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ORIGINAL ARTICLE

Interaction of *H. pylori* PAMPs with TLR2 and TLR4 in an in-silico and expression-based study in *H. pylori* positive gastric disorders

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ABSTRACT
TLRs play a key role in the pathogenesis of H. pylori infection via the recognition of PAMPs. TLR2 and TLR4 are important in initiating immune responses against H. pylori. The present study aims to elucidate the interaction between TLR2 and TLR4 with H. pylori (pathogen-associated molecular patterns) PAMPs (Flagellin A, Flagellin B, CagA, VacA). Along with the in-silico interaction study, it aims to relate it in terms of mRNA expression in H. pylori positive gastric diseases such as mild gastritis (MG), chronic gastritis (CG), peptic ulcer disease (PUD) and gastric cancer (GC). Docking study was carried out using the available protein data resources in various databases. Expression study was interpreted in terms of fold change. In-silico study showed an affirmative strong interaction by both TLR2 and TLR4 for all of the PAMPs under consideration in terms of negative weighted binding score and amino acid cluster. TLR4 showed higher values for all the PAMPs compared to TLR2 suggesting a stronger and more stable response. In addition, the mRNA expression studies in four patient groups (MG, CG, PUD and GC) showed a downregulated TLR2 and TLR4 expression in CG and PUD compared to MG. However, in GC the expression of TLR2 was downregulated and TLR4 was upregulated indicating a more possible role of TLR4 and H. pylori PAMPs. The TNFα/IL10 ratio showed a more pro-inflammatory state with highest in GC followed by PUD and CG compared to the MG.
Keywords: *H. pylori, PAMPs, TLR2, TLR4, docking, mRNA expression.*

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INTRODUCTION

TLR stands for Toll-like Receptor, a class of proteins that play a key role in the innate immune system by recognizing molecules typically found on pathogens. Studies have shown that TLRs, particularly TLR2 and TLR4, can recognize components of *H. pylori* and initiate immune responses against the bacterium (5; 15). TLR2 and TLR4 are more significant than other members because of their bacterial ligands (20). TLRs play a crucial role in recognizing pathogen-associated molecular patterns (PAMPs), including those derived from *H. pylori*, and triggering immune responses. *H. pylori* PAMPs include components lipoproteins, lipoteichoic acid, peptidoglycan LPS, flagellin and other cytotoxins like CagA and VacA (4). Flagellin, a protein component of bacterial flagella is recognized by TLR. It can bind to flagellin from various bacterial species, including *H. pylori*. *H. pylori* can modify its flagellin to evade TLR recognition or manipulate TLR signalling pathways to promote bacterial survival (6). CagA refers to cytotoxin-associated gene A, a protein produced by the bacterium *Helicobacter pylori*. *H. pylori* is a bacterial species that infects the stomach lining and is associated with various gastrointestinal disorders, including gastritis, peptic ulcers, and even stomach cancer. CagA is one of the major virulence factors of *H. pylori*, and it's delivered into host cells via a type IV secretion system (1). Once inside the host cell, CagA can manipulate various signalling pathways, leading to alterations in cell morphology, proliferation, and inflammation. The interaction between *H. pylori*, particularly its CagA protein, and Toll-like Receptors (TLRs) has been of interest in understanding the immune response to *H. pylori* infection (18). TLR2,

