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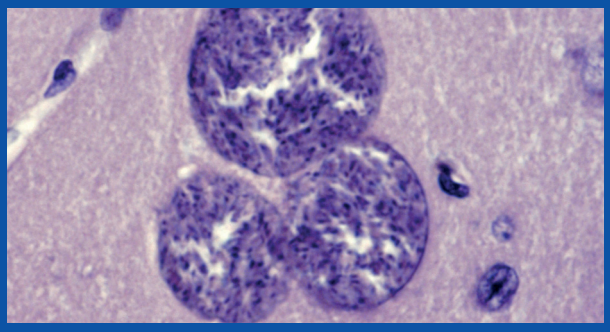


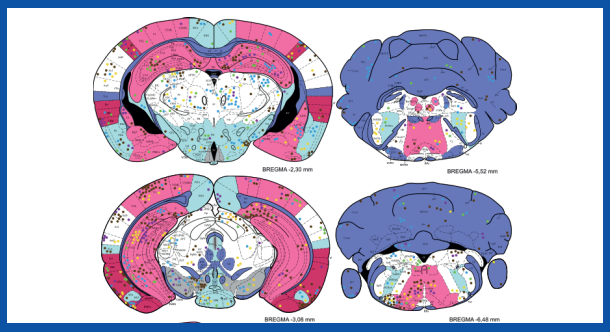

Special Issue

Effects of Latent Toxoplasmosis: Three Decades of Studies

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Special Issue on Toxoplasmosis
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Chronic inflammation in pregnant women with latent toxoplasmosis and explanation of discordant results of serological tests for toxoplasmosis

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Abstract: *Toxoplasma gondii* (Nicolle et Manceaux, 1908), an intracellular parasite that causes toxoplasmosis, infects a third of the human population. Latent toxoplasmosis has been linked to altered immune responses, including elevated proinflammatory cytokines. In early pregnancy, the immune system adapts to balance inflammation and foetal tolerance. This study assessed whether pregnant women in the first trimester infected with *Toxoplasma gondii* have different cytokine levels than uninfected women. This study also examined whether women with discordant test results for toxoplasmosis represent a distinct group or a mixed group composed of infected women with unusually low levels of anti-*Toxoplasma* antibodies and uninfected women with high levels of cross-reacting antibodies. We measured 18 cytokines (IL-1β, IL-1ra, IL-2, IL-4, IL-7, IL-9, IL-17A, Eotaxin, FGF basic, G-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α) in 78 pregnant women, classified as *Toxoplasma*-positive, *Toxoplasma*-negative or *Toxoplasma*-discordant (negative by IgG ELISA, positive by complement fixation test [CFT]). Using exploratory factor analysis, we identified two factors, the first explaining 29.6% and the second 24.9% of the total variability in cytokine concentrations. *Toxoplasma*-positive women scored significantly higher in the second factor, primarily associated with cytokines linked to Th1-driven inflammation and cellular immunity. Specifically, these women exhibited elevated levels of IL-1β, IL-1ra, IL-2, FGF basic and PDGF-BB compared to *Toxoplasma*-negative women. This finding suggests that pregnant women with latent toxoplasmosis experience some degree of chronic inflammation. Additionally, our results indicate that *Toxoplasma*-discordant women are likely *Toxoplasma*-negative individuals with detectable anti-*Toxoplasma* IgM antibodies. However, as this study focused on pregnant women, further research is necessary to validate these conclusions in broader populations.

Keywords: cytokines, *Toxoplasma gondii*, pregnancy, immunity

Toxoplasma gondii (Nicolle et Manceaux, 1908) is an intracellular parasite that causes a disease called toxoplasmosis. The prevalence of toxoplasmosis in the human population varies from 20 to 80% depending on various factors such as age, hygiene, environment, and cultural habits (Tenter et al. 2000). Definitive hosts of *Toxoplasma gondii* (*T. gondii*) are representatives of any feline species, and its intermediate hosts can be any warm-blooded vertebrate, including humans. A person can become infected with this parasite by eating insufficiently washed root or leafy vegetables or insufficiently heat-processed meat of infected intermediate hosts (Dubey et al. 1998). In the Czech Republic, toxoplasmosis was significantly associated with consuming raw meat, gardening, currently living in a small village, and living in a village during childhood (Kolbeková et al. 2007).

After infection with the parasite in humans, the acute phase of toxoplasmosis occurs first, during which the tachyzoites (a proliferative developmental form of the parasite) multiply rapidly in various host body cells. In a person at this phase of the disease, we can observe symptoms like a bacterial or viral infection. In immunocompetent individuals, this phase transitions to a latent phase in which bradyzoites (another developmental form of the parasite) multiply slowly in tissue cysts. Tissue cysts can occur in various organs, including nerve or muscle tissue (Tenter et al. 2000). If a person becomes infected, he/she remains infected for life, as there is no treatment for the latent toxoplasmosis.

One group of people who should take special care to avoid infection with *T. gondii* is pregnant women. If a woman becomes infected shortly before becoming pregnant or during pregnancy, the foetus can be infected (Robert-Gangneux et al. 2011). The probability of transmission

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to the foetus varies depending on when in pregnancy the woman becomes infected: it increases from 6% in the first trimester to 72% in the third trimester. However, infection acquired in the first trimester has the most severe clinical impact on the foetus (Dunn et al. 1999). Some infections may be asymptomatic at first in newborns but, later in life, lead to slower mental development, blindness or epilepsy (Lopez et al. 2000).

In the most severe cases, we can observe symptoms of the classic Sabin's triad (chorioretinitis, hydrocephalus or intracerebral calcification) in infected children (Wolf et al. 1939). The prevalence of prenatally acquired (congenital) toxoplasmosis ranges from 0.1 to 3 per 1000 pregnancies (Varella et al. 2009). In acutely infected pregnant women, treatment should be initiated to accelerate the transition from the acute phase to the latent phase to reduce the likelihood of transmission of the infection to the foetus (Dunay et al. 2018).

In addition to the impact of acute toxoplasmosis on the foetus of pregnant women, the effects of latent toxoplasmosis on women's fertility, the course of pregnancy and the development of children were also investigated. A higher prevalence of toxoplasmosis has been observed in infertile women compared to fertile women (Li et al. 2011, El-Tantawy et al. 2014). In the study by Kaňková et al. (2015), *Toxoplasma*-positive women reported taking significantly longer to conceive, becoming pregnant at an older age, and overall having more fertility problems and requiring artificial insemination more often than *Toxoplasma*-negative women.

