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Tryptophan metabolism and immune alterations in pregnant Hispanic women with chronic *Toxoplasma gondii* infection

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Abstract

Pregnancy markedly modifies women's metabolism and immune functions. We hypothesized that pregnancy might alter the immune and metabolic responses to chronic *T. gondii* infection in pregnancy. A population of 690 pregnant Hispanic women were screened for antibodies to *T. gondii* and 158 women were positive (22% positivity) with 83% showing high avidity indices. These seropositive women were followed through their pregnancies with 4 data collection time points and a postpartum collection at two clinics in Tampa, Florida. A *T. gondii* seronegative group (N=131) was randomly selected to serve as a control group and measured along pregnancy in the same way. Serum levels of tryptophan, kynurenine, and their ratio, phenylalanine, tyrosine and their ratio, neopterin and nitrite were measured through pregnancy and the postpartum. A plasma cytokine panel (IFN-γ, TNFα, IL-2, IL-10, IL-12, IL-6, IL-17) was analyzed in parallel. The major findings suggest that indoleamine 2,3-dioxygenase (IDO-1) was less activated in *T. gondii* seropositive pregnant Hispanic women with chronic infection. Evidence for IDO-1 suppression was that tryptophan catabolism was less pronounced and there were lower levels

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of multiple inflammatory cytokines including IFN-γ, which is the major inducer of IDO-1, and higher nitrite concentration, a surrogate marker for nitric oxide, an inhibitor of IDO. In contrast, TNF-α increased over time in the T. gondii positive women. We also observed lower titers of *T. gondii* IgG across pregnancy compared to the postpartum, suggesting that immune suppression in pregnancy differs from the postpartum period of chronically infected women.

Keywords

Toxoplasma gondii; pregnancy pathway chronic infection; immunity; tryptophan pathway

Introduction

Toxoplasma gondii Infection

Toxoplasma gondii (T. gondii) is a highly successful parasite, infecting about a third of the world population (Montoya & Liesenfeld, 2004), yet is still considered a neglected parasitic infection by the Centers for Disease Control (CDC). *T. gondii* is considered “neglected” due to its association with poverty, its high prevalence, despite its significant pathological effects (Hotez, 2014). It is well known that acute infection during pregnancy increases the risk of miscarriage and is associated with damage to multiple organs in the fetus (i.e., congenital toxoplasmosis) (Dubey et al., 2021). After the acute phase of infection, the parasite persists as a chronic, lifelong infection that is associated with the development of intracellular cysts containing the bradyzoite form of the parasite in immune privileged sites such as the eyes, the brain and heart muscle (Xiao & Yolken, 2015). Chronic *T. gondii* infection during pregnancy is generally regarded as having no obstetrical risk, although reactivation has been reported in pregnant women with severe immunocompromise such as HIV disease (Kodym et al., 2015).

There is a vigorous microglial, innate and Th-1 polarized $CD8^+$ T-cell dependent immune response to this parasite during both the acute and chronic phases of infection. The innate immune response involves toll like receptors (TLRs) and other pathogen recognition receptors on macrophages and dendritic cells (DCs). IL-12 production and antiparasitic action, including phagocytosis, against the parasite by these activated innate antigen presenting cells ensues and can result in the death of infected cells (Sasai et al., 2018). Most infected individuals retain a persistent lifelong chronic infection of intracellular cysts containing bradyzoites, the dormant form of the parasite. Cells of the adaptive immune system patrol cysts and directly kill parasites that leak from the cysts or secrete antibodies to keep the infection under control and essentially asymptomatic. Throughout the life of the host, immunoglobulins against various *T. gondii* antigens are produced, and in general IgG titer and avidity indices rise with aging, suggesting that there is some exit of bradyzoites from cysts constantly stimulating B cell defenses (Roiko et al., 2018) (Weilhammer et al., 2012). IFN-γ is released during acute infection largely through $CD4^+$ T cells and Natural Killer (NK) cells, but $CD8^+$ T cells are the major effectors during chronic infection by secreting perforin to rupture and destroy cysts directly (Suzuki, 2021), and by releasing IFN-γ and thus preventing reactivation of acute infection from the leak of bradyzoites from cysts. $CD4^+$ cells help the vigorous $CD8^+$ effector response through Th-1 cytokine release.

IFN-γ, along with other proinflammatory cytokines, such as IL-1β, IL-6, IL-18 and tumor necrosis factor alpha (TNF-α), induce indoleamine 2,3-dioxygenase-1 (IDO-1) expression (Duan et al., 2018). IDO-1and TDO (tryptophan 2,3 -dioxygenase) are enzymes that catabolize tryptophan into kynurenine, and other downstream catabolites (see Figure 1). TDO is found in the liver and placenta and is activated by corticosteroids, while IDO-1 is ubiquitous, and present in many types of cells. (Sedlmayr et al., 2014) Shunting of tryptophan into the kynurenine pathway by both pathways is thought to play a large protective role in infection, perhaps by decreasing the availability of this amino acid that is essential for invading microbes, as well as increasing downstream kynurenine metabolites which are immunosuppressive during pregnancy (Tatsumi et al., 2000) as well as in other conditions such as autoimmune disease and cancer (Hjortsø et al., 2015). Tryptophan is an amino acid required by many cells, and is required for *T. gondii* parasite growth (Pfefferkorn et al., 1986). The multiple functions of tryptophan and the tryptophan catabolite pathway in pregnancy appear to be protective. The placenta also produces TDO and IDO-1 and the role for tryptophan depletion and catabolite production during pregnancy is thought to be immunosuppression of T-cells and macrophages, thereby protecting against maternal immune attack on semi-allogeneic fetal tissues (Munn et al., 1998) (Kudo et al., 2004) (Silvano et al., 2021). Tryptophan levels decrease throughout the course of pregnancy, but there is controversy as to why this occurs. The decline could be related to the increasing metabolic demand by the mother and the fetus for protein synthesis or the result of accelerated breakdown resulting from the inflammation associated with pregnancy. However, it may be that the decline is in albumin-bound tryptophan and free tryptophan is actually increased in pregnancy (Badawy, 2015).