TLR4, have been involved in the *H. pylori* recognition in the stomach, with bacterial factors such as CagA modulating the interaction of the bacterium with TLR, eventually leading to activation of nuclear factor-kappaB (NF-kB) and secretion of inflammatory cytokines (11; 13). However, the specific interactions between TLRs and CagA are still an area of ongoing research. It's suggested that CagA might modulate TLR signalling pathways to evade or subvert the host immune response, contributing to the persistence of *H. pylori* infection and associated diseases. *H. pylori* VacA (Vacuolating cytotoxin A) is another important virulence factor produced by *Helicobacter pylori*. This protein is secreted by the bacterium and can target various cells in the stomach lining, leading to cellular alterations and contributing to the pathogenesis of *H. pylori*-associated diseases. *H. pylori* VacA has been shown to interact with TLRs, particularly TLR2 and TLR4, which are expressed on the surface of various immune cells, including macrophages and dendritic cells. Studies have demonstrated that VacA can activate TLR signalling pathways, leading to the production of pro-inflammatory cytokines and chemokines. The activation of TLRs by VacA may contribute to the host immune response against *H. pylori* infection by promoting the recruitment of immune cells to the site of infection and enhancing the clearance of the bacterium(12). However, chronic activation of TLR signalling by *H. pylori* components, including VacA, can also lead to chronic inflammation and tissue damage, contributing to the development of *H. pylori*-associated diseases such as gastritis, peptic ulcers, and gastric cancer. Overall, the interplay between *H. pylori*, CagA, VacA and Toll-like Receptors represents a complex interaction between bacterial virulence factors and host-pathogen interaction during *H. pylori* infection, with implications for understanding the pathogenesis of *H. pylori*-associated diseases and developing therapeutic strategies to target the immune response against the bacterium. This study aimed to understand the role played by TLR2 and TLR4 in different *H. pylori* positive gastric disorders including mild gastritis, chronic gastritis and peptic ulcer disease.

MATERIAL AND METHODS

For Docking study: The study involved the identification of standard *H. pylori* strains available in the database. For this study the strain *H. pylori* 26695 was chosen for a uniform output. PAMPs considered for the study were Flagellin A, Flagellin B, CagA and VacA. These PAMPs then underwent docking with TLR 2 and TLR 4. Information on the structure of these molecules under consideration were downloaded from various available protein structure database. The structural data in PDB format was obtained from Protein Data Bank for TLR 2 (1FYW) and VacA (6NYF). PDB files for CagA (P55980), Flagellin A (P0A0S1), Flagellin B (Q07911) was obtained from AlphaFold protein structure database. Structural data in PDB format for TLR 4 was constructed using SWISS-MODEL. Protein-protein dockings for each of the Toll like receptor (TLR 2, TLR 4) with Flagellin A, Flagellin B, CagA and VacA considered to be established interactions in in-vivo studies was performed using rigid docking program PIPER based clustering program ClusPro. Finally, the docking results were visualized and analyzed using PyMol version 2.5.7.

TLR expression study: Biopsy samples were collected from *H. pylori* positive patients undergoing endoscopy for gastrointestinal issues such as gastritis including both mild gastritis (MG) & chronic gastritis (CG), peptic ulcer disease (PUD) and gastric cancer (GC) patients All patients provided written informed consent prior to the collection of biopsy samples, and all experiments were approved by the Institutional Ethics Committee of Guwahati Metro Hospitals (Ref. No. GMH/Research/001 dated 20.02.2021). A total of 73 patients with 10 healthy controls were enrolled for the study which included 20 (MG), 19 (CG), 23 (PUD) and 11 (GC). For the expression studies, cDNA was prepared from the RNA isolated from the biopsy samples. Real time PCR analysis was carried out for TLR2, TLR4, TNFα and IL10 as per (8; 16).

Results and discussion:

The results of protein-protein docking analysis in PyMol 2.5.7 showed positive docking for all the PAMPs and TLR combination. The study considered the best model from ClusPro based on the values of Negative Weighted Energy Score and number of members in Cluster. The model analysis in PyMol generated all the polar contacts, Van der Waals and π-interactions for the receptor-ligand docking (Fig. 1, Fig. 2, Fig. 3 & Fig 4).

