Effect of *Toxoplasma gondii* on the Expression of Cytotoxic T-Lymphocyte-Associated Protein 4 in Women with Toxoplasmosis

Sura Abbas Sultan[1], Azhar Hatif Al-Kuraishi[1], Ali Abdul Hussein Mahdi[2]

[1]Department of Microbiology, College of Medicine, Al-Mustansiriyah University, Baghdad, Iraq, [2]Medical Laboratory Science Technology, College of Health and Medical Technology, Middle Technical University, Baghdad, Iraq

Abstract

Background: Toxoplasmosis is a disease caused by an obligatory intracellular protozoan parasite (*Toxoplasma gondii*). Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a protein receptor that acts as an immunological checkpoint and reduces immune responses. **Aims:** The present study aimed to evaluate the expression of CTLA-4 protein in women with different toxoplasmosis stages and observed its role in the occurrence of abortion. **Materials and Methods:** Anti-*Toxoplasma* immunoglobulin M (IgM) and IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) test in the sera of 116 patients with toxoplasmosis (46 aborted infected groups, 35 pregnant infected groups, and 35 married infected groups). Furthermore, this test was done for 70 control samples (35 aborted women without toxoplasmosis infection and 35 healthy women). As a final point, all samples were examined to measure the level of CTLA-4 using ELISA kits (MyBioSource. Com., Ltd., USA). **Results:** Results showed that a higher level of CTLA-4 was seen in the aborted women group 34 (73.9%) than in other infected groups, and the highest CTLA-4 levels were seen in IgG positive, 20 (43.47%) of 46 in the same group, than other types of antibodies. Furthermore, the highest concentration (948.2 ± 269.4 ng/ml) was indicated in the same group and type of antibodies, with a cutoff value of 597.0 ng/ml (71.7% sensitivity and 97.1% specificity). **Conclusions:** It can be concluded that the CTLA-4 level can be used as a predicted and supported factor for the occurrence of abortion in women infected with toxoplasmosis, especially in the chronic type of the disease (IgG-positive) with a cutoff value of 597.0 ng/ml with 71.7% sensitivity and 97.1% specificity.

Keywords: Abortion, cytotoxic T-lymphocyte-associated protein 4, toxoplasmosis

Introduction

The parasite *Toxoplasma gondii* may infect a wide range of warm-blooded hosts including humans, making it a highly zoonotic parasite. When it comes to humans, this illness is normally asymptomatic in healthy people, but it can potentially cause severe eye or brain damage. The baby is most at risk if the mother becomes infected in the third trimester, but the earlier in the pregnancy the infection occurs, the more serious the outcome for the baby. Many early infections end in stillbirth or miscarriage.[1] Acute toxoplasmosis is a leading cause of miscarriage, affecting anywhere from 14% to 77% of those who are pregnant.[2] Infants who survive may have issues such as seizures, enlarged liver, and spleen, yellowing of skin and eyes (jaundice), or severe eye infections. Some effects are not seen at birth and may occur in their teen years or later.[1]

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Detection of *T. gondii* infection was done using serological tests that identify specific antibody immunoglobulin M (IgM) and IgG titer to distinguish between active and previous infection. Furthermore, specific IgG antibodies which usually persist for life, while IgM antibodies begin to decline after several months, but may in some cases persist for over a year.[3]

T. gondii has successfully established strategies to evade the host's immune response and reach immune-privileged

Address for correspondence: Ms. Sura Abbas Sultan, Department of Microbiology, College of Medicine, Al-Mustansiriyah University, Baghdad, Iraq.
E-mail: sura.abbsultan@gmail.com