Pregnancy

There is much evidence that chronic *T. gondii* can reactivate in states of immune suppression, such as in HIV disease. (Manuel et al., 2020). It is reasonable to suggest that the normal immune suppression seen during pregnancy might impede the immune response to chronic *T. gondii* infection and lead to reactivation (Rezende-Oliveira et al., 2020). A recent systematic review of association of miscarriage with *T. gondii* IgG seroprevalence in cross-sectional and case-control studies showed a pooled odds ratio of 1.65 (95% CI: 1.31–2.09) and 2.26 (95% CI: 1.56–3.28) (Nayeri et al., 2020). It is likely that chronic infection and possibly reactivation occurred in these pregnant women. The co-occurrence of chronic *T. gondii* infection and pregnancy may alter immunologic and metabolic states as host metabolism such as the tryptophan kynurenine pathway, or nitric oxide metabolism, plays a role in reactivation (Weilhammer et al., 2012). The immune system in pregnancy promotes maternal-fetal tolerance but may threaten cell-mediated containment of bradyzoites within chronic *T. gondii* cysts. The old concept of a Th-1/Th2 negative skew is simplistic and has been replaced with models that show a strong T regulatory (Treg) bias in pregnancy, associated with Th-1 and Th-17 suppression or a Th-17/Treg balance (Moldenhauer et al., 2022). Tregs recruited from maternal blood to the fetal-maternal interface secrete anti-inflammatory cytokines such as TGF-beta and IL-10 at the decidua upon implantation, and induce peripheral antigen presenting cells to secrete reduced amounts of IFN-γ and TNF-α, and higher levels of IL-4, IL-5, and IL-10 (Piccinni et al., 2021). Pregnancy induced changes

in immune tolerance may therefore potentially interfere with the dominant Th-1 and Treg homeostatic mechanisms that normally restrain cysts from producing and releasing *T. gondii* bradyzoites.

The current study was a large prospective examination of *T.gondii* seropositive compared to seronegative pregnant Hispanic women, chosen for their vulnerability to chronic infection with the parasite (Pappas et al., 2009). We sought to characterize the course of chronic *T. gondii* infection in pregnancy and hypothesized that chronic *T. gondii* infection could reactivate during pregnancy and would be associated with alterations in immunity and tryptophan catabolism. We evaluated this possibility through serial retinoscopy, PCR, *T. gondii* IgG and IgM titers, serotypes, avidity, and plasma levels of tryptophan, kynurenine, and their ratio, phenylalanine, tyrosine and their ratio, neopterin and nitrite and a plasma cytokine panel (IFNγ, TNFα, IL-2, IL-10, IL-12, IL-6, IL-17), measured throughout the pregnancy and once in the postpartum.

Methods

The USF Institutional Review Board approved the study, and all women gave written informed consent. Pregnant women who self-identified as Hispanic were recruited in two prenatal clinics in Tampa, Florida. Exclusion criteria included age under 18 years old, HIV infection, drug and/or alcohol dependency, autoimmune disease or cancer, steroid medications, BMI<20, plans to terminate the pregnancy, or presence of congenital anomalies. All screened women (N=690) provided a 15 mL venous blood sample at the first prenatal visit (usually first trimester). Blood was collected in tubes with EDTA as anticoagulant and were kept cold until brought to lab for processing. Plasma was extracted and aliquots stored at −80°C. IgG titers to *T. gondii* (IU/mL) were measured with the *Toxoplasma gondii* IgG ELISA kit (Cat. No. IB79841, IBL America) following the manufacturer's instructions with a cutoff of 10 IU/ml. A seronegative control group (N=124) was randomly selected from the sample at the first prenatal visit and followed through pregnancy. All seropositive women (N=158) were enrolled and quantitative IgG titers, as well as the cytokine and metabolites were measured in as many as 4 pregnancy visits and once in the postpartum. The data were divided into first trimester (3-12 weeks), early (13-18 weeks) and late (19-23 weeks) second trimester, and early (24-30 weeks) and late (31-42 weeks) third trimester. In cases where 2 visits occurred in the same interval, the data were averaged. The sample size having blood draws was 178 at the first trimester visit women, 236 for the late second trimester, 227 for the late third trimester and 202 for the postpartum visit.

T. gondii serotypes (Types I/II, II, atypical, and non-reactive) were analyzed with a peptide-based ELISA assay adapted from Kong et al. (Kong et al., 2003) and Shobab et al. (Shobab et al., 2013). An avidity ELISA was performed on all plasma samples from T. gondii seropositive women, using a kit from Euroimmun (Lubeck, Germany), following kit instructions, to determine if any primary infections were present in the sample and to estimate duration of chronic infection. Results were grouped as low avidity, borderline avidity and high avidity. To determine serotype, *T. gondii* positive IgG serum samples were tested for reactivity against GRA6 and GRA7 strain-specific allelic peptide motifs (Kong et

al., 2003; Shobab et al., 2013). Each serotyping ELISA contained three *T. gondii* negative control sera (*T. gondii* IgG levels of less than 2 IU/mL) for calculation of the threshold values for each serotype, i.e., above which normalized assay values were positive. Threshold values were determined for each serotype-specific peptide by averaging the optical density (OD) values of the three normalized *T. gondii* negative samples and then adding two standard deviations (Shobab et al., 2013).