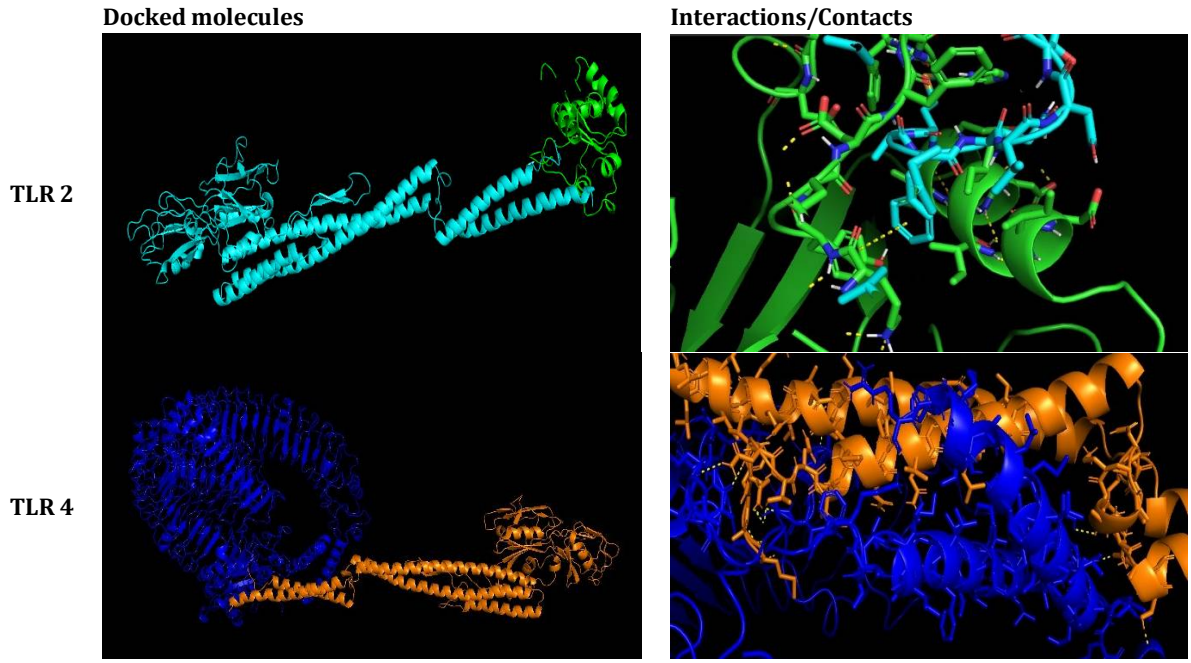

Fig. 1: TLR-Flagellin A interaction (yellow dashes depict interactions)