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places. These immune evasion strategies are contributing factors to parasite dissemination throughout the host to reach the brain and retina, crossing crucial hosts' barriers. Both parasite and host have developed several strategies to decrease damage immediately after infection such as interfering with cell-autonomous immunity, cell signaling, and also blocking apoptosis allowing infected host cells to remain alive. Furthermore, controlling the dissemination of parasites over metastatic-simile mechanisms, allows the parasite to spread to immune-restricted sites, where it remains in a low proliferative state, with little damage to the host.[4] Furthermore, *T. gondii* can manipulate host immunity through the control of host gene transcription and dysregulation of signaling pathways that result in the modulation of cell adhesion and migration, the secretion of immune-regulatory cytokines, the production of microbicidal molecules, and apoptosis to avoid immune clearance.[5] One of the immune regulatory molecules is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), CD152, a protein receptor that functions as an immune checkpoint and downregulates immune responses.[6] CTLA-4 is constitutively expressed in regulatory T-cells, but only upregulated in conventional T-cells after activation. It acts as an "off" switch when bound to CD80 or CD86 on the surface of antigen-presenting cells.[7] CTLA-4 attach to CD80/CD86 co-stimulatory molecules with antigen-presenting cell, which inhibits stimulatory signals.[8,9] In response to antigen stimulation, the protein is elevated on T-cells. However, constitutive expression among CD4+T-cells is restricted to regulatory CD4+ and CD25+T-cells.[10] Its expression is favorably linked with the release of anti-inflammatory cytokines, showing the immunosuppressive activity of CTLA-4 in the maternal–fetal interface.[11] CTLA-4 deficiency reduces the activity of T-regulatory cells.[12]

Several reports suggest that CTLA-4 acts as a negative regulator of T-cell activation rather than as a positive co-stimulatory molecule. Indeed, cross-linking of CTLA-4 molecules on the cell surface results in decreased T-cell proliferation and IL-2 production. Conversely, blockade of CTLA-4 interactions using anti-CTLA-4 mAbs increases T-cell responses *in vitro* and leads to the rejection of implanted tumors in murine models *in vivo*. Furthermore, in previously activated human T-cells, cross-linking of CTLA-4 has been reported to induce apoptosis, perhaps upon binding to a ligand different from B7-1 to B7-2.[13]

CTLA-4 expression is downregulated in T-regulatory cells from pregnant mice infected with *T. gondii*, whereas inflammatory cytokines are upregulated.[14] Anti-*T. gondii* antibody-positive pregnant women showed a large increase in CTLA-4 cells, but this increase did not pose a danger of reinfection, as there have been no reports of reactivation of the infection in the final weeks of pregnancy.[15]

Therefore, the present study aimed to evaluate the expression of CTLA-4 protein in women with different toxoplasmosis stages, in another meaning with different anti-*Toxoplasma*-positive antibodies.

Materials and Methods

The study was planned as a case–control study.

Subjects and samples

One hundred sixteen blood samples were collected from women with toxoplasmosis; the *T. gondii* IgM/IgG cassette test was used as a screening test for infection with *T. gondii*, through the period from December 2021 to April 2022. Blood samples were collected from women who had reviewed the Obstetrics and Gynecology Department of Al-Hakeem Hospital and many private laboratories in Baghdad City (based on geographic area). The 116 blood sample cases between ages ranging from 20–30 years were divided into three groups: 46 aborted groups, 35 pregnant groups (none aborted), and 35 married groups (none pregnant-none aborted). In addition, 35 were obtained from aborted women (without toxoplasmosis infection) used as the aborted control group, and 35 samples from apparently healthy women were used as a healthy control group. Five ml of venous blood were collected from each subject (patients and control), and the serum was separated and then stored at −20°C until it was used.

Serological tests

The sera of all samples (patients and control) were tested for the presence of specific IgM and IgG antibodies of *T. gondii*, through enzyme-linked immunosorbent assay (ELISA) kits that had supported by MyBioSource. Com., Ltd., USA and applied the test according to the manufacturer's instructions. Furthermore, serum levels of CTLA-4 were measured in each serum of patients and controls using an ELISA kit (MyBioSource. Com., Ltd., USA). Finally, statistical analysis was applied.

Statistical analysis

The data were carried out using the available statistical package of SPSS-27 (Statistical Package for the Social Sciences version 28) (https://www.ibm.com/spss), whenever the $P \leq 0.01$, and the receiver operating characteristic (ROC) curve technique to determine the "cutoff value" which of optimum sensitivity and specificity for diagnosing disease. Area under the curve of the ROC was explanted as follows, perfect (0.9), good (0.8), fair (0.7), poor (0.6), and failure 0 > 0.6. The sensitivity and specificity were calculated according to the following equations:

Sensitivity = (True Positive/Total Disease) × 100

Specificity = (True Negative/Total Healthy) × 100

The significance of differences between of different means (quantitative data) was tested using the Students' *t*-test for the difference between two-independent means or the ANOVA test for the difference between more than two-independent means. The significance of differences of different percentages (qualitative data) was tested using

the Pearson Chi-square test with the application of Yate's correction or Fisher exact test whenever applicable. Statistical significance was considered whenever the $P \leq 0.05$.