Reactivation

We assessed *T. gondii* reactivation in women with positive titers at each visit. First, we used a Digital Retinopathy System (DRS)-equipped retinoscope camera (ICare, Revenio Group, Finland) to capture digital retinal images at each study visit. A consulting ophthalmologist examined the retinal images for the characteristic retinochoroiditis or scars associated with *T. gondii*. Next, we spot checked IgM levels by ELISA (IBL) in 75 samples, and *T. gondii* DNA in 18 cord bloods and 20 postpartum samples using qPCR. The Toxoplasma ELITe MGB assay (ELITechGroup, France) was performed according to manufacturer's instructions with a Bio-Rad real-time PCR system. We used the *T.gondii* RH repeat region for designing the primers (F; 5'-AGA GAG ACC GGA ATG CGA TCT-3', R; 5'-TTC GTC CAA GCC TCC GAC T-3') and probe-fam (5'-FAM-TCG TGG TGA TGG CGG AGA GAA TTG A-3') from Invitrogen (Waltham, MA).

Plasma aliquots were kept frozen at −80°C until analysis. Tryptophan, kynurenine, phenylalanine, tyrosine, neopterin and nitrite were measured in all samples from each visit. Amino acids and the derivate kynurenine were analyzed using high-performance liquid chromatography (HPLC)–based methods as reported earlier (Geisler et al., 2015). In brief, 3-nitro-L-tyrosine was added as internal standard and plasma samples were deproteinized with trichloroacetic acid. Separation was performed on a reversed-phase LiChroCART 55-4 C18 column (3 μm particle size, Merck, Darmstadt, Germany) using sodium acetate buffer (15 mmol/L, pH 4.0) for analysis of tryptophan and kynurenine, or potassium dihydrogen phosphate (0.015 mol/L) for analysis of phenylalanine and tyrosine on a Varian ProStar liquid chromatography system with autosampler Model 400 (Varian, Palo Alto, CA). Kynurenine was monitored by its ultraviolet light-absorption at 360 nm (UV-detector SPD-6A, Shimadzu, Japan) and tryptophan by detection of its natural fluorescence at 286 nm excitation and 366 nm emission wavelengths (ProStar fluorescence-detector Model 360, Varian). Peaks were identified by comparing peak retention time to an albumin-based calibrators. Neopterin levels were measured by enzyme-linked immunoassay (BRAHMS, Hennigsdorf, Germany) according to the manufacturer's instructions, with a detection limit of 2 nmol/L. Nitrite concentrations were measured with the modified Griess-Ilosvay diazotization reaction assay (Merck KGaA, Darmstadt, Germany) as reported previously (Geisler et al., 2015).

Cytokines

Cytokines (IFN-γ, TNFα, IL-2, IL-6, IL-10, IL-12, IL-17) were measured in plasma aliquots in duplicate using the Luminex MagPix multiplexing system. Coefficients of

variation were under 10, and cytokines that measured below the limit of detection (LOD) were assigned a value between zero and the LOD.

Demographics

An investigator-developed questionnaire available in Spanish and English was used to collect data on age, parity, number of people living in the household, country of origin, time in the US, health problems, medications, smoking and drinking habits, income, occupation, and education. This information was updated as needed over the course of the pregnancy. Access to the electronic medical record was also used for additional health related data collection.

Statistics

SPSS 28 was used for demographic analysis and GraphPad Prism version 9.5.0 for Mac OS 13.0, GraphPad Software, San Diego, California USA, www.graphpad.com, was used for immunoglobulins, metabolites, and cytokines. Robust nonlinear regression and false discovery rate (ROUT, q=1%) was used to detect significant outliers at each time point for each dataset. (Motulsky & Brown, 2006). After outlier removal, mixed effects models were fitted with Geisser-Greenhouse correction for non-sphericity (Bathke et al., 2009), if required, to examine relationship across time for the tryptophan, inflammation biomarkers (neopterin, nitrite, kynurenine), immunoglobulins, and proinflammatory and anti-inflammatory cytokines by *T. gondii* seropositivity status. Multiple comparisons were controlled for false discovery using the original Benjamini and Hochberg method with a desired false discovery rate of < 0.1 (Bathke et al., 2009). Country of birth and age were included in these models initially as covariates using SPSS, version 28, as *T. gondii* positivity was different by these variables (see Table 1), but controlling for these variables had no effect on the results. Interaction terms were excluded from the models as only one variable had an interaction with *T. gondii* status and time.

Results

Demographics

The population under study consisted of women who self-identified as Hispanic. Most of these women were immigrants, having been born in countries or territories outside of mainland US. The more common origins were Mexico, Puerto Rico, Cuba, and Honduras. These women were receiving prenatal care in clinics for socioeconomically deprived populations. Many women spoke only Spanish and were in families with members employed as agricultural workers in Florida. The women had an average number of 2 children, with a range from 0 to 8. Demographic details comparing the *T. gondii* seropositive to seronegative group are shown in Table 1. The incidence of seropositivity was 22% in the population in contrast to the seroprevalence of the U.S. national population, which the CDC reports as 11% (https://www.cdc.gov/parasites/toxoplasmosis/epi.htm).

Toxoplasma Reactivation

Only one *T. gondii* ocular lesion in the 158 *T. gondii* positive women was found, indicating previous chorioretinitis scarring. There was no evidence by PCR of *T. gondii* DNA in any of the random plasma samples (N=38).