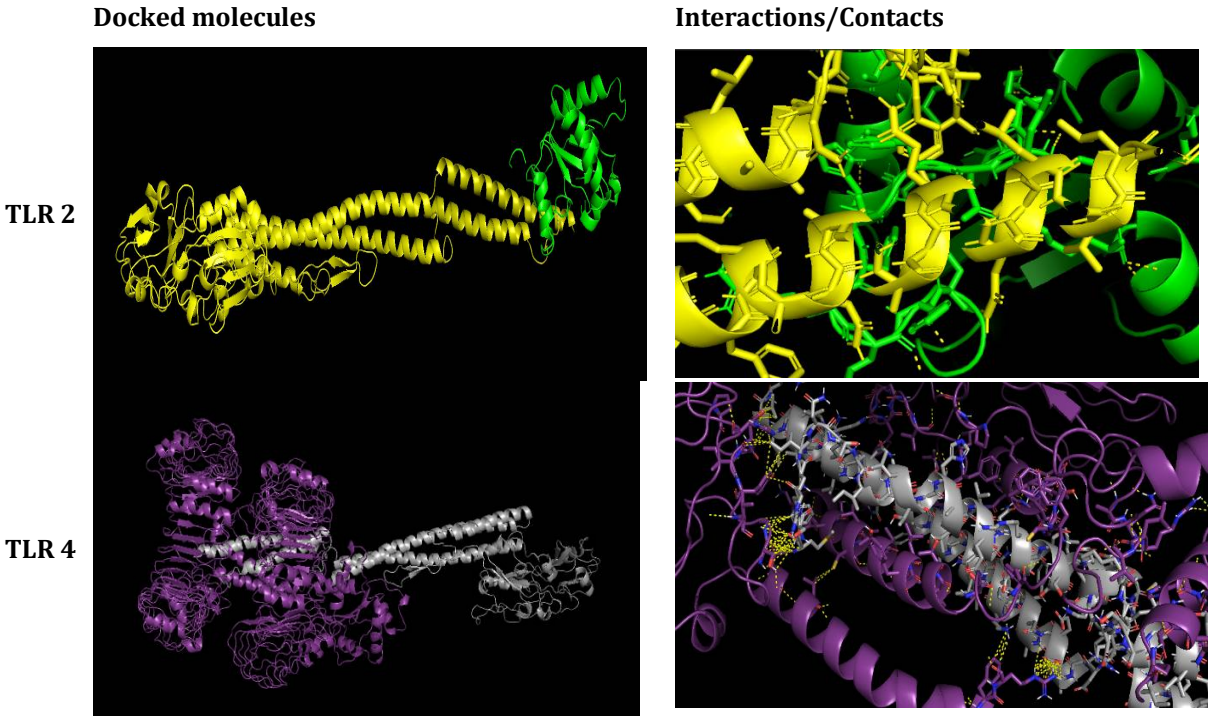

Fig. 2: TLR-Flagellin B interaction (yellow dashes depict interactions)

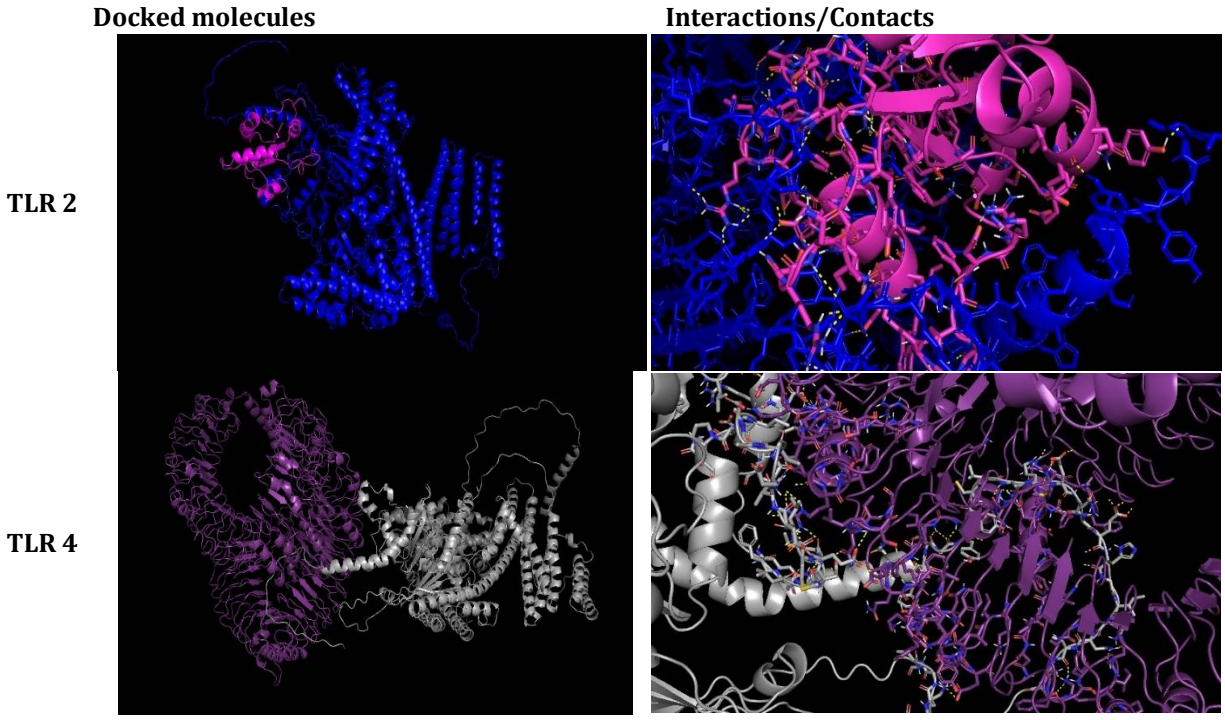

Fig. 3: TLR-CagA interaction (yellow dashes depict interactions)

