks [803]. Moreover, Schober et al. [794] reported that the increased risk of this abnormality cannot be caused by carbonic anhydrase inhibitors, as previously suggested [804], because treatment with other antiepileptic medications also was associated with metabolic acidosis, which was observed even in patients without anticonvulsive treatment. The patients with epilepsy were younger at onset of diabetes and shorter as compared with those without epilepsy [794].

Palmer [805] showed a highly significant association between the seroprevalence rates for chronic *T. gondii* infection and prevalence rates of cryptogenic epilepsy ($P < 0.001$) (log-odds ratio of 4.8, CI 2.6 to 7.8). Three children aged 5 to 11 years with Landau-Kleffner syndrome (aphasia plus epilepsy; the speech disorders appeared after epileptic seizures started) and EEG abnormalities, also had increased titers of IgG antibodies against *T. gondii* [806]. Furthermore, Stommel et al. [803] suggested that chronic *T. gondii* infection with brain cysts may be a cause of cryptogenic epilepsy because they found a statistically significant 59% elevation of the parasite antibodies among these patients as compared to controls (P=0.013).

Interestingly, it was suggested [807] that the syndrome of *epilepsia partialis continua* represented symptoms of hyperglycemia and was the first symptom leading to the diagnosis of diabetes mellitus. However, the majority of the 22 patients analyzed had evidence of localized structural brain lesions and therefore it seemed that the hyperglycemia was not the main cause of the epileptic abnormalities [807].In a German population-based case-control study including 366 glioma and 381 meningioma cases, and 1494 controls Berg-Beckhoff et al. [808] found the positive association between epilepsy and particularly glioma suggesting that epilepsy is an early symptom of the disease. As the association was observed also for epilepsies occurring more than a decade before the diagnosis of glioma, the authors suggested that this might indicate an etiological role of epilepsy, or a relatively long preclinical state [808]. This finding and reasoning is consistent with the recent suggestion that chronic latent *T. gondii* infection of the central nervous system may be responsible for development of ependymoma and glioma [809].

| Drug | Solvent | $ID_{50}$[a] (µg/ml) | $TD_{50}$[b] (µg/ml) | TI[c] |
|---|---|---|---|---|
| Valproic acid | ethanol | 4.5 | 62.4 | 13.9 |
| Sodium valproate | ethanol | 4.1 | 52 | 12.7 |
| Carbamazepine | ethanol | 72 | 100 | 1.3 |
| Litium carbonate | 1 N HCl | > 100 | > 100 | |
| Haloperidol | ethanol | 5.6 | 103 | 18.4 |
| 9-OH-Risperidone | tartaric acid | 20.1 | 134 | 6.7 |
| Risperidone | tartaric acid | 74 | 129 | 1.7 |
| Fluphenazine HCl | Toxo CGM | 3.5 | 17.9 | 5.1 |
| Clozapine | ethanol | 5.8 | 20 | 3.4 |
| Olanzapine | DMSO | 33.2 | 100 | 3.0 |
| Chlorpromazine HCl | DMSO | 2.6 | 6 | 2.3 |
| Quetiapine fumarate | DMSO | 18.6 | 33 | 1.8 |
| Trimethoprim | DMSO | 5.3 | 63.8 | 12.1 |

[a]Median inhibitory dose, a measure of tachyzoite inhibition; [b]Median toxicity dose,a measure of cytotoxicity; [c]Therapeutic index, a measure of efficacy determined by $TD_{50}/ID_{50}$ ratio. DMSO, dimethylsulfoxide; Toxo CGM, Toxoplasma cell growth medium. Valproic acid at a concentration of 1 µg/ml inhibited 7% of the tachyzoites and trimethoprim at 3.2 µg/ml produced 2% inhibition, but the combination of these two compounds at those concentrations resulted in a potentiating effect inhibiting 55% of the tachyzoites

**Table 43:** Drugs tested for *in vitro* activity against *T. gondii* (according to Jones-Brando et al. [801]; with own modification).

## Possible association between diabetes, migraine/other type of headaches and *T. gondii* infection

The prevalence of migraine and tension-type headache in the patients with diabetes mellitus was found to be higher as compared with controls without diabetes [810-812]. The disease was more common amongst teenagers with migraine compared with those without migraine [813]. Also, within a headache clinic population Tietjen et al. [814] identified diabetes mellitus as a migraine comorbidity constellation. However, in a large population-based cross-sectional study performed in Norway, Aamodt et al. [810] found the inverse relationship between migraine and diabetes. Prevalence odds ratios of migraine was lower amongst persons with diabetes compared with those without diabetes, the OR being 0.4 (95% CI: 0.2-0.9) for T1DM, and 0.7 (CI: 0.5-0.9) for T2DM. It appeared that OR of headache were lower amongst those with duration of diabetes ≥ 13 yrs compared with those who were diagnosed with diabetes during the last 3 years, OR 06 (0.4-0.9) [810].