Regarding pregnancy, more prolonged pregnancy and slower foetal development were observed in infected women than in uninfected ones (Kaňková and Flegr 2007). Based on a questionnaire study, it was found that children of *Toxoplasma*-positive mothers show slower postnatal motor development during the first 18 months of life than children of *Toxoplasma*-negative mothers (Kaňková et al. 2012). According to reports from mothers, children of *Toxoplasma*-positive women developed the ability to control the head position significantly later, rolled from supine to a prone position later, and began to crawl slightly later than children of *Toxoplasma*-negative women. However, latent toxoplasmosis did not affect the time they started to sit and walk. These results are compatible with the hypothesis of reduced stringency of embryo quality control in partially immunosuppressed *Toxoplasma*-positive mothers (Kaňková and Flegr 2007, Kaňková et al. 2012), which may result in a higher proportion of infants with genetic or developmental disorders in offspring (Hostomská et al. 1957, Neuhäuser and Krackow 2007).

Recent studies suggest that early pregnancy is characterised by activating a complex network of immune-related factors (Sharma et al. 2016, Peterson et al. 2020). While placental implantation and development are associated with elevated levels of proinflammatory cytokines, the immune response to inflammation, and possibly to the implantation process itself, involves the production of regulatory and anti-inflammatory cytokines, helping maintain a balanced environment for pregnancy (Curry et al. 2008, Jarmund et al. 2021, Spence et al. 2021). The immune system generates a robust immune response in the presence of infection (Racicot et al. 2014). However, this activity is carefully regulated to prevent excessive inflammation that could harm the developing foetus.

Toxoplasma gondii infection disrupts this balance by inducing a Th1 response with increased production of proinflammatory cytokines such as IFN-γ and TNF-α, which are necessary for developing resistance to the parasite (Denkers and Gazzinelli 1998, Moghaddami et al. 2024). Furthermore, in response to *T. gondii* infection, a range of pro- and anti-inflammatory cytokines and mediators (including IFN-γ, TNF-α, TGF-β, nitric oxide, IL-1, IL-6, IL-4, and IL-10) are released (Kazemi Arababadi et al. 2024). *Toxoplasma gondii* infection has also been associated with a higher production of the proinflammatory cytokines IL-1β and IL-18 (Yoon et al. 2022).

For the diagnosis of *Toxoplasma* infection, either indirect (serological) tests or direct detection of *T. gondii* are used. The highly sensitive polymerase chain reaction (PCR) directly detects *T. gondii* in the examined samples. Indirect tests are used to analyse blood serum antibodies. One of the tests is the complement fixation test (CFT), which detects those classes and subclasses of antibodies against *T. gondii* that bind complement (IgM and some subclasses of IgG). Another test is ELISA (Enzyme-Linked ImmunoSorbent Assay), which can distinguish between individual classes of specific antibodies. Other indirect tests include indirect immunofluorescence or the Western blot. IgG class antibody tests are used for diagnosing latent toxoplasmosis, while IgM and IgA class antibody tests are used for diagnosing acute toxoplasmosis (Montoya 2002).

Although both CFT and ELISA have been found to have high test sensitivity and specificity (Mohamed et al. 2012), during our studies, we repeatedly encountered discordant results in diagnosing toxoplasmosis when using both tests simultaneously. These results suggest that groups of people may be falsely identified as *Toxoplasma*-positive or *Toxoplasma*-negative. An exceptionally high occurrence of samples with discordant results from diagnostic tests was observed in pregnant women, possibly due to the specific immune shifts they undergo, as discussed above.

The primary objective of this study was to determine whether pregnant women infected with *T. gondii* exhibit different levels of cytokines than uninfected women by examining the levels of a broad spectrum of cytokines during the first trimester of pregnancy. The secondary aim was to investigate whether individuals with discordant diagnostic test results form a distinct group. Therefore, we compared cytokine profiles among women with discordant, negative and positive test results to determine if the discordant group comprises infected individuals with low levels of specific IgG antibody subclasses and uninfected individuals with high levels of cross-reactive antibodies.

MATERIALS AND METHODS

Participants and procedures

This study took place between March 2018 and June 2019 at the private gynecology clinic Profigyn in Prague on the Czech

Table 1. Serum cytokine levels (pg/ml) in women during the first trimester of pregnancy depending on toxoplasmosis status

Toxoplasmosis status	Negative		Positive		Discordant	
N	51		12		13	
	Mean +−	SD	Mean +−	SD	Mean +−	SD
IL-1β	0.46	0.17	0.55	0.13	0.43	0.11
IL-1ra	175.23	127.97	210.88	80.41	141.07	35.46
IL-2	4.58	2.27	5.97	1.99	4.23	2.45
IL-4	2.60	0.91	3.11	1.18	2.58	1.01
IL-7	17.22	5.04	19.50	6.39	15.90	6.09
IL-9	137.98	18.43	142.89	20.09	133.22	9.79
IL-17A	15.71	3.26	17.78	3.65	14.84	2.02
Eotaxin	56.59	20.69	66.16	29.02	50.36	18.40
FGF basic	27.83	6.11	31.10	2.95	25.22	2.74
G-CSF	271.47	69.22	292.62	85.35	263.00	69.57
IFN-γ	4.93	2.77	5.39	1.57	3.89	1.00
IP-10	801.89	416.55	739.89	260.61	762.38	610.93
MCP-1	43.78	23.65	57.29	37.34	38.59	13.58
MIP-1α	2.28	1.63	2.05	0.59	1.70	0.38
MIP-1β	134.07	15.92	132.32	15.01	130.45	11.07
PDGF-BB	5109.6	1984.9	6323.9	1784.3	3939.1	1751.3
RANTES	27132	30659	45990	49650	17748	7592
TNF-α	24.80	6.58	27.27	5.67	19.96	3.37

population. It was part of a larger longitudinal project focusing on women's nausea during pregnancy (Kaňková et al. 2022, Dlouhá et al. 2023). When entering the study, the women received information about the study, signed an informed consent form and filled out a demographic and health questionnaire. Seventy-eight pregnant women aged 22 to 41 (mean age = 32.2 years, SD = 4.4) without serious health problems participated in this study. Of these women, 68 became pregnant naturally, and 10 through artificial insemination. Forty-two women (54%) were primiparous, and the same fraction of women (54%) were expecting a male foetus. Between the 9th and 14th week (mean = 10.3, SD = 0.9) of pregnancy, the women's blood was taken to examine antibodies against *Toxoplasma gondii* and the levels of 27 cytokines. Blood sampling was carried out uniformly for all women as part of the first-trimester screening – all women came to the gynecology clinic in the morning on an empty stomach.