Titers, Avidity and Serotypes

One sample from 75 randomly selected plasma samples had a positive IgM along with IgG to *T. gondii*, and the IgM declined at the next study visit, suggesting that she did not have acute infection, so her data were retained. IgG titers in *T.gondii* positive women were lower during pregnancy compared to postpartum titers. The mean avidity index (A.I.) was 82.2+−2.79, and range was between 37.4 and 373. The majority (88.2%) of the women had high A.I. (>60), 11.1% had borderline A.I. (50-60), and only 1 (0.06 %) had low A.I. (<50). The distribution of serotypes across the Hispanic *T. gondii* positive women showed that the atypical (25%) and the non-reactive (40%) serotypes were the most prevalent in those born outside the U.S., while the U.S.-born *T. gondii* positive women had an equal distribution of the 4 known *T. gondii* serotypes. There were differences by both IgG titers and avidity indices by serotype, with the atypical serotype showing both highest titer and avidity. This was not statistically significant.

Tryptophan and Inflammation Biomarkers

The drop in tryptophan levels over pregnancy in all women was highly significant ($F=39.96$, $p<0.001$) and tryptophan was significantly higher across pregnancy in the *T. gondii* seropositive women ($F=7.97$, $p<0.005$) (Figure 2). The ratio of kynurenine to tryptophan rose over pregnancy ($F=62.31$, $p<0.001$) but *T. gondii* seropositive women had lower ratios ($F=3.81$, $p=0.052$) (Figure 3). Neopterin also rose significantly throughout pregnancy ($F=32.28$, $p<0.001$), but did not differ in women with *T. gondii* (data not shown). Nitrite rose in both groups through pregnancy ($F=9.473$, $p<0.001$) and was significantly higher across time in the *T. gondii* positive group ($F=6.057$, $p=0.0015$) (Figure 4). Phenylalanine and tyrosine both differed across pregnancy but did not show any differences by *T. gondii* status (data not shown).

Cytokines

Tested cytokines varied significantly over time throughout pregnancy (some rising, some falling) and several inflammatory cytokines were significantly lower in the *T.gondii* positive group, IFN-γ over time ($F= 3.51$, $p=0.005$), by *T.gondii* status ($F= 0.68$, $p<0.001$ (Figure 5a); IL-6 over time ($F= 14.45$, $p<0.001$), by *T. gondii* status ($F=14.86$, $p=0.02$) (Figure 5b; IL-17 over time ($F= 9.8$, $p<0.001$), by *T.gondii* status ($F=10.21$, $p=0.0016$) (Figure 5c); and IL-12 over time ($F = 8.406$, $p<0.001$, by *T. gondii* status ($F=10.7$, $p=0.0012$ (Figure 5d). The interaction of time and *T.gondii* status was only significant in IL-12 ($F=6.236$, $p<0.001$ (Figure 5e). IL-12 varied significantly over time ($F = 8.406$, $p<0.001$, and was lower in *T. gondii* women $F=10.7$, $p=0.0012$. 5e). IL-10 did not differ significantly across pregnancy or by *T.gondii* serostatus (data not shown). TNF-α, however, showed significant increases across time ($F = 9.685$, $p< 0.001$, and by *T. gondii* status ($F=7.44$, $p=0.007$) (Figure 5f).

Discussion

In our prospective sample, we confirmed the presence of chronic infection through avidity titers and absence of IgM. We saw no evidence of *T.gondii* reactivation in these chronically infected pregnant women by PCR or retinoscopy. While IgG titers were lower during pregnancy compared to postpartum levels, titers alone may not reflect parasite burden and serological titers are not considered a surrogate of clinical disease (Beazley & Egerman, 1998). Antigen stimulation leading to elevation of the IgG titers may occur in the presence of tachyzoites or bradyzoites (Deshmukh et al., 2021). The bradyzoite cysts were, until recently, considered immunologically silent during chronic infection but recent study in animal models suggest that the cysts are more dynamic and $CD8^+$ T cells are capable of cyst destruction even during the chronic phase (Suzuki, 2021). This model also suggests that the constant $CD4^+$ and memory $CD8^+$ T cell recruitment and activation in chronic infection produces a mild but constant reactivation of the Th-1 axis leading to T cell exhaustion (Khan & Moretto, 2022).

T. gondii serotype is known to be associated with virulence, and the most common serotypes in our sample were the atypical and nonreactive types. There was both higher IgG titer and avidity index in those with the atypical serotype, and this serotype is considered more virulent than the more common Type II. The non-reactive (NR) serotype prevalence in this Hispanic population is a novel finding that mimics previous studies (de-la-Torre et al., 2013; Shobab et al., 2013). It was reported in sera from ocular toxoplasmosis patients in Columbia (de-la-Torre et al., 2013). Individuals with NR serotype (43%) were also observed in a study of German patients with ocular toxoplasmosis (Shobab et al., 2013). The NR serotype may have a genetic background as it represents the inability to react to archetypal *T. gondii* allelic peptide motifs. It may also be that the nonreactivity may indicate suppression of the antibody response to less abundant serotype-specific antigens. The roles of serotypes in adverse pregnancy events deserves further study.

In exploring the relationships among tryptophan metabolites, inflammatory biomarkers, cytokines, and the IDO-1 pathway, we found lower levels of many inflammatory cytokines including IFN- γ, a key cytokine in *T. gondii* parasite control. There was evidence of reduced activation of the IDO-1 pathway, which is normally activated by IFN-γ, with corresponding higher tryptophan levels and lower kynurenine/tryptophan ratios in the seropositive women, and higher levels of nitrite, a proxy for NO, which inhibits IDO-1, and subsequent immunomodulation by the parasite that can result in suppression of *Toxoplasma*-directed antibody response (Saeij et al., 2007). Our findings of reduced levels of certain Th-1 and proinflammatory cytokines (IFN-γ, IL-17, IL-12, IL-6) either throughout pregnancy or at certain times during pregnancy suggest that T cell function in chronically infected pregnant women may be altered. Throughout the life of the chronically infected host there is an equilibrium between the parasite survival and the Th-1, Th2, Th-17, and T regulatory (Treg) host responses to the parasite (Sana et al., 2022). Maintenance of quiescence involves a balance between pro- and anti-inflammatory cytokines. Overproduction of proinflammatory cytokines, such as IFN-γ could be harmful to the host but is balanced by anti-inflammatory cytokines, such as IL-10, produced by T regs. *T. gondii* bradyzoites can evade host immune effects by suppressing IFN-γ signaling (Zimmermann et al., 2006),