Split & Szydlowska [812] reported that amongst their cohort of 154 patients with T2DM, 95 (61.7%) had migraine, and 32 (20.8%) tension-type headaches. In 50 individuals, the onset of migraine headaches occurred after diagnosis of diabetes, while in 45 individuals migraine was diagnosed before the onset of diabetes. Interestingly, in this group of patients the onset of diabetes caused a significant increase in the average number of headache days per year. In the control group of 106 persons, migraine was diagnosed in 17 (16%) subjects, while 28 (26.4%) individuals suffered from tension-type headache [812]. Recently, Cavestro et al. [815] showed that both blood glucose and insulin levels were higher in migraineurs than in healthy controls ($P < 0.0001$) suggesting altered metabolism of insulin in this clinical entity. Moreover, Okada et al. [816] demonstrated higher levels of lactic acid and pyruvic acid in the plasma of patients with migraine as compared with controls, which indicated mitochondrial function abnormalities in carbohydrate and fats metabolism, including tricarboxylic acid cycle. In addition, insulin sensitivity was found to be impaired in the patients with migraine as compared with normal individuals ($P < 0.001$) [817,818]. Therefore, its seems that there is a relationship between migraine/other types of headache and T1DM/T2DM, especially that many patients with recurrent headaches/migraine were found to be seropositive for *T. gondii* [819,820] and about two billion of people worldwide are chronically infected with the parasite.

## Possible association between T1DM, autism spectrum disorders (ASD) and *T. gondii* infection

Freeman et al. [821] reported an increased prevalence of ASD in their group of 984 pediatric patients with T1DM in Toronto (Canada) than in general population (0.9% [95% CI: 0.3-1.5 vs. 0.34-0.67]). The median age at diagnosis of ASD was 4.8 yrs (range 3.3-6.8), while that for T1DM was 8.2 years (range 0.8-13.5). In mothers of patients with autism, Comi et al. [822] reported higher incidence of autoimmune diseases compared with controls, and specifically autoantibodies implicated in autoimmune thyroid disorders also have been found with an increased prevalence in the patients with T1DM [823-825]. Although the studies performed by Harjutsalo & Tuomilehto [826] in northern Finland did not support their suggestion about the link between T1DM and ASD, Iafusco et al. [827] in their cohort of patients

with T1DM (aged <14 years) from several Italian centers of pediatric diabetology (0.72% [0.69-0.75] found a pattern similar to that observed by Freeman et al. [821]. They suggested that these differences may be linked to the incidence of diabetes, and emphasized that, for example, Sardinia (Italy) has a very high incidence of T1DM epidemiology (42.4/100 [95% CI: 40.5-44.4]), while the peninsular Italy has on overall incidence similar to other Mediterranean regions (8.4/100 [7.9-8.9]; only 2 of 1373 analyzed patients aged < 14 years from Sardinia were diagnosed with autism [827]. These discrepancies emphasize importance of geoepidemiological differences in the incidence of diabetes and the fact that some environmental factors play an important role in development of both T1DM and ASD in the same person. Recently, a significantly lower occurrence of seropositive titers against the parasite was found in the patients with T1DM and their close family members [20] as compared with healthy controls. Similar results were obtained in 83 autistic children aged 1-18 yrs (mean age 6 yrs) in whom only two patients had positive serum anti-*T. gondii* IgG levels (unpublished results, Magdalena Cubala-Kucharska, personal information, November 2012).These unexpected findings may be explained by the suppression of cytokine IL-2 generation, decreased activation of lymphocyte B cells responsible for immunoglobulin secretion and markedly lower levels of immunoglobulins IgG, IgA, and IgM due to *T. gondii* infection [21]. In addition, persistent and prolific primary autoimmune-induced generation of many antibodies characteristic for the patients with ASD directed against own proteins (the so called a *perpetuum mobile*-like biomachinery [434,828] may be associated with their exhausted secondary innate and acquired immune responses directed against foreign *T. gondii* antigens [23], in which host-endoplasmic reticulum-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *T. gondii*-infected dendritic cells [829,15]. This explanation may be at least in part responsible for the markedly low occurrence of specific antibodies directed against the parasite found in the autistic children, as well as for the positive relationship found between the proportion of children who received the recommended vaccines by age 2 years and the prevalence of autism or speech/language impairment across the U.S. population [830].