After collection, the samples were centrifuged for 2 minutes at 3,000 rpm, and the blood serum was transferred to several eppendorf tubes and frozen at -18°C. Blood serum was taken for examination for toxoplasmosis every week due to the possible rapid detection of acute toxoplasmosis in pregnant women. Fifty-one women (65%) were *Toxoplasma*-negative, 12 (15%) were *Toxoplasma*-positive and 15 (19%) were *Toxoplasma*-discordant (for more information, see *Toxoplasma* analysis). Twenty women reported that they had recent health problems (runny nose, cough, cold, flu, and angina) in the last two weeks before blood collection.

This study was approved by the Institutional Review Board of the Faculty of Science, Charles University (Approval No. 2018/6 and 2019/10).

Toxoplasma analysis

Examination for toxoplasmosis was carried out at the National Reference Laboratory for Toxoplasmosis at the National Institute of Public Health in Prague using two serological tests simultaneously, the complement fixation test (CFT) and the ELISA IgG (Immunoglobulin G) test (both tests from TestLine Clinical Diagnostics, Brno, Czech Republic). In the case of the ELISA IgG test, positivity meant an optical density above 200. Positivity in the case of the CFT test was a titre of 1 : 8 and greater. We classified individuals with negative results on both tests as *Toxoplasma*-negative, those with positive results on both tests as *Toxoplasma*-positive, and those with contradictory results as *Toxoplasma*-discordant. In all discordant cases, the result of the CFT was positive, and the result of the ELISA test was negative. If a woman had an inconclusive result, her blood was retaken a few months later for repeated testing. The results of these repeated tests were the same as before, except for two women who had negative results for both tests during the retesting. Based on the retesting results, these two women were excluded from the analysis.

In the case of high levels of IgG antibodies or discordant results of both tests, tests for IgM and IgA antibodies (ELISA, TestLine Clinical Diagnostics) were also performed since high levels of IgM and IgA antibodies indicate acute toxoplasmosis and recent infection. In our study, no women had acute toxoplasmosis.

Cytokine analysis

Cytokines were analysed from blood serum at the Institute of Endocrinology in Prague using a multiplex bead-based suspension array system (xMAP Technology, Luminex Corp.) with the Bio-Plex Pro Human Cytokine 27-Plex panel (Cat. No.: M500KCAF0Y, Bio-Rad, Prague, Czech Republic). Blood serum was stored uniformly at the same temperature (-18°C) for an average of 508 days (293–703 days, SD = 116) before cytokine testing. All samples were analysed on a single plate in a single run. Concentration data were evaluated by five-parameter logistic regression in Bio-Plex Manager software v. 6.1.1 (Bio-Rad). Nine out of 27 cytokines were excluded from the analysis (IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, GM-CSF, and VEGF) because their levels were below or above the detection limit in most examined women. In some samples, the cytokine levels were very low but detectable (IL-1β: N = 8; IL-2: N = 21; IL-7: N = 1; FGF-basic: N = 2; RANTES: N = 9). Concentration readings for these samples were done by extrapolation of the calibration curve for low values. For cytokine level characteristics used in the analysis, see Table 1.

Statistics

The data were analysed using Jamovi 2.3.28 (The Jamovi project 2022) and are available in the online open-access repository Figshare (https://doi.org/10.6084/m9.figshare.28099748.v1).

The Kendall correlation was used to test the relationship between anti-*Toxoplasma* antibody levels. In the analysis of the associations between parity, child sex, method of conception (natural conception or assisted reproduction), and recent health problems with toxoplasmosis status, Fisher's exact test was applied. The association between the age of women and toxoplasmosis status was analysed using the ANOVA. The relationships between the age of women, gestational age at the time of blood sampling, and storage time, parity, child sex, recent health problems and conception method with cytokine levels were tested using the Kendall correlation.

Exploratory factor analysis with oblimin rotation and maximum likelihood extraction method was used to determine the main factors in cytokine levels. The number of factors was determined based on parallel analysis. The assumptions of tests (Barttlet's test of sphericity and KMO measure of sampling adequacy) have been met. The partial Kendall correlation controlling for