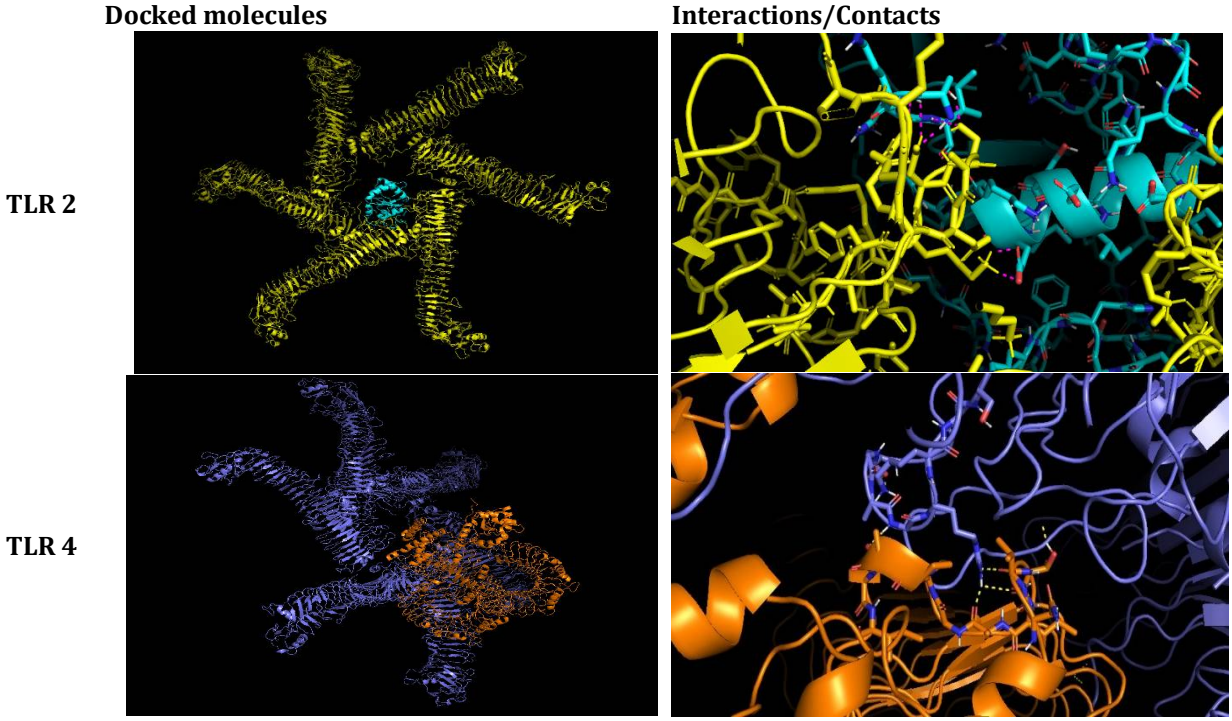

Fig. 4: TLR-VacA interaction (yellow dashes depict interactions)

The TLR-PAMPS interaction yielded a variety of results as given in Fig6 and Fig7. The interactions observed between the TLR receptor and the *H. pylori* PAMPs involved polar as well as pi interactions as given in fig 1, 2, 3 & 4. During TLR-Flagellin A docking, TLR 2 had comparatively higher amino-acid binding clusters (365) with a negative binding energy score of 896.9 than TLR 4 with 77 amino-acid binding clusters with a negative energy of 1030.9. For TLR-Flagellin B docking also, TLR 2 showed highest amino-acid binding cluster (125) with negative energy score of 856.3 compared to 80 amino-acid binding

clusters of TLR 4 with a negative energy score of 1073.9. Docking of the *H. pylori* toxin CagA and VacA with TLRs, also revealed that TLR2 expressed a negative binding energy score for CagA (1666.4) and VacA (985.6) with 63 and 44 amino-acid binding clusters respectively. The interaction between CagA and VacA with TLR4 showed a negative energy score of 1688.30 and 1480.4 respectively but with a less amino-acid binding cluster of 40 for CagA and 16 for VacA. All the data for negative weighted energy score and related members in clusters are shown in fig. 5 & 6.