blocking STAT-1 mediated transcription of inflammatory cytokines (Lima & Lodoen, 2019), reducing NF-kB signaling (Shapira et al., 2004) and producing effector molecules such as granule proteins which mediate iNOS expression and nitric oxide thereby reducing IDO-1 mRNA, with downstream immuno-protective effects (Bando et al., 2019). IDO-1 activation results in degradation of tryptophan which is required for T-cell activation (Gostner, Becker, Überall, et al., 2015; van Baren & Van den Eynde, 2015{{Zamanakou, 2007 #498; Zamanakou et al., 2007) but also for *T. gondii* intracellular growth (Pfefferkorn et al., 1986). Latency is maintained not only through $CD8^+$ T cell IFN-γ production, but also by induction of interferon-stimulated genes (ISGs) that enhance immune suppression, although exact mechanisms for disrupting *T.gondii* infection through these genes are unknown (Rinkenberger et al., 2021). Others have observed lower levels of these cytokines during pregnancy with *Toxoplasma* infection. In a study of Brazilian pregnant women, those with chronic *T. gondii* infection had lower levels of IFN-γ, TNF-α, and nitrite (Marchioro et al., 2018). A similar observation was made in the U.S. (Pernas et al., 2014). These researchers found 23 lower cytokine levels in U.S. pregnant chronically infected women. The ability of *T.gondii* to produce host immune suppression is well known (Kaňková et al., 2010), and one mechanism is by the parasite inducing immune cell microRNAs (de Faria Junior et al., 2021). MicroRNAs act on messenger RNA, resulting in degradation or repressing mRNA translation, resulting in less effective inflammatory responses.

The reduction in circulating cytokines could also reflect loss of recognition and effector functions in certain T cell populations, including reduced IFN-γ production by $CD4^+$ and $CD8^+$ T cells during chronic infection such as the population in this study. Chronic exposure of T cell receptors (TCR) responding over long periods of time to persistent *T.gondii* antigen stimulation may exacerbate the T cell exhaustion seen in pregnancy (Slutsky et al., 2019). T cell exhaustion is marked by increased expression of inhibitory receptors, loss of the ability to secrete cytokines, and development of a different transcriptional program (Catakovic et al., 2017). If $CD4^+$ T cell exhaustion occurs over the time of *T. gondii* infection, and is combined with pregnancy related T cell alterations, such as reduced levels of IFN-γ, the Th-17/Treg balance may shift towards excessive dampening of the effector immune response, so that low grade parasitemia may occur (Khan & Moretto, 2022). Excessive fetal antigen exposure at the placenta also can produce T cell exhaustion, with greater risk associated with older age (Catakovic et al., 2017). T reg function is also important in pregnancy as these cells regulate the $CD8^+$ effector T cells, and maintain tolerance, but little is known about their role in *T. gondii* infection. Miscarriage in *T. gondii* seropositive women is associated with reduced T reg numbers (Koucký et al., 2014). We found that TNF-α differed from the other cytokines in that it was present at higher plasma concentrations in the *T. gondii* seropositive women. This cytokine is largely produced by macrophages (Parameswaran & Patial, 2010) so this innate immune branch may be more active in the chronically infected women.

In our sample, *T. gondii* positive women had a significantly higher number of miscarriages ($p=0.008$). At the uterus, inflammatory signals are necessary preparation for labor, and if inadequate may influence the natural progression of the pregnancy. These T cell related dynamics need to be explored further in chronic *T. gondii* infection and pregnancy. We have recently published our findings of both increased preterm birth and small for gestational age

infants in the chronic *T. gondii* positive women in the study (Mutka et al., 2022). The role of these immune differences in adverse pregnancy outcomes represents a future challenge.

Our metabolic findings are in line with previous observations showing generally decreasing tryptophan levels and an increasing kynurenine to tryptophan ratio over the course of pregnancy (Schröcksnadel et al., 1996) (Schröcksnadel et al., 2003). (Savitz, 2020) (Cervenka et al., 2017) (Jenny et al., 2011) (Wang et al., 2010). Tryptophan catabolism stands at the crossroads of multiple metabolic, neuroendocrine, and immune processes (Gostner, Becker, Ueberall, et al., 2015). IDO-1 is primarily induced by IFN-γ (Duan et al., 2018). The first stable product of IDO-1 enzymatic action is kynurenine, and activity of the IDO-1 enzyme may be estimated by the ratio of kynurenine to tryptophan, in the presence of other markers of immune activation such as neopterin (Fuchs 1990), which is elevated in pregnancy. Neopterin synthesis occurs in activated macrophages and dendritic cells (Mangge et al., 2014), is a marker of immune system activation (Murr et al., 2002) and increases across pregnancy, as was observed in our data, but did not differ by *T. gondii* positivity. Kynurenine downstream metabolites are involved in downregulating inflammation, immune function, and neurotransmission and their ratios and concentrations are carefully regulated in health but can be toxic under conditions of activated immunity (Song et al., 2017). The drop in tryptophan across pregnancy was less pronounced in *T. gondii* seropositive women compared to seronegative individuals suggesting less activation of IDO-1. Shunting of tryptophan into the kynurenine pathway is thought to play a large protective role in infection, perhaps by decreasing the availability of this essential amino acid required by infecting pathogens. Tryptophan can also decline due to low dietary intake or increased metabolic demand, which can occur in pregnancy (Duan et al., 2018). The lower kynurenine/tryptophan ratio observed in the *T. gondii* seropositive women suggests that there may be less functional kynurenine pathway catabolites in T. *gondii* pregnancy.