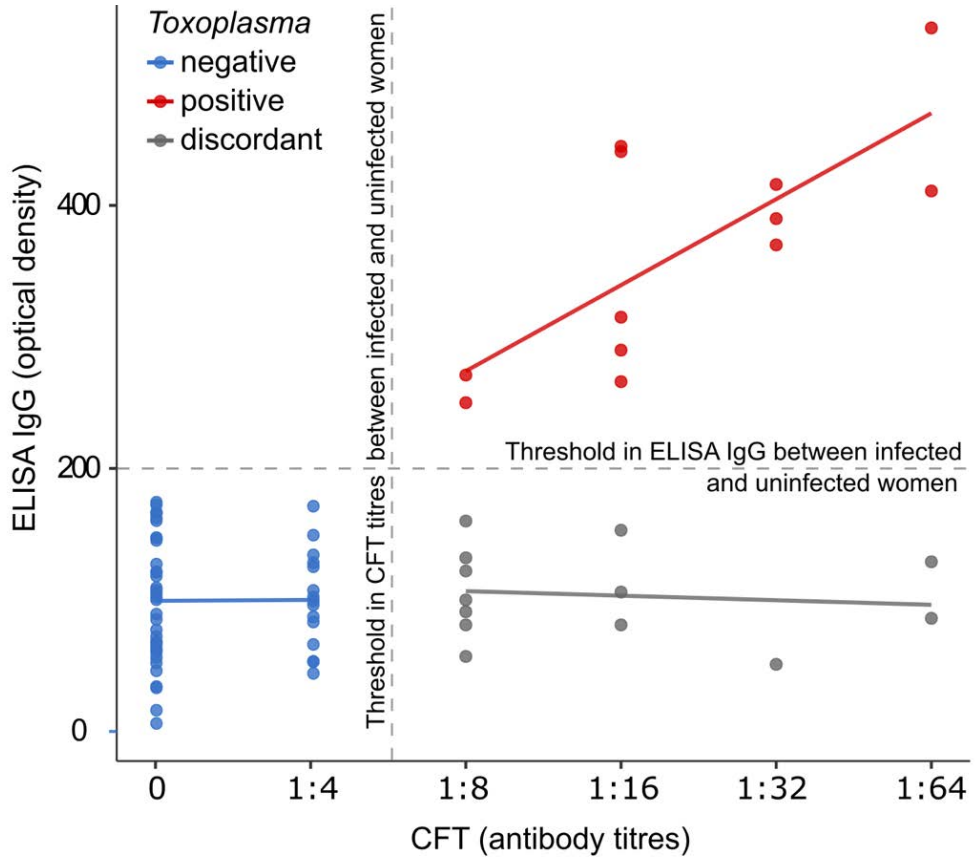

Fig. 1. Correlation between CFT titres and ELISA IgG concentration in *Toxoplasma*-negative, *Toxoplasma*-positive, and *Toxoplasma*-discordant women.

age, method of conception and storage time was used to analyse the relationship between factor scores and toxoplasmosis status and the associations between toxoplasmosis status and cytokine levels. Stouffer's method, which considers the significance of individual tests, was used to determine the overall significance of differences in cytokine levels. Correction for multiple testing was applied using the Benjamini-Hochberg procedure with a false discovery rate pre-set to 0.1.

RESULTS

Descriptive statistics

Antibody levels measured by ELISA IgG and CFT were significantly positively correlated in *Toxoplasma*-positive women (Tau = 0.534, p = 0.016) but not in *Toxoplasma*-negative (Tau = 0.001, p = 0.990) or *Toxoplasma*-discordant women (Tau = -0.094, p = 0.655) (Fig. 1).

Parity (p = 0.486), child sex (p = 0.486), method of conception (p = 1.000) and recent health problems (p = 0.635) were not significantly associated with toxoplasmosis status. However, association between the age of women and toxoplasmosis status approached statistical significance (F = 2.92, df = 2, p = 0.060), with *Toxoplasma*-discordant women being non-significant younger (mean age = 29.9 years, SD = 3.0) than *Toxoplasma*-negative (mean age = 32.6 years, SD = 4.5, t = 2.101, df = 73, p_{tukey} = 0.097, Cohen's d = 0.653) and *Toxoplasma*-positive women (mean age = 33.7 years, SD = 3.9, t = 2.241, df = 73, p_{tukey} = 0.071, Cohen's d = 0.897). Due to the large effect sizes, the woman's age was controlled in the following analyses.

Age of women, parity, child sex, gestational age at the time of blood sampling and recent health problems were not significantly associated with cytokine levels after correction for multiple testing (Tables S1 and S2). All cytokine levels were lower in women who conceived artificially, with six nominally significant differences and two additional near-significant trends, but only TNF-α remained significant after correction for multiple testing (Tau = –0.280, p = 0.004; Table S2).

Storage time was significantly positively correlated with the levels of 16 out of 18 cytokines (all except IP-10 and MIP-1β) after correction for multiple testing (Table S1). The method of conception and storage time were controlled in the following analyses.

Testing the main hypothesis – does latent toxoplasmosis affect the concentration of cytokines?

To reduce the dimensionality of the dataset, which included concentrations of 18 cytokines, we first identified two factors using Exploratory Factor Analysis (Table 2). The stronger Factor 1, explaining 29.6% of the variability in cytokine concentrations, was primarily loaded by cytokines associated with Th2 immunity (the antibody-mediated branch of the immune response). The slightly weaker Factor 2, explaining 24.9% of the variability in cytokine concentrations, was primarily loaded by cytokines invol-

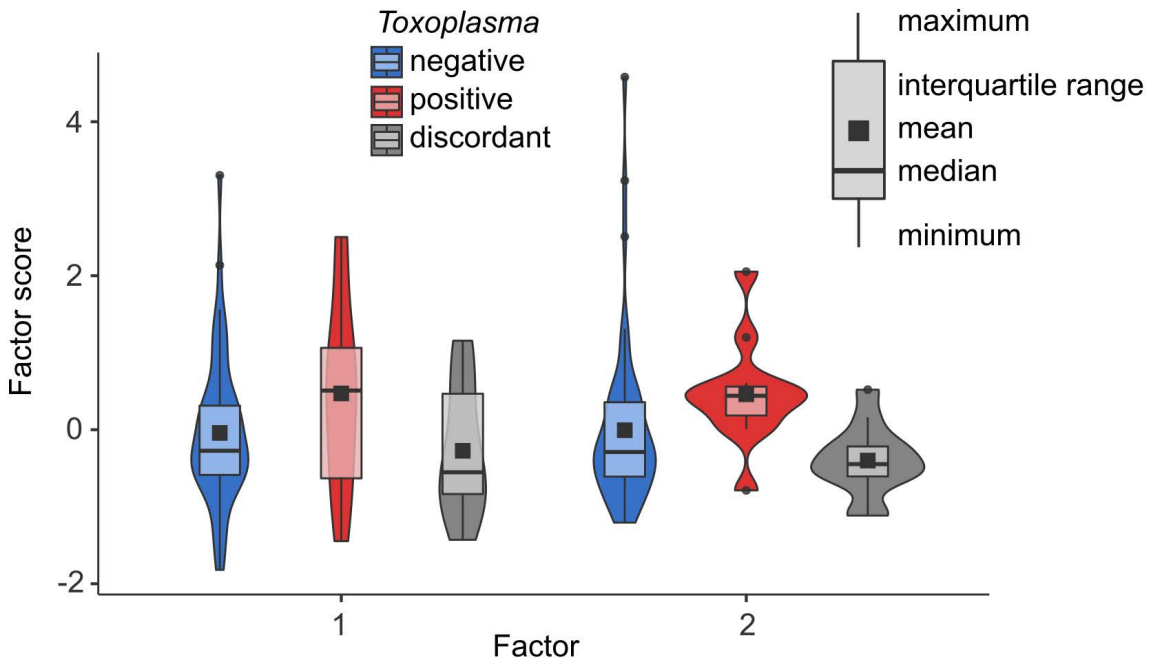

Fig. 2. The differences in factor scores depending on toxoplasmosis status.