Patients with autism have significantly increased NO production [579,831-833]. Higher plasma nitrite and nitrate (NOx) levels were also found in children with autism compared with controls [832]. Higher NOx concentrations demonstrated in red blood cells of autistic patients compared to age- and sex-matched normal controls, along with enzymatic evidence of NO-related oxidative stress [831], were associated with mitochondrial dysfunction [834]. The induction of a high-output inducible enzyme NOS (iNOS) is triggered primarily by IFN-γ, in combination with TNF- and IL-1β, or endotoxin [579,835,836]. It must be noted that autistic children showed enhanced production of the cytokines IFN-γ, TNF-α and IL-1 compared to controls [837,838]. Given the key role of these cytokines in the induction of iNOS that resulted in the elevated NO generation in autism one may suggest that iNOS was also involved in the enhanced NO production in diabetes mellitus due to a profuse secretion of these proinflammatory cytokines in this clinical entity (Table 6) [83-87]. Recently, it was demonstrated *in vitro* that exogenous NO released by different doses of sodium nitroferricyanide (III) dehydrate could trigger egress of *T. gondii* tachyzoites from infected peritoneal macrophages collected from C57BL/6 mice, which then underwent necrosis [839]. This finding is very important because it may represent a novel approach for treatment of neuroinflammation caused by the parasites located in astrocytes and other eukaryotic cells.

## Possible association between T1DM, celiac disease (CD) and *T. gondii* infection

CD occurs in children and adolescents with T1D with the prevalence ranging from 4.4 to 11.1% (mean 8%) as compared with 0.5% in general population [839-841]. The mechanism of association of these two diseases involves HLA genotypes DR3-DQ2 and DR4-DQ8 (T1DM), and DR3-DQ2 (CD) [839]. It is striking that T1DM and celiac disease share 13/52 (25%) risk loci outside the HLA gene complex (http://www.t1dbase.org) [842]. Both diseases T1DM and CD have an abnormal small intestinal immune response with inflammation and a variable grade of enteropathy [841]. Patients with T1DM often have only few and mild symptoms of CD or are asymptomatic (silent), and rarely present with severe manifestation of CD. The mean age at diagnosis of classical CD is usually around 2-3 yrs, while the age for T1DM is 7-8 yrs. In the patients with T1DM, diabetes is usually diagnosed first, CD precedes diabetes onset only in 10-25% of individuals [839,843,844].

Cronin et al. [845] reported that of 177 patients attending a seizure clinic, four individuals had celiac disease (1 in 44). In a control group of 488 pregnant women only 2 samples were positive for celiac disease (1 in 244). In previous hospital records, 16 patients (10 F/6 M) suffered from both celiac disease and epilepsy (mean age at diagnosis of epilepsy was 23 yrs (range from < 1 to 67); mean age at diagnosis of celiac disease was 28.5 yrs (range < 1 to 73 yrs) [845].

Sandberg-Bennich et al. [846] demonstrated that the most evident risk factor for development of celiac disease was associated with neonatal infections (OR =1.52, confidence limits 1.19; 1.95). *T. gondii* infection may therefore play an important role in triggering development of both diabetes and celiac disease, especially that the increased percentage of antigliadin IgG antibodies was found to be associated with T1DM [20] (Table 2). Moreover, Rostami Nejad et al. [847] found that amongst 827 pregnant women, 154 (31%) and 58 (7%) of them had positive total IgG and IgM for *T. gondii* serology (blood sample were taken at mean pregnancy duration of 5.5 months). In addition, 27 women (mean age 27 yrs; mean pregnancy duration 4.8 months) were simultaneously diagnosed with celiac disease, and 16 out of 27 (59%) had infection with the parasite as compared with 257 out of 800 (32%) non-celiac disease pregnant women (OR = 3.07, 95% confidence limits 1.4-6.7) (P = 0.04). The authors suggested that celiac disease during pregnancy increase the risk of *T. gondii* infection [847]. Critical analysis of the literature data suggests however that on the contrary, chronic latent infection with the parasite increases the risk of celiac disease development [809]. This reasoning is strongly supported by the recent report of Severance et al. [848] that in mice receiving a standard wheat-based rodent chow, peroral, intraperitoneal and prenatal *T. gondii* exposure launched a highly significant generation of anti-gluten IgG antibodies in all infected animals compared to uninfected controls ($P \leq 0.00001$). Perorally-infected females showed higher concentrations of anti-gluten IgG than males ($P \leq 0.009$) indicating that the parasite-generated gastrointestinal infection led to a marked anti-gluten response in a sex-dependent manner [848]. These findings may be explained by the facts that: 1) transepithelial migration of *T. gondii* is linked to its active motility and virulence [849], 2) involves an interaction of human ICAM-1 with the parasite adhesin MIC2 resulting in its immunoprecipitation [317], and 3) the parasite targets the paracellular pathway to invade the intestinal epithelium and affects epithelial tight junction-associated proteins [10], thus finally affecting host intestinal wall permeability. Beneficial ameliorative effects of breastfeeding and human colostrum, which contains large quantities of lactoferrin, administered in neonatal autistic rats with celiac disease, may support the above-presented reasoning [850].