Authorizations and ethical approvals

All authorizations and permissions were obtained before starting work in hospitals including patients' ethical approval for samples and data collection.

Results

According to the type of *Toxoplasma* antibodies in the blood serum, the results showed the presence of IgM antibodies in 12 (10.3%) out of 116, IgM and IgG antibodies in 29 (25.0%) out of 116, and IgG antibodies in 75 (64.7%) out of 116, in different toxoplasmosis infected women groups [Table 1]. The presence of toxoplasmosis antibodies in each group, the results exhibited that IgM was present in12 (26.1%) of 46 aborted groups, and no sample gave positive results in both pregnant and married groups. While the presence of both IgG and IgM was present in 12 (26.1%) of 46 aborted groups, 11 (31.4%) of 35 pregnant groups, and 6 (17.1%) of 35 married groups. Furthermore, the presence of IgG alone was indicated in 22 (47.8%) of 46 aborted groups, 24 (68.6%) of 35 pregnant groups, and 29 of 35 (82.9%) married groups, as explained in Table 1.

Concerning to the CTLA-4 levels, the results showed that CTLA-4 with a normal level in toxoplasmosis women was seen in 70 (60.3%) as compared with 69 (98.6%) in controls groups, while a high level of CTLA-4 was seen in 46 (39.7%) in toxoplasmosis groups and 1 (1.4%) in the control groups. These differences were highly significant ($P < 0.0001$) [Table 2].

According to the toxoplasmosis groups, the result showed the normal levels of CTLA-4 in the aborted group were seen in 12 (26.1%), 25 (71.4%) in the pregnant group, and 33 (94.3%) in the married group, while the high levels were seen in 34 (73.9%) of the aborted group, in 10 (28.6%) of the pregnant group, in 2 (5.7%) of the married group, and in 1 (2.9%) of the in the healthy control group. The results indicated that the level of CTLA-4 was higher in the aborted infected group (73.9%) than in other studying groups. This correlation was a highly significant difference ($P < 0.0001$), as notated in Table 2.

As regards to the types of *Toxoplasma* antibodies, the result disclosed the normal level of CTLA-4 in toxoplasmosis women with IgM antibodies was seen in 9 (19.56%) aborted groups and no sample gave positive results in both pregnant and married groups. Samples with IgM and IgG antibodies showed 15 with normal values, which were distributed as follows: 1 sample (2.17%) in the aborted group, 8 (22.86%) in the pregnant group, and 6 (17.14%) in the married group. Furthermore, the normal level was also present in 48 samples with IgG positive, which were distributed as follows: 2 (4.34%) in the aborted group, 19 (54.29%) in the pregnant group, and 27 (77.14%) in the married group [Table 3].

The high level of CTLA-4 in toxoplasmosis women with IgM antibodies was present in 3 (6.5%) samples, all of them in the aborted group only. While women with IgG and IgM positive, the results indicated that 14 samples were with high levels distributed as follows: 11 (23.91%) in the aborted group, 3 (8.57%) in pregnant women, and no sample gave high levels in the married group in this type of antibody. While in women with IgG-positive antibodies, there were 27 samples with CTLA-4 high levels that were distributed as follows: 20 (43.47%) aborted, 5 (14.29%) pregnant, and 2 (5.71%) married infected groups. All these results showed that CTLA-4 had the highest levels in the aborted group with IgG-positive antibodies than the other toxoplasmosis groups with different antibody types, and this correlation was highly significant ($P < 0.0004$) [Table 3].

There was a highly significant increase in CTLA-4 serum level 766.4 ± 300.2 ng/ml ($P < 0.01$) in the aborted group, as compared with the levels of the pregnant group 479.1 ± 104.0 ng/ml, married group 433.2 ± 65.2 ng/ml, aborted control 320.3 ± 7.6, and healthy control group 420 ± 81.7 ng/ml, respectively [Figure 1].