Further evidence for a reduction in IDO −1 activity in the *T. gondii* positive women is the higher levels of nitrite in the seropositive women. Nitrite is an oxygenated derivative of nitric oxide (NO) and plasma levels could reflect diet (Mattila et al., 2020) or cellular production of NO, a metabolite with a very short plasma half-life (minutes) (Ignarro, 1990). NO levels rise during pregnancy (Sutton et al., 2020) and inducible NOS (iNOS) is a major factor in maintaining a low resistance placental vascular circuit (Zullino et al., 2018). High levels of NO can inhibit IDO-1 mediated tryptophan degradation, and vice versa, thus suppressing the shunting of tryptophan to kynurenine (López et al., 2006; Samelson-Jones & Yeh, 2006).

The lower IgG titers across pregnancy compared to the postpartum may support a more general immune suppression during pregnancy. These data suggest that chronic *T. gondii* infection may impact the normal metabolic and immune characteristics of pregnancy. Our recent report of increased risks for preterm births, miscarriages and small for gestational age infants in chronically infected women suggests that further investigations into immune and metabolic changes in chronicity during pregnancy deserve further study (Mutka T. & Jacques A, 2023). In addition, we have preliminary evidence for different cytokine patterns over time in *T. gondii* seropositive women who gave birth preterm.

Limitations

All the measurements in the study may have been affected by the hemodilution effects of plasma volume expansion during pregnancy, but this would be a consistent effect across all measures. While our sample size was large and data were collected prospectively, we did not have a non-Hispanic control group or non-pregnant control groups. Many more women born outside the US were *T. gondii* positive (N=148) compared to U.S. born (N=10) in our sample. There were likely many cultural, microbial, and dietary differences that could impact infection, metabolism, and immunity by women born or raised in other countries, but we could not control these many variables. Our population consisted of economically disadvantaged, immigrant women and may not, therefore, be generalizable to populations not experiencing the stresses encountered uniquely in this population.

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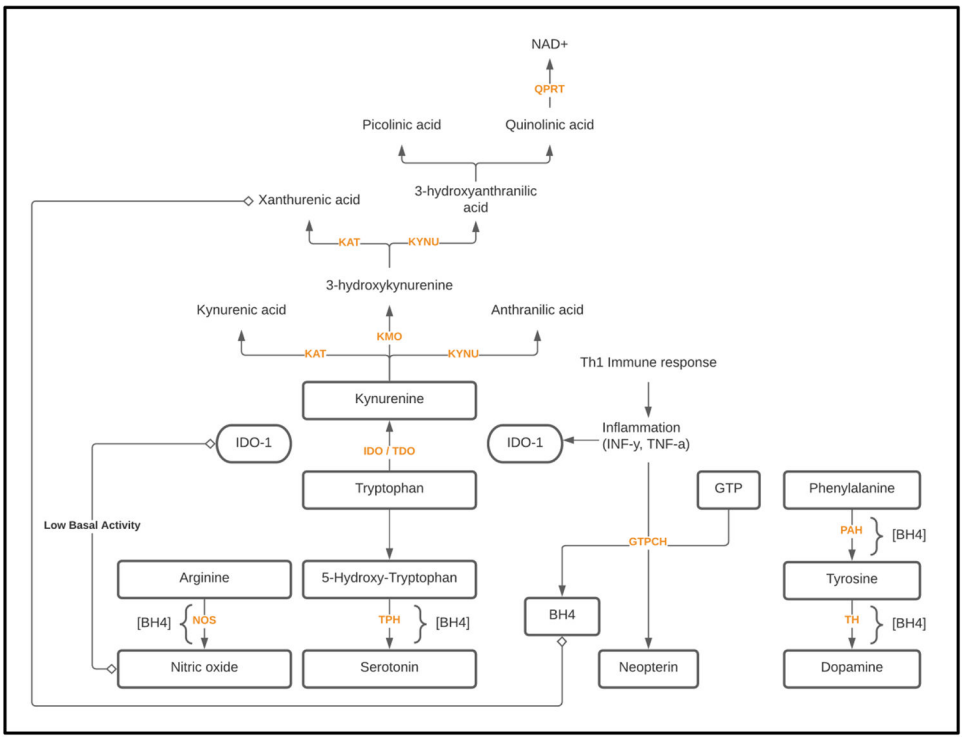

Figure 1. Biogenic amines and their metabolites

IDO, indolamine 2,3-dioxygenase; *TDO*, tryptophan 2,3-dioxygenase; *KAT*, kynurenine aminotransferase; *KMO*, kynurenine 3-monooygenase; *KYNU*, kynureninase; *QPRT*, quinolinate phosphoribosyltransferase; *NAD*, nicotinamine adenine dinucleotide; *BH4*, tetrahydrobiopterin; *TPH*, tryptophan hydroxylase; *NOS*, nitric oxide synthase; *GTP*, guanosine triphosphate; *GTPCH*, GTP cyclohyrdolase; *PAH*, phenylalanine hydroxylase; *ThH*, tyrosine hydroxylase.