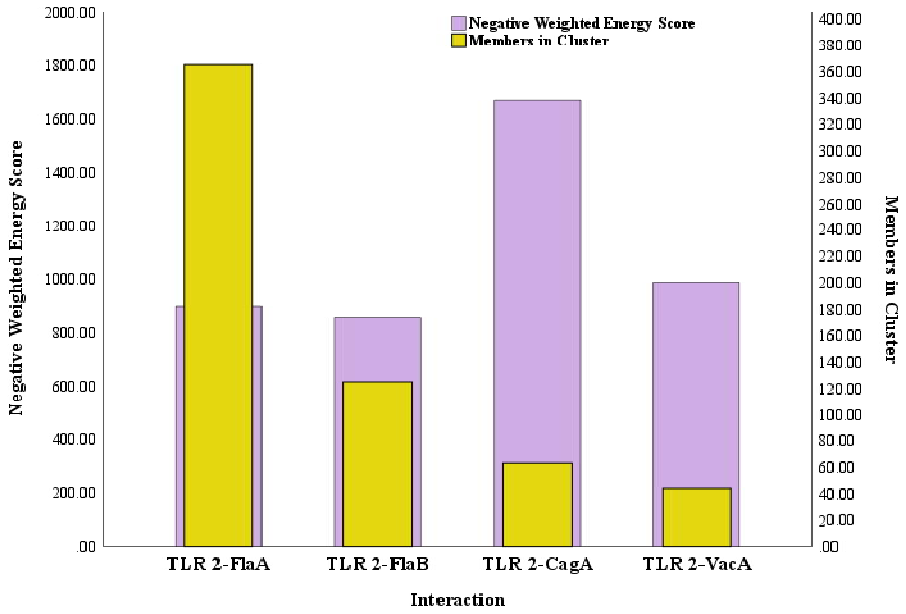

Fig. 5: Negative Weighted Energy Score and Members in Cluster for TLR 2- PAMPs interaction

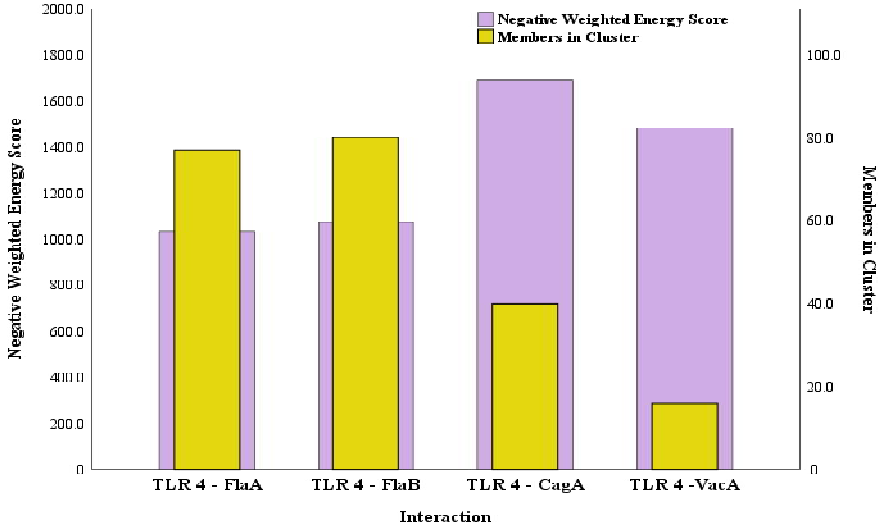

Fig. 6: Negative Weighted Energy Score and Members in Cluster for TLR 4- PAMPs interaction

The patient characteristics recruited for the TLR expression studies are summarized in Table 1.