Furthermore, the mean values of CTLA-4 concentrations in the studying groups according to the presence of types of toxoplasmosis antibodies (IgM and IgM and IgG and IgG). The high means of CTLA-4 were present in the aborted group; 423.9 ± 119.5, 775.6 ± 147.3, and 948.2 ± 269.4 ng/ml (IgM+Ve, IgM, and IgG+Ve and IgG+Ve, respectively), as compared with controls and other toxoplasmosis infected groups. In addition, the highest one (948.2 ± 269.4 ng/ml) was

Table 1: Distribution of anti-*Toxoplasma* antibodies in different study groups

Groups	Total number	IgM +ve, *n* (%)	IgM +ve IgG +ve, *n* (%)	IgG +ve, *n* (%)	Control, *n* (%)
Toxoplasmosis	116	12 (10.3)	29 (25.0)	75 (64.7)	-
Controls	70	-	-	-	70 (100)
Patient's groups					
Aborted group	46	12 (26.1)	12 (26.1)	22 (47.8)	-
Pregnant group	35	-	11 (31.4)	24 (68.6)	-
Married group	35	-	6 (17.1)	29 (82.9)	-
Control groups					
Aborted control	35	-	-	-	35 (100)
Healthy control	35	-	-	-	35 (100)

seen in IgG+Ve aborted group and these results with highly significant differences ($P < 0.0001$) [Figure 2].

The result in Table 4 suggested the cutoff value of 597.0 ng/ml with 71.7% sensitivity and 97.1% specificity.

Discussion

T-cells produce the protein CTLA-4, to maintain a healthy immune system, which plays a role in immunological control. Thus, cancer cells as well as T-cells are less likely to be destroyed. Competition for ligands, intercellular signals, or both may reduce T-cell activation through the intracellular domain.[16] So far, it is the first study exploring the CTLA-4 concentration level in the serum of toxoplasmosis-infected women by ELISA in Iraq. The present results indicated that there were highly significant differences in that CTLA-4 with a normal level in toxoplasmosis-infected women groups were seen in 60.3%, while a high level of CTLA-4 was seen in 39.7% in the same groups. In addition, the high level was concentrated in 73.9% of aborted toxoplasmosis women group than in other groups. The Allami[17] study was the first to investigate the influence of CTLA-4 +49A/G polymorphism on the susceptibility to toxoplasmosis in Iraq and found that the AG genotype and G allele protect against the disease. There have been several studies showing the influence of intracellular parasites on the expression of CTLA-4, such as those by Martins *et al*.,[18] who found a substantial increase in CTLA-4 expression in infected spleen cells from noninfected mice compared to those from infected animals. The mechanisms bearing the increased and sustained CTLA-4 expression of T-cells induced by *Toxoplasma* infection are not fully understood. The increased CTLA-4 expression could simply be a consequence of T-cell activation caused by the infection. It could also result from the presence of soluble factors secreted by the infected cells, such as cytokines.[18]

When the distribution of CTLA-4 levels was compared according to the type of *Toxoplasma* antibodies in different study groups, a high level of CTLA-4 was seen in the aborted women group with all types of anti-*Toxoplasma* antibodies. As well as, the highest levels were obtained in the aborted women group with IgG-positive antibodies (43.47%) than in the other toxoplasmosis groups with different antibody types. Up to now, there is no research showing the effect of the type of antibodies on the concentration of CTLA-4, especially with toxoplasmosis infection. Most researches that deal with CTLA-4 show its relationship to cancers. There is a strong correlation between

Table 2: The cytotoxic T-lymphocyte-associated protein 4 level in different study groups

Groups	CTLA 4 (ng/mL) (300-550)		
	High (>550 ng/mL), *n* (%)	Normal (300-550), *n* (%)	Total (*n*)
Toxoplasmosis	46 (39.7)	70 (60.3)	116
Controls	1 (1.4)	69 (98.6)	70
P	0.0001*		
Patient's groups			
Aborted group	34 (73.9)	12 (26.1)	46
Pregnant group	10 (28.6)	25 (71.4)	35
Married group	2 (5.7)	33 (94.3)	35
Control groups			
Aborted control	-	35 (100.0)	35
Healthy control	1 (2.9)	34 (97.1)	35
P	0.0001*		

*Highly significant difference at 0.05 level. CTLA 4: Cytotoxic T-lymphocyte-associated protein 4