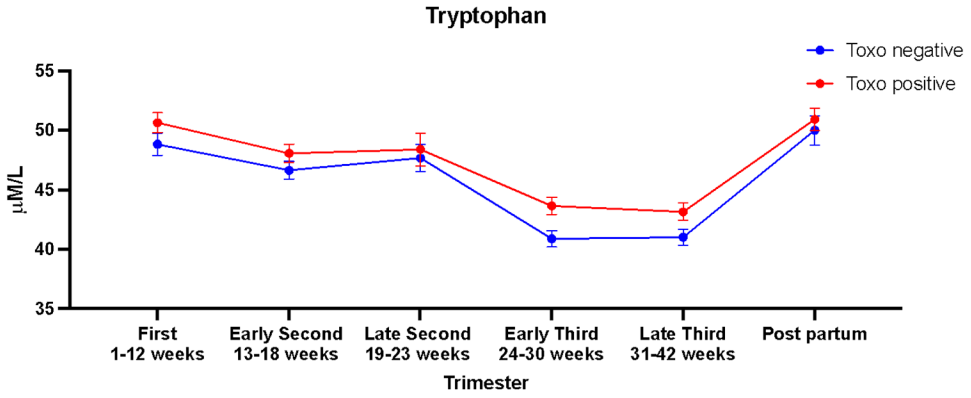

Figure 2. Plasma tryptophan levels were higher across pregnancy in the seropositive women

After outliers at each timepoint were removed by robust nonlinear regression and false discovery rate (ROUT, q=1%), mixed effects modeling revealed significantly increased tryptophan levels across ($F=39.89$. $p<0.001$) and by T. gondii seropositive status ($F=7.969$, $p<0.005$), interaction ns.

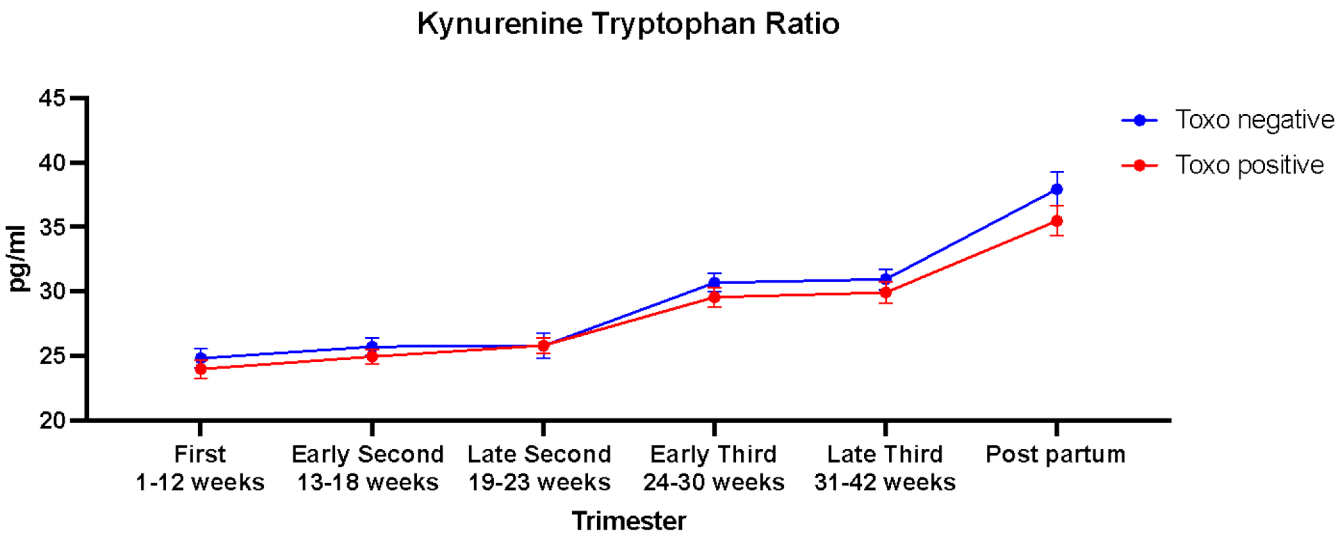

Figure 3. Kynurenine Tryptophan ratio rises throughout pregnancy, but trended lower in *T.gondii* positive women

After outliers at each timepoint were removed by robust nonlinear regression and false discovery rate (ROUT, q=1%), mixed effects modeling revealed that the kynurenine to tryptophan ratio rose over pregnancy (F=10.57, p=0.0013) indicating more conversion of tryptophan to kynurenine in later pregnancy, and *T. gondii* seropositive women had a trend towards lower ratios across pregnancy (F)=3.814, p=0.052), interaction ns.

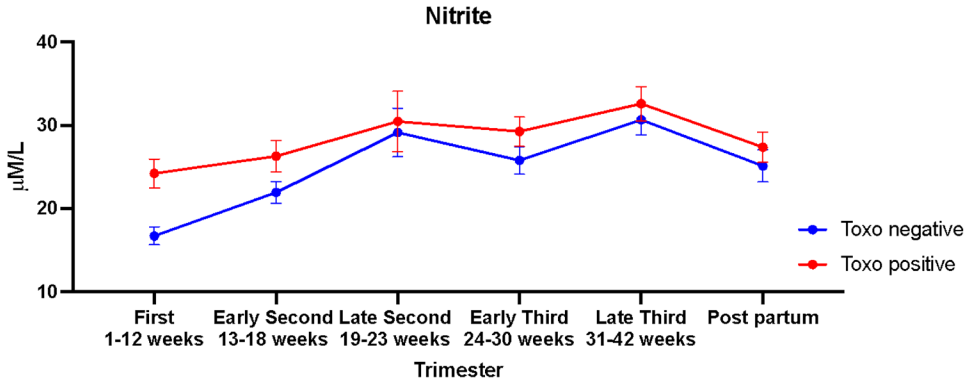

Figure 4. Plasma Nitrite levels were higher across pregnancy in *T. gondii* seropositive women

After outliers at each timepoint were removed by robust nonlinear regression and false discovery rate (ROUT, q=1%), mixed effects modeling revealed that nitrite rose in both groups through pregnancy ($F=9.36$, $p<0.001$) and was significantly higher across time in the *T. gondii* positive group ($F=7.4$, $p=0.007$), interaction ns.