Table 2. Loadings of individual cytokines on the factors extracted by factor analysis.

	Factor 1	Factor 2
IL-1β	0.471	**0.547**
IL-1ra	-0.189	**0.756**
IL-2	0.418	**0.509**
IL-4	**0.916**	0.078
IL-7	**0.677**	0.204
IL-9	0.022	**0.380**
IL-17A	**0.859**	0.067
Eotaxin	**1.056**	-0.186
FGF basic	0.329	**0.446**
G-CSF	**0.856**	0.056
IFN-γ	0.017	**0.864**
IP-10	-0.063	**0.325**
MCP-1	**0.465**	0.463
MIP-1α	0.085	**0.324**
MIP-1β	0.055	**0.370**
PDGF-BB	**0.374**	0.195
RANTES	-0.108	**0.797**
TNF-α	0.191	**0.673**
Partial % of variance	29.6	24.9
Cumulative % of variance	29.6	54.5

The highest loading for each cytokine is presented in bold.

ved in Th1 immunity (the cell-mediated branch of the immune response).

The results of a partial Kendall correlation controlled for age, method of conception and storage time between factor scores and *Toxoplasma* groups showed that *Toxoplasma*-positive women did not differ in Factor 1 (Tau = 0.052, p = 0.557) but differed significantly from *Toxoplasma*-negative women in Factor 2 (Tau = 0.203, p = 0.022). Similarly, the discordant group significantly differed from *Toxoplasma*-positive women in one of two factors (Factor 2 score: Tau = -0.432, p = 0.005). No significant differences were found between *Toxoplasma*-negative and *Toxoplasma*-discordant women. All results are shown in

Table 3. Partial Kendall correlation between factor scores of factor analysis and toxoplasmosis status.

Toxoplasmosis status	Negative/positive		Negative/ discordant		Positive/ discordant	
N	63		64		25	
	Tau B	p-value	Tau B	p-value	Tau B	p-value
Factor 1 score	0.052	0.557	-0.087	0.322	-0.102	0.508
Factor 2 score	0.203	**0.022***	-0.103	0.243	-0.432	**0.005***

Controlling for age, method of conception, and storage time. *Toxoplasma* groups were coded as 0 for negative, 1 for positive, and 2 for discordant. Significant p-values are presented in bold.
*p-values significant after correction for multiple tests.

Table 3. The differences in factor scores depending on toxoplasmosis status are graphically represented in Fig. 2.

Exploratory *post hoc* analysis – which cytokines are responsible for the observed effect?

Toxoplasma-positive women had significantly higher levels of IL-1β, IL-1ra, FGF basic and PDGF-BB compared to *Toxoplasma*-negative women. Additionally, IL-2 showed a similar trend (p = 0.078), approaching significance (Table 4). The Stouffer test indicated a significant increase in cytokine levels in *Toxoplasma*-infected women (Z = 3.16, p = 0.002), suggesting a systemic inflammatory response to the infection.

Toxoplasma-discordant women had significantly lower levels of FGF basic, MIP-1α, PDGF-BB and TNF-α than *Toxoplasma*-negative women, and RANTES displayed strong trend (p = 0.051) approaching significance (Table 4). However, the Stouffer test did not show overall lower cytokine levels in *Toxoplasma*-discordant women (Z = -1.57, p = 0.117).

Toxoplasma-discordant women had significantly lower levels of 7 out of 18 cytokines (IL-1β, IL-1ra, FGF basic, IFN-γ, PDGF-BB, RANTES, and TNF-α) than *Toxoplasma*-positive women. Notably, these numerous significant

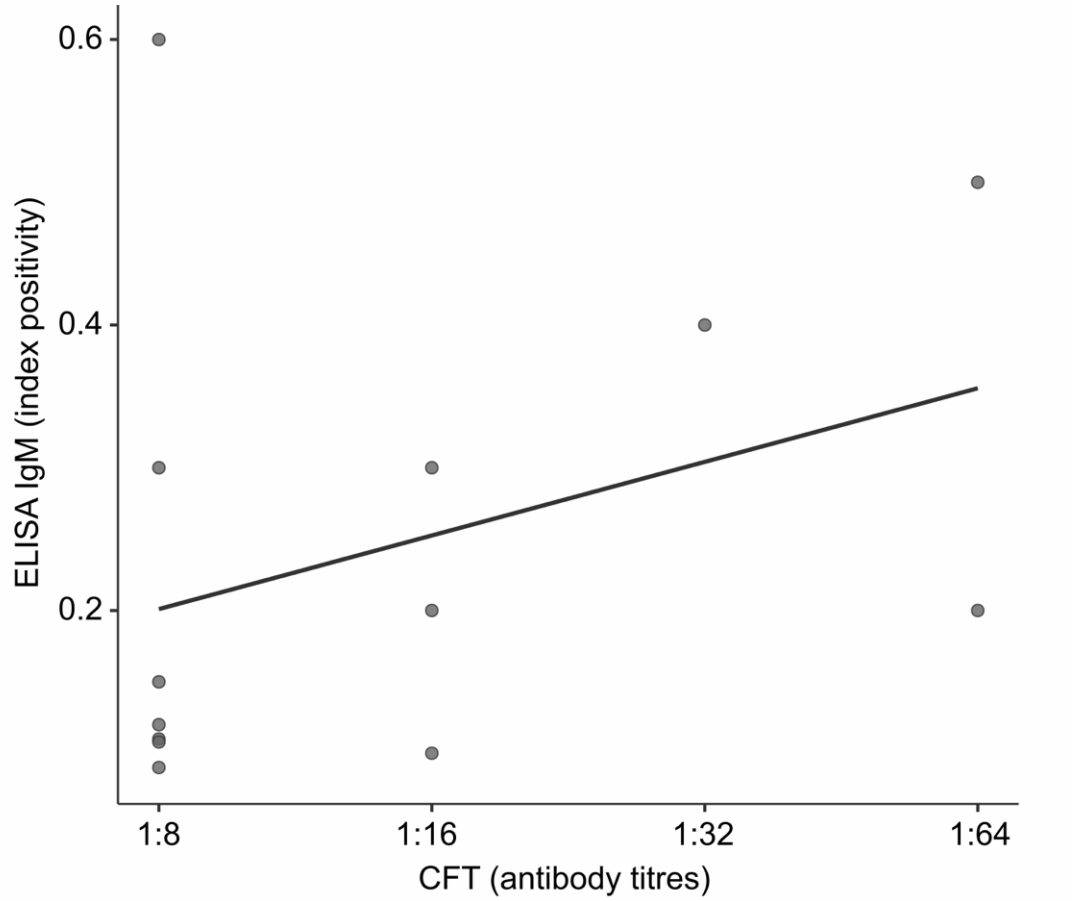

Fig. 3. Correlation between CFT titres and ELISA IgM concentrations in *Toxoplasma*-discordant women.