## Concluding Remarks

Maternal and/or fetal-derived islet infiltration by the cells infected with *T. gondii* may induce increased production of proinflammatory cytokines, such as TNF-α, IL-1β, and IFN-γ, which can result in development of insulitis. They also modify the antigen presenting cells toward a more immunogenic phenotype, thus triggering the offspring T cells to react against pancreatic self antigens, like it was found during induction of diabetes by viral or bacterial infections. This reasoning is consistent with the reports that elimination of maternally transmitted autoantibodies prevented development of diabetes in nonobese diabetic mice, and that oral administration of autoantigen induced autoimmune diabetes. Moreover, *in vitro* studies showed that insulin and D-glucose had a dose-responsive mitogenic effect on intracellular *T. gondii* replication and development in 3T3-L1 cells. One cannot therefore exclude that the additive/synergistic effect of insulin/glucose on multiplication of the parasite in pancreatic islet beta-cells may be at least in part responsible for triggering autoimmune defense reaction of the host that induce insulitis, and finally cause diabetes. TGF-β, a multifunctional cytokine, also was found to favour growth of the parasite through suppressing IFN-γ toxoplasmastatic activity. The pancreatic islet β cells hypertrophy and fibrosis characteristic for the patients with T2DM may be caused by the increased levels of TGF-β due to *T. gondii* infection because the elevated expression of the cytokine in vitreous, retina and retinal pigment epithelium was closely correlated with retinal fibrosis and choroidal neovascularization. In addition, TGF-β played a key role of in mediating diabetic renal hypertrophy, tubulointerstitial and vaginal tissue fibrosis. Increased membrane metalloproteinases secretion induced by the parasite may also contribute to the pancreatic islet fibrosis. Diabetes caused marked changes in the function and metabolism of neutrophils, for example glutamine oxidation and glutaminase activity were markedly decreased in the neutrophils from diabetic rats, and glutamine plays an important role in protein (as an amino acid source), lipid (by NADPH production) and nucleotide synthesis (by purine and pyrimidine production), as well as in NADPH oxidase activity. It must be emphasized that the tachyzoite stage of *T. gondii*, responsible for an acute infection, rapidly metabolizes glucose via glycolysis However, it was also demonstrated that glucose was nonessential for *T. gondii* tachyzoites because host-derived glucose and its transporter in the parasite were dispensable by glutaminolysis. Thus, eventually increased requirements for glutamine and competition for this amino acid between *T. gondii* and neutrophils (and probably other cells) may result in diminished sources of glutamine and development of disturbances in maintaining regular metabolic and immune processes in many host cells. Moreover, this amino acid raises the *in vitro* bacterial killing activity and the rate of ROS generation by neutrophils, and delays spontaneous apoptosis of these cells. These findings may be further supported by the morphological abnormalities of myenteric neurons in experimental animals during *Toxoplasma gondii* infection, including hypertrophy/atrophy of the neurons and changes in the cell body areas depending on the parasite genotype, its form, dose, route of inoculation, animals studied, and part of the gastrointestinal tract involved. Virulence of the parasite was also associated with distinct dendritic cell responses and reduced numbers of activated $CD8^+$ T cells. Impaired vascular endothelial function and abnormal metabolic fate of NO in patients with diabetes mellitus may be at least in part due to the preferential *T. gondii* infection of endothelial cells. Finally, vitamin D and minocycline exerted beneficial effects on development and clinical course of diabetes mellitus probably because of their immunomodulating and antitoxoplasmatic activities. In addition, a strong correlation was found between the HSP65 expression and protection against *T. gondii* infection, suggesting that this protein significantly contributed to development of the host defense system. It seems therefore that infection with this protozoan plays an important role in the etiopathogenesis of both types of diabetes.

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