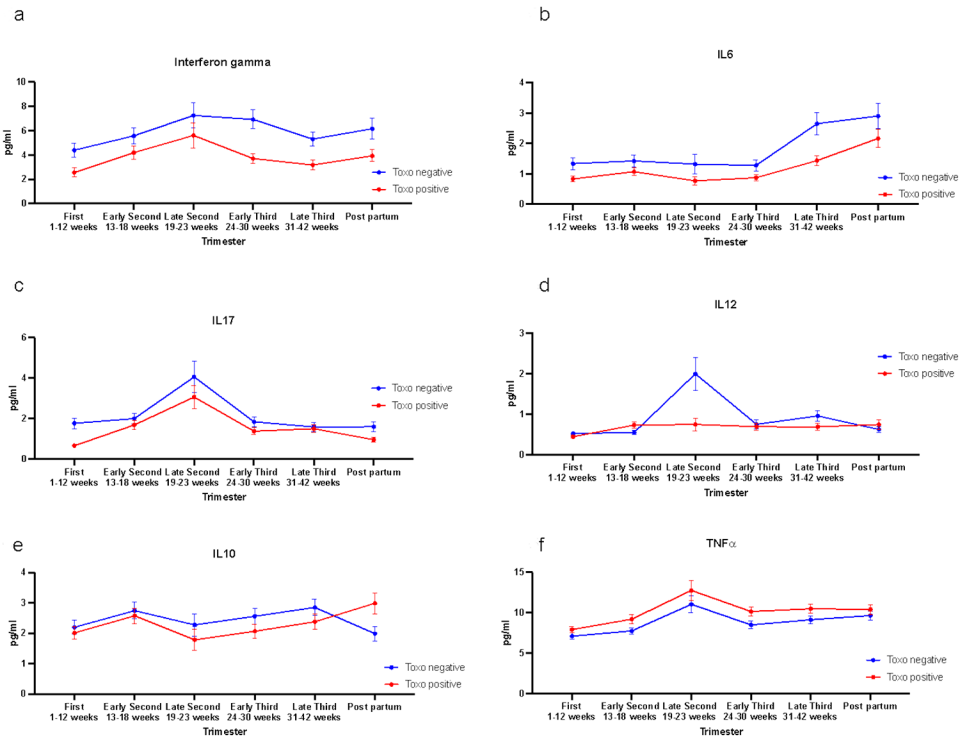

Figure 5. Cytokines across pregnancy by *T.gondii* status

After outliers at each timepoint were removed by robust nonlinear regression and false discovery rate (ROUT, q=1%), mixed effects modeling revealed that, 5a) IFN-γ varied significantly over time (F= 3.45, p=0.005,) and was lower in *T.gondii* positive pregnant women (F= 25.95, p<0.001); 5b). IL-6 increased over time (F = 14.27, p<0.001, and was lower in T. *gondii* positive women F=8.44, p=0.004; 5c) IL-17 varied significantly over time (F = 9.71, p<0.001, but was lower in *T.gondii* women F=8.44, p=0.004; 5d) IL-12 varied significantly over time (F = 8.406, p<0.001, and was lower in *T. gondii* women F=10.7, p=0.0012. 5e). 5f), TNF-α, showed significant increases across time (F = 9.835, p< 0.001, and by *T. gondii* status F=8.07, p<0.001 (Figure 5f).

Interactions of time and cytokines ns except IL12 (F=6236 P<0.001).

Table 1.

Demographic variables by *T. gondii* seropositivity status.

	T. *gondii* negative	**T. *gondii* positive**	***P value***
Number of Women	131	158	
Maternal Age (mean, range)	30 .7(19-45)	32 .2 (18-47)	0.033 *
Country of Birth			0.000 ***
United States	40	10	
Puerto Rico	18	8	
Mexico	50	58	
Dominican Republic	4	8	
Cuba	4	21	
Honduras	7	23	
Guatemala	5	14	
Other	3	16	
Number of pregnancies (mean, range)	3 (1-9)	4 (1-15)	0.111
Maternal BMI at visit 1 (mean, range)	30.6 (19-56)	31.4 (21-57)	0.160
Marital Status			0.100
Single/unmarried	76	81	
Married	49	59	
Divorced/Separated	6	18	
Education			0.073
Grammar school	4	5	
Middle school	25	53	
High school graduate	74	69	
College graduate	26	27	
Postgraduate	2	4	
Income			0.041 *
<$4,999	25	31	
$5,000-$14,999	22	43	
$15,000-$24,999	45	60	
$25,000-$39,000	21	16	
$40,000-$69,000	13	5	
$70,000+	1	3	
Toxoplasma gondii serotype		Type 1/III: 31 (20%); Type II: 25 (16%); atypical: 41 (26%); nonreactive: 60 (38%)	
Infant weight in kg (mean, range)	3.39 (.91-4.8)	3.19 (0.4-4.6)	0.093
Gestational Age at Delivery in weeks (meann, range)	38.2 (23-41.1)	37.3 (21.4-41)	0.085
Miscarriage	2	14	0.008 **
Preterm Birth (excluding miscarriage)	17	30	0.198

	T. *gondii* negative	**T. *gondii* positive**	***P value***
Preeclampsia	10	7	0.453
Gestational diabetes	19	28	0.266

Chi Square or t-test analyses: *p<0.05; **p <0.01, ***p<0.001. Data are given as means with ranges or as percentages