Table 4. Partial Kendall correlation between toxoplasmosis status and cytokine levels.

Toxoplasmosis status	Negative /positive		Negative /discordant		Positive /discordant	
N	63		64		25	
	Tau B	p-value	Tau B	p-value	Tau B	p-value
IL-1β	0.206	**0.020**	-0.001	0.988	-0.379	**0.014**
IL-1ra	0.197	**0.026**	-0.044	0.615	-0.410	**0.008**
IL-2	0.156	0.078	-0.043	0.627	-0.090	0.556
IL-4	0.067	0.452	-0.023	0.796	-0.019	0.901
IL-7	0.052	0.556	-0.065	0.457	-0.069	0.653
IL-9	0.050	0.569	-0.039	0.658	-0.221	0.150
IL-17A	0.095	0.282	-0.087	0.319	-0.179	0.244
Eotaxin	0.028	0.756	-0.107	0.223	-0.122	0.426
FGF basic	0.224	**0.012**	-0.214	**0.015**	-0.586	**<.001**
G-CSF	-0.015	0.865	-0.055	0.531	-0.050	0.746
IFN-γ	0.110	0.214	-0.110	0.210	-0.382	**0.013**
IP-10	-0.027	0.762	-0.112	0.203	-0.086	0.575
MCP-1	0.107	0.229	-0.004	0.966	-0.156	0.310
MIP-1α	-0.082	0.354	-0.177	**0.044**	-0.117	0.445
MIP-1β	-0.051	0.567	-0.028	0.749	0.018	0.906
PDGF-BB	0.199	**0.025**	-0.196	**0.025**	-0.354	**0.021**
RANTES	0.119	0.179	-0.171	0.051	-0.389	**0.011**
TNF-α	0.060	0.495	-0.269	**0.002**	-0.404	**0.009**

Controlling for age, method of conception and storage time. *Toxoplasma* groups were coded as 0 for negative, 1 for positive, and 2 for discordant. Significant p-values are presented in bold. No corrections for multiple tests (Althouse 2016).

differences were observed even though the comparison between *Toxoplasma*-positive and *Toxoplasma*-discordant groups was conducted on a much smaller sample (25 vs. 64 participants) than the comparison between *Toxoplasma*-negative and *Toxoplasma*-discordant groups (Table 4). The Stouffer test showed that cytokine levels in *Toxoplasma*-discordant women were significantly lower than in *Toxoplasma*-positive women (Z = -4.20 p = 2.70e-5).

Do IgM molecules drive CFT positivity? A correlation analysis

If IgM, not IgG, is the primary contributor to CFT positivity in *Toxoplasma*-discordant women, its levels would be expected to correlate positively with CFT titres. To test this one-sided hypothesis, we examined whether mildly elevated IgM levels below the diagnostic threshold for acute toxoplasmosis were linked to higher CFT titres. A moderate correlation (Tau = 0.333) was found, approaching significance (p = 0.056) in one-sided test. This correlation is graphically represented in Figure 3.

DISCUSSION

In this study, we divided pregnant women into three groups (*Toxoplasma*-negative, *Toxoplasma*-positive and *Toxoplasma*-discordant) according to the relationship between anti-*Toxoplasma* antibody levels measured by ELISA IgG and CFT. The underlying factors explaining variability in cytokine concentrations were identified using exploratory factor analysis of individual cytokine levels. The resulting factor scores were then analysed for their relationship to toxoplasmosis status. *Toxoplasma*-

positive women scored significantly higher on the factor, indicating chronic Th1-type inflammation than *Toxoplasma*-negative and *Toxoplasma*-discordant women. In contrast, *Toxoplasma*-negative women did not differ significantly in their scores on either factor from *Toxoplasma*-discordant women.

Factor 1 (accounting for 29.6% of the variability) does not appear to be associated with latent toxoplasmosis. Its cytokine profile, characterised predominantly by IL-4 and eotaxin, is more consistent with a Th2-driven immune response (Harvanová et al. 2023). While specific cytokines, such as IL-17A, which is not a Th2 cytokine but rather the hallmark of Th17 cells (Bettelli et al. 2007), introduce additional complexity, these variations likely reflect unique immunological adaptations occurring during pregnancy.

In contrast, Factor 2 (accounting for 24.9% of the variability) correlates with latent toxoplasmosis and is primarily loaded with cytokines typically associated with a Th1-driven inflammatory response directed at intracellular parasites (Harvanová et al. 2023). However, not all cytokines contributing to Factor 2 correlated significantly with latent toxoplasmosis. For example, although IP-10, MIP-1α and MIP-1β loaded positively on Factor 2, their correlations with toxoplasmosis status were not statistically significant. It is important to note that these three cytokines are not classical representatives of the Th1 immune response. This suggests that the inflammatory process can be intentionally modulated, potentially through parasite-mediated interventions at key points in the host immune response.

Overall, the cytokine profile observed in pregnant women with latent toxoplasmosis suggests a mild, chronic, cell-mediated inflammatory state maintained in balance by regulatory mechanisms. Elevated levels of pro-inflammatory cytokines (e.g., IL-1β, IL-2, TNF-α) and IFN-γ indicate active Th1-type cellular immunity consistent with a response to an intracellular parasite such as *Toxoplasma gondii*. These cytokines promote the mobilisation and activation of immune cells necessary to keep the parasite under control. At the same time, the presence of IL-1ra (an IL-1 antagonist), elevated levels of certain growth factors (e.g., FGF basic), and chemokines (RANTES) suggest a well-regulated process. IL-1ra attenuates excessive inflammation, FGF basic and other cytokines facilitate tissue maintenance and repair, and RANTES, together with MCP-1, directs the recruitment and positioning of immune cells (Khan 2016, Harvanová et al. 2023).