Table 1: Characteristics of the patients

Group	C	MG	CG	PUD	GC
Total patients	10	23	19	20	11
Total Male (%)	7 (70)	13 (56.52)	13 (68.42)	15 (75)	7 (63.63)
Total Female (%)	3 (30)	10 (43.47)	10 (52.63)	5 (25)	4 (36.36)
Age (mean) ± SD	41.3 ± 12.97	40.15 ± 16.85	40.21 ± 14.76	43.21 ± 13.32	41.5 ± 10.88
Range	27-65	19-73	20-60	26-61	20-54

TLR2 and TLR4 mRNA expression was shown in terms of fold change. The results as in fig. 7 showed that the TLR2 and TLR4 expression was downregulated starting from MG, CG to PUD. However, TLR4 expression was upregulated compared to TLR2 expression in GC patient group. TNFα expressions were similar in all the patient groups however, IL10 expression was decreasing in the order from MG, CG, PUD and GC (fig. 8). The TNFα/IL10 ratio was elevated in GC as well as in CG and PUD compared to MG (fig. 8). Pearson correlation was carried out for finding out the correlation between the expression of TLR2, TLR4, TNFα and IL 10. For TLR2, a significant correlation (r=1.000 & p=0.005) was found between TNFα and TNFα/IL10 ratio in MG group; in PUD group a significant correlation (r= 0.999 & p=0.021) was obtained between TLR2 and TNFα/IL10 ratio; in GC patient group TLR2 was significantly positively correlated (r=1.000 & p<0.01) to TNFα. For TLR4 interaction in MG group, a positive significant correlation (r=1.000 & p=0.005) was obtained between TNFα and TNFα/IL10 ratio; in the GC patient group, TLR4 was significantly positively correlated (r=1.000 & p< 0.01) to TNFα.

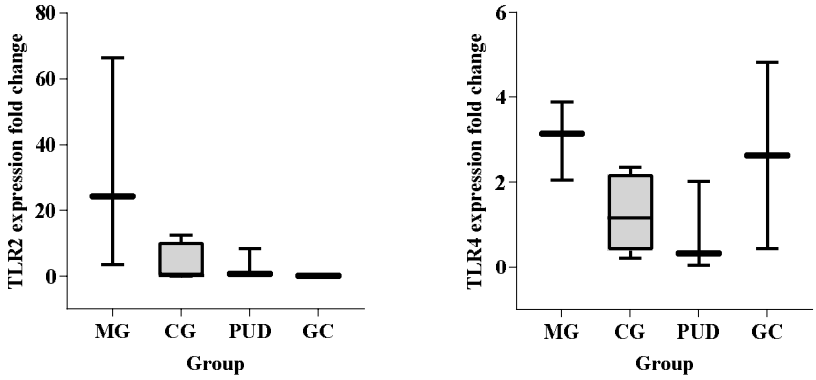

Fig 7: Expression fold change of TLR 2 and TLR 4 in Mild gastritis (MG), Chronic Gastritis (GC), Peptic ulcer disease (PUD) and Gastric cancer (GC)