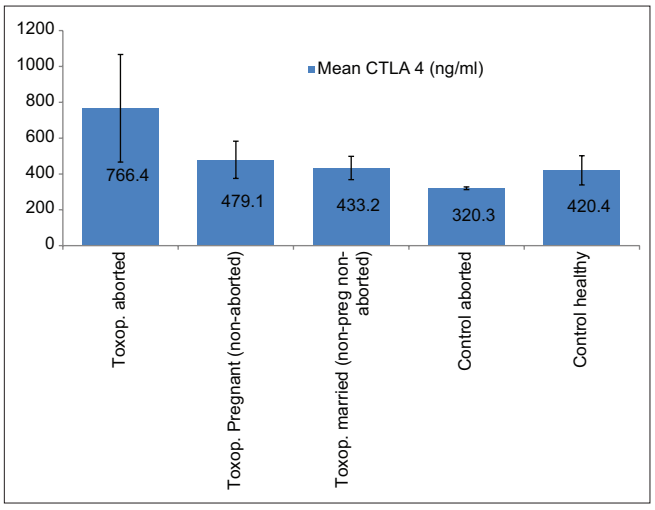

Figure 1: Comparison of mean of CTLA-4 level in different study groups. CTLA: Cytotoxic T-lymphocyte-associated protein 4

Table 3: Distribution of cytotoxic T-lymphocyte-associated protein 4 level according to the type of antibodies of *Toxoplasma gondii* in different study groups

Groups	CTLA 4 (ng/mL) (300-550)							
	Total number	Normal (300-550 ng/mL)			High (>550 ng/mL)			*P*
		IgM +ve, *n* (%)	IgM and IgG +ve, *n* (%)	IgG +ve, *n* (%)	IgM +ve, *n* (%)	IgM and IgG +ve, *n* (%)	IgG +ve, *n* (%)	
Aborted group	46	9 (19.56)	1 (2.17)	2 (4.34)	3 (6.50)	11 (23.91)	20 (43.47)	0.004*
Pregnant group	35	-	8 (22.86)	19 (54.29)	-	3 (8.57)	5 (14.29)	0.674
Married group	35	-	6 (17.14)	27 (77.14)	-	-	2 (5.71)	-
Total number	116	9	15	48	3	14	27	0.0004*

*Highly significant difference at 0.05 level. CTLA 4: Cytotoxic T-lymphocyte-associated protein 4

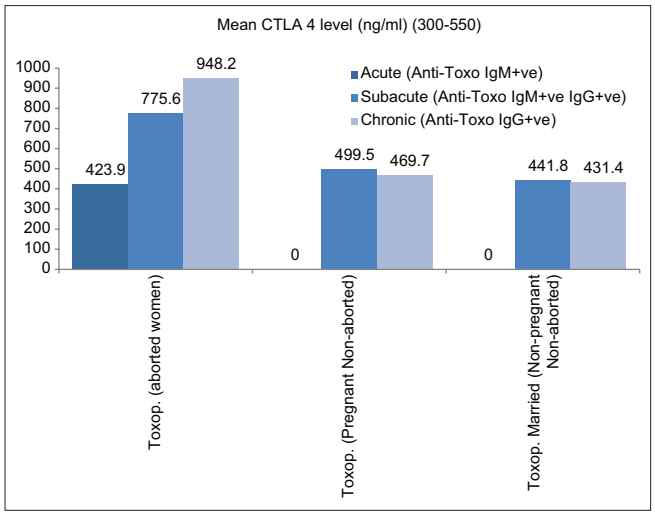

Figure 2: Comparison of the mean values of CTLA-4 concentrations in the infected groups according to the presence of type of *Toxoplasma* antibodies. CTLA: Cytotoxic T-lymphocyte-associated protein 4

cancer and CTLA-4 in the majority of studies. It has recently been shown that activated T lymphocytes produce checkpoints on their surface that inhibit the body's ability to fight cancer. For example, several checkpoint molecules that serve as negative regulators of activated T-cells have been identified, such as CTLA-4, PD-1, TIM-3, LAG-3, B, T-cell lymphocyte attenuator, and others.[11] As combinatorial approaches of CTLA-4 blockade with other therapeutic agents, such as radiation and chemotherapy, have proven to be effective in the regression of tumors in various malignancy murine models, a wide range of cancers, including melanoma, small cell lung cancer, nonsmall cell lung cancer, and prostate cancer, are now being studied using these approaches.[18]