This scenario reflects a controlled, long-term state of mildly elevated immune activity rather than representing an acute, unrestrained inflammatory response. This balance allows the host to defend itself against the parasite while minimising damage to the mother and foetus. *Toxoplasma gondii* is kept under control allowing the host and parasite to coexist for extended periods of time (Racicot et al. 2014).

Our findings of elevated pro-inflammatory cytokine levels in individuals with latent toxoplasmosis align with those reported in previous studies. For example, the study of the patients of the immunology clinic (Flegr and Stříž 2011) showed that *Toxoplasma*-positive women had a higher number of leukocytes, natural killer cells and monocytes than *Toxoplasma*-negative women. Monocytes are recruited to the site of *T. gondii* infection and participate in resistance against this parasite. Another major mediator of resistance to *T. gondii* is IFN-γ produced by natural killer cells which is involved in killing the parasite and inhibiting its replication (Dupont et al. 2012). Our results also agree with the information from the study about the immune response against toxoplasmosis (Sana et al. 2022). That study reports that IL-1β, IL-2, IFN-γ and TNF-α are in synergistic relationships and play a role in resistance to *T. gondii*.

Evidence of ongoing inflammation has also been observed in animal models in the late stages of infection. For example, the study by Kaňková et al. (2010) demonstrated significant changes in cytokine production and immune response modulation in experimentally infected mice during latent toxoplasmosis. Mice with latent toxoplasmosis showed increased production of IL-12 and IFN-γ and decreased production of IL-2, IL-4, IL-6 and IL-10. The cytokines IL-6, IL-10 and IL-12 were not measured in our study, and while the decreased levels of IL-2 and IL-4 reported in the mouse study are not consistent with our findings, the increased level of IFN-γ corresponds with our results.

These discrepancies may result from key differences between the studies. First, our study focused on pregnant women, whereas Kaňková et al. (2010) examined non-pregnant mice. While latent toxoplasmosis is mainly asymptomatic in humans, mice show pathological changes that often appear in the late stages of chronic infection (Flegr 2010). Second, our study measured baseline cytokine concentrations directly from serum, reflecting systemic immune status, whereas the mouse study assessed cytokine production after *in vitro* stimulation of immune cells. Specifically, only IL-12 levels were measured from serum in the mouse study, while the other cytokines (IL-6, IL-10, and IL-12) were measured from peritoneal macrophages and IL-2, IL-4 and IFN-γ from splenocytes.

This methodological difference could explain the discrepancies, as prolonged exposure of immune cells to elevated cytokine concentrations in vivo may lead to the downregulation of cytokine production capacity. Thus, the results of these two studies are not necessarily contradictory but instead reflect different aspects of cytokine regulation under steady-state *in vivo* conditions versus *in vitro* stimulated conditions.

The authors of another study (Pernas et al. 2014) came to different results. They compared cytokine levels between pregnant women with acute and latent toxoplasmosis and uninfected women from the USA and Colombia. They reported lower levels of cytokines (e.g., IFN-γ, TNF-α, IL1-α, IL-2, IL-4, IL-15, IL-17, Eotaxin, G-CSF) in US women with acute toxoplasmosis compared to uninfected women. Colombian women with acute toxoplasmosis had lower levels of IL-8 and higher levels of IL1-α than uninfected women. There was no significant difference in cytokine levels between latently infected and uninfected US or Colombian women. The authors discuss extensive limitations of that study, including different sample storage and unknown gestational age at which blood was collected.

It is important to have comparable baseline conditions to compare sensitive pregnancy parametres, such as changing cytokine levels.

Another study conducted in Florida (Prescott et al. 2023) measured cytokine levels four times in pregnancy and once in the postpartum period. The study's authors observed lower levels of IFN-γ, IL-6, IL-17 and IL-12 but higher levels of TNF-α in women with latent toxoplasmosis compared to uninfected ones throughout pregnancy. The authors suggest that reduced cytokine levels could reflect a loss of T-cell recognition and effector functions during pregnancy in women with latent toxoplasmosis. However, these results are not consistent with our observation of higher cytokine levels in pregnant women with latent toxoplasmosis, and we currently have no clear explanation for this discrepancy.

As observed in various studies mentioned above, cytokine levels and other immune markers differ between *Toxoplasma*-positive and *Toxoplasma*-negative non-pregnant humans and animals. However, given that pregnancy induces major immunological changes (Peterson et al. 2020), it cannot be excluded that these changes might influence the results of serological tests, including complement-fixation assays. Since this area remains understudied, future research should explore whether pregnancy-related changes influence serological test results in *Toxoplasma*-infected individuals. A particularly interesting approach would be to longitudinally assess immune markers and serological test results in women before and after conception to determine whether pregnancy-related immune modulation contributes to the discordant findings.

An interesting finding of our study was that serum storage time positively correlated with measured levels of 16 of the 18 cytokines (Supplementary Table S1). We currently do not have a definitive explanation for this phenomenon. However, one possible explanation is the gradual release of cytokines from damaged cells or molecular complexes present in frozen serum. The practical implications of this observation are twofold: first, storage at −18°C is unlikely to result in substantial degradation of cytokine molecules; second, it highlights the need to include serum storage time as a covariate in all relevant statistical analyses.

Another factor that likely influenced cytokine levels in our study is the conception method. All 18 proinflammatory cytokines were consistently lower in women who conceived artificially, with five nominally significant differences and two additional near-significant trends, although only TNF-α remained significant after correction. This pattern probably reflects immunosuppressive effects of hormonal treatments or other medical interventions routinely administered during preparation for assisted reproduction, highlighting the importance of accounting for conception method in cytokine analyses.

One aim of this study was also to determine whether *Toxoplasma*-discordant women – diagnosed as infected by CFT but uninfected by IgG ELISA – represent a distinct population or a mixed group of *Toxoplasma*-positive women with low anti-*Toxoplasma* antibody levels and *Toxoplasma*-negative women with high cross-reacting antibody levels. If *Toxoplasma*-discordant women were a mixture, their cytokine levels would fall between those of infected and uninfected women. However, *Toxoplasma*-positive women exhibited elevated levels of five cytokines (one of them approaching significance) compared to *Toxoplasma*-negative women, while *Toxoplasma*-discordant women showed lower levels of five cytokines (one of them approaching significance) compared to *Toxoplasma*-negative women. This suggests that *Toxoplasma*-discordant women are not a mixture of infected and uninfected individuals.