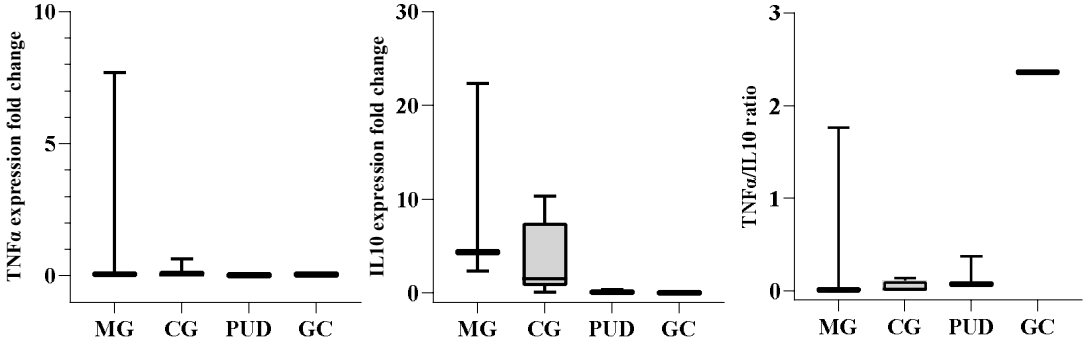

Fig 8: TNFα, IL10 fold change and TNFα/IL10 ratio for MG, CG, PUD and GC

Bacterial lipoproteins and peptidoglycan are recognized by TLR2 and LPS from the majority of Gram-negative bacteria is recognized by TLR4 (18). The in-silico interaction study between TLR and *H. pylori* PAMPs flagellin A and flagellin B showed that TLR4 has more negative weighted binding energy score than TLR2. This indicates that TLR4 has a more stable and stronger interaction with the flagellin A and flagellin B compared to TLR2. For, TLR- CagA and VacA interaction study, it was seen that TLR4 again showed higher negative weighted energy binding score compared to TLR2. Thus, the in-silico results indicated that TLR4 has a comparatively more stable and stronger binding with the *H. pylori* PAMPs. The components of *H. pylori*, such as LPS, flagella, vacuolating toxin A, and cytotoxin associated gene pathogenicity island, have been attributed to the positive link between *H. pylori* and GC. These substances are thought to contribute to the pathogenicity of *H. pylori* by altering host gene expression, causing infection-induced cell proliferation, elongating epithelial cells, breaking down cell-cell junctions, and reducing the production of stomach acid (19). TLRs are expressed in the pattern of Type I transmembrane proteins, and when they are recognized, nuclear factor-kappa β (NF-κβ) is activated, cytokine production is induced, and antigen-presenting molecules are generated. These signalling cascades trigger a number of co-stimulatory molecules that are necessary for immune responses that are

adaptive (3). TLRs are some of the most well-known receptors that detect bacterial components, particularly TLR2 and TLR4 (7). Our expression study showed a higher expression of TLR2 as well as TLR4 in *H. pylori* positive MG patients followed by *H. pylori* CG patients. The GC group showed elevated expression for TLR4 compared to no significant change for TLR2 expression. In a study, the mRNA and expression levels of TLR2, TLR4, IL-1β, TNF-α, and NF-κB in human GC were examined experimentally using qRT-PCR. According to the findings, the GC samples had around twice as much TLR4 expression as the normal stomach samples. But when GC samples were compared to the control group, TLR2 transcripts showed no discernible alterations, which highlights the potential involvement of the interaction between *H. pylori* LPS and TLR4 (20). High levels of pro-inflammatory cytokine production, upregulation of PRRs and co-stimulatory molecules including MD2, and activation of the CXCR7 signalling pathway are all signs of inflammation conditions caused by TLR4 activation (9;14).This can also be said for our results as both in-silico and expression studies TLR4 had stronger binding and upregulated expression. The usual hex acylated form of LPS, which *Campylobacter* produces, can trigger inflammation by binding to TLR4 (2). The development of tumours depends on the generation of inflammatory mediators like IL-8 and TNF-α, which are produced when LPS binds to TLR4 in gastric epithelial cells and activates TLR4 signalling. According to studies, TLR4 is overexpressed in gastric epithelia and is also higher in superficial gastritis monocytes and macrophages in GC patients (17). The degree and duration of an inflammatory response can be influenced by the ratio of TNF-alpha to IL-10 generated in response to TLR2 activation (10).

CONCLUSION

The present study has shown the individual interaction between TLR2 and TLR4 with *H. pylori* PAMPs. The in-silico study was supported by the mRNA expression study which showed the role played by both TLR2 and TLR4 in the stages of gastric pathogenesis from mild gastritis to gastric cancer supported by the downstream TNFα and IL 10 expression leading to a more pro-inflammatory state.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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