Moreover, there is a highly significant increase in this parameter 766.4 ± 300.2 in the aborted group, as compared with its levels in other infected and control groups when comparing study groups. In fact, this is the first study that measures CTLA-4 serum levels in women with different toxoplasmosis types and comparison that level with each other and with control groups. Gupta *et al*.[19] showed an association of mutant homozygous genotype GG of CTLA4 +49A/G, and AA genotype to play predisposing roles in recurrent miscarriage patients but the limitation of the study remains as they have not estimated the CTLA-4 level and functional component of these polymorphisms. At the maternal–fetal interface in mouse abortion-prone models, Zhou *et al*.[20] detected increased expression of CD80/86 and CD28 and reduced expression of CTLA-4. Immune rejection is caused by the activation of T lymphocytes by co-stimulatory signals sent by CD80/CD86 and their interaction with CD28. In addition, the current study showed a relationship between the level of CTLA-4 and the type of antibodies (IgM and IgM and IgG and IgG), wherever this study recorded a high means of CTLA-4 in the aborted women group as compared with controls and other toxoplasmosis groups. Furthermore, the highest one (948.2 ± 269.4 ng/ml) was seen in IgG antibodies in the same group. As mentioned above, *T. gondii* infection induces both innate and adaptive immune responses. During the late phases of parasite replication, adaptive immunity is essential for parasite replication control. CD8+T-cells are the major defenders of long-term immunity, whereas CD4+T-cells play a crucial role in preventing relapses in the immune response.[21] Anyhow, a strong CD8+T-cell immunity induced during *T. gondii* persists in a chronic state, while CD4+T-cells appear to be involved during the acute phase of infection.[22,23] Since this is the first study that links between CTLA-4 concentration in the aborted and toxoplasmosis, there was only one study conducted by Jin *et al*.,[24] their study analyzed the changes in the quantity of CD4+CD25bright regulatory T-cells and the expression of stimulatory molecules, CTLA-4 and CD28, in the peripheral blood and deciduas in the nonpregnancy, normal early pregnancy, and miscarriage (with any reason). They see an increase in the CD28 mRNA expression was accompanied by a decrease in the CTLA-4 mRNA expression in decidual tissues from human miscarriage. The ratios of CTLA-4+/CD28+ in miscarriage were significantly lower than that of the normal pregnancy both in the peripheral and in deciduas. This may be because Treg cells might play a role in the maintenance of pregnancy through the upregulation of CTLA-4 expression. This study was completely differ from the present study. Furthermore, CTLA-4 intracellular expression was measured by Leng *et al*.[25] and found to be significantly higher in HIV-infected individuals with advanced clinical symptoms or AIDS than in asymptomatic patients, and this was found to be strongly associated with the HIV disease stage (CTLA-4 molecules and CTLA-4+CD4+ cells). Antigen-specific human T-cell death is mediated by CTLA-4 expression, which plays a critical role in maintaining peripheral CD4+ and CD8+ homeostasis. T-cell anergy and apoptosis are significant features of advanced HIV infection and persistent immunological activation, and CTLA-4 plays a crucial role in their development. It may deduce that the level of CTLA-4 in chronic infection is high because of the existence of IgG antibodies.

Eventually, it can be concluded that these three parameters can be used as predicted and supported parameters for the occurrence of abortion in women infected with toxoplasmosis, especially in the chronic type of the disease (IgG-positive)

Table 4: The cutoff values, sensitivities, and specificities for early detection of cytotoxic T-lymphocyte-associated protein 4 levels in the aborted group as compared with the other infected groups

Test result variables	Positive if greater than or equal to	Sensitivity	Specificity
CTLA-4 (ng/mL)	327.5	100	2.9
	454.5	80.4	71.4
	579.0	71.7	97.1
	602.0	69.6	100

CTLA 4: Cytotoxic T-lymphocyte-associated protein 4

with a suggested cutoff value of concentration 597.0 ng/ml with 71.7% sensitivity and 97.1% specificity.

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Conflicts of interest

There are no conflicts of interest.

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