Our results showed that the cytokine levels in *Toxoplasma*-discordant women were similar to those in uninfected women. However, some cytokine levels were lower in *Toxoplasma*-discordant women compared to negative women. A closer examination of the violin plots (see Fig. 2) points to an explanation for these lower cytokine levels. Specifically, it is not the discordant group but the *Toxoplasma*-negative group that appears to be a mixed population. This group consisted primarily of women with cytokine levels similar to those of *Toxoplasma*-discordant women, along with a small subset – possibly just three women – who exhibited much higher cytokine levels, resulting in elevated factor scores. It is important to note that we used non-parametric tests in all our analyses. Therefore, the heterogeneity of the populations had no impact on the results of our statistical tests.

It is highly probable that *Toxoplasma*-discordant women are indeed *Toxoplasma*-negative and share the same (low) cytokine concentration as the main subpopulation of *Toxoplasma*-negative women. However, it remains unclear why they tested positive in the CFT. The CFT differs from the IgG ELISA test by detecting the presence of antibody classes that bind and activate complement, namely IgM and specific subclasses of IgG antibodies (Dimech et al. 2016). It is unlikely that IgG antibodies are responsible for the positivity in the CFT because while there is a strong positive correlation between antibody levels measured by the ELISA IgG test and the CFT in *Toxoplasma*-infected women, this correlation is absent in the *Toxoplasma*-discordant group.

However, there appears to be a non-significant but moderately strong positive correlation (Tau = 0.333) between IgM levels and CFT antibody titres in the group of 13 *Toxoplasma*-discordant women. Despite the small number of women, this correlation was almost significant ($p = 0.056$) in the one-sided test. The contrasting results for IgG and IgM support the one-sided hypothesis that elevated IgM levels are responsible for the increased CFT titres in *Toxoplasma*-discordant women. It is important to note that IgM antibody levels detected by the ELISA IgM test in *Toxoplasma*-discordant women without acute infection are significantly lower (positivity index 0.09–0.6) than in individuals with acute toxoplasmosis, for whom the test is primarily intended.

The binding sites of IgM antibodies typically exhibit 2–3 orders of magnitude lower affinity and much lower specificity compared to IgG antibodies (Eisen 2014). It is plausible to assume that cross-reactive IgM antibodies, potentially targeting unrelated ongoing infections rather than

anti-*Toxoplasma* antibodies, are responsible for false-positive results in CFT. However, this hypothesis is inconsistent with the low cytokine concentrations observed in this subset of women, as well as with the observation that *Toxoplasma*-discordant individuals consistently remain in the discordant category during repeated testing, even months or years after the initial testing.

A more likely explanation is that the IgM antibodies detected are specific anti-*Toxoplasma* antibodies produced by a specialised subpopulation of memory B cells (Weisel and Shlomchik 2017). We can speculate that *Toxoplasma*-discordant individuals were exposed to the parasite in the past but successfully eliminated the infection at an early stage – before the immune system initiated a full IgG response and tissue cyst formation. These individuals may represent a naturally resistant group of people who do not develop chronic *Toxoplasma* infection. This intriguing possibility warrants further investigation, ideally using experimental mouse models to explore the underlying mechanisms of such early immune control.

In this context, it is necessary to note that our analysis testing the association between CFT titres and concentration of anti-*Toxoplasma* IgM antibodies in *Toxoplasma*-discordant women was intended to provide a deeper understanding of the observed phenomena. However, we are aware that we worked with sera in which IgM levels did not reach the values characteristic for acute toxoplasmosis, and that the IgM positivity index can easily be influenced by minor inaccuracies in the testing and evaluation procedure, as well as by non-specific reactions, especially when not all samples were tested in one series.

The main limitation of the present study is the relatively small number of participants. For example, the small sample size of *Toxoplasma*-positive and *Toxoplasma*-discordant women does not allow us to determine why women with very high Factor 2 scores appear in the *Toxoplasma*-negative group but not in the *Toxoplasma*-discordant group. Given the circumstances, chance is the most likely explanation. Only three women had very high scores, and the *Toxoplasma*-negative group was twice as large (51) as the other two groups combined (25). However, it is essential to emphasise that a small sample size can lead only to a failure to detect an existing association (a Type II error). Still, it cannot identify a non-existent association (a Type I error). Therefore, all our findings concerning the significantly higher levels of cytokines in *Toxoplasma*-positive women remain robust, even given the relatively limited number of infected women.

Another issue with this study is that it was conducted on a specific population– pregnant women. While there is an increased proportion of *Toxoplasma*-discordant individuals among pregnant women, they also appear in comparable numbers in other populations. Until this study is replicated in different groups, it is difficult to determine how much our findings can be generalised.

Another limitation of this study is that IgM antibody levels were only measured in the *Toxoplasma*-discordant women or *Toxoplasma*-positive women with high levels of IgG antibodies or elevated CFT titres (to rule out acute toxoplasmosis). As a result, IgM antibody levels were measured in only 13 *Toxoplasma*-discordant and two *Toxoplasma*-positive women in our study. In future studies specifically designed to address the nature of specific subpopulations of *Toxoplasma*-negative individuals with specific anti-*Toxoplasma* IgM antibodies in sera, it would be valuable to measure IgM antibody levels across all groups. Comparing IgM levels between these groups could further clarify the findings of our study.

In this study, we revealed that the enigmatic subpopulation of individuals who test negative in IgG ELISA and positive in CFT for latent toxoplasmosis consists of *Toxoplasma*-negative individuals with detectable levels of IgM antibodies that react with *Toxoplasma* antigens. These levels correlate with CFT titres but do not reach the values typically observed in individuals with acute toxoplasmosis. In the future, it would be interesting to verify whether a similar subpopulation exists in rodents and to what extent these individuals are protected against infection by the parasite *T. gondii*.

However, the most significant finding of our study was that women with latent toxoplasmosis had markedly elevated levels of many studied cytokines despite showing no apparent symptoms. This suggests that they may be experiencing a state of chronic inflammation. Chronic inflammation has numerous adverse effects on both physical and mental health. It could help explain why individuals with latent toxoplasmosis exhibit poorer health across several parameters (Flegr and Escudero 2016), have a more severe course of COVID-19 (Flegr 2021), and show a higher incidence of a wide range of physical and mental health disorders (Flegr et al. 2014).

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