Virulent Gamma-Glutamyltranspeptidase Expression by *Helicobacter saguini*,
an Enterohepatic Helicobacter Species Isolated from Cotton Top Tamarins with Chronic Colitis and Colon Cancer

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Virulent Gamma-Glutamyltranspeptidase Expression by Helicobacter saguini, an Enterohepatic Helicobacter Species Isolated from Cotton Top Tamarins with Chronic Colitis and Colon Cancer

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Abstract

*Helicobacter saguini* is a novel enterohepatic *Helicobacter* species isolated from the colon and feces of captive cotton top tamarins with chronic colitis and colon cancer. Mono-associated *H. saguini* infection in gnotobiotic IL-10 knockout mice causes inflammatory bowel disease and pro-carcinogenic changes to the large intestine. Genomic and biochemical characterization suggest *H. saguini* may express gamma-glutamyltranspeptidase (GGT), a constitutively expressed periplasmic enzyme that metabolizes extracellular glutamine and glutathione for import as nutrients. GGT activity by other pathogenic *Helicobacter* spp. and *Campylobacter* spp., including *H. pylori*, exhibits virulent effects including inhibition of cellular proliferation, immunomodulation, host colonization persistence, and promotion of inflammatory-mediated pathologies including cancer. In this paper, we use molecular biology, biochemical, and cell culture methods to demonstrate that *H. saguini* expresses an enzymatically active and virulent functional *ggt* gene. Multi-sequence alignments indicate that GGT from *H. saguini* is most homologous to the GGT expressed by *H. bilis*. GGT from *H. saguini* inhibited gastrointestinal epithelial and lymphocytes proliferation without evidence of cell death. Additionally, GGT from *H. saguini* induced pro-inflammatory gene expression in colon epithelial cells. This data indicates *H. saguini* may utilize GGT expression as an important virulence factor to enable infection and elicit chronic gastrointestinal inflammation.
Introduction

Cotton top tamarins (Saguinus oedipus; CTTs) are an endangered new world primate species that develop a high incidence of idiopathic chronic colitis and colonic adenocarcinomas while held in captive conditions\(^1\)-\(^5\). The clinical and histopathological manifestations of large bowel inflammation in CTTs strongly resemble those of human ulcerative colitis\(^1\)-\(^5\). Thus, CTTs are regarded as an ideal animal model to study the etiology and pathogenesis of human idiopathic inflammatory bowel disease (IBD).

Our lab isolated *Helicobacter saguini*, a novel intestinal *Helicobacter* species, from colonic biopsy and fecal samples of captive CTTs living in colonies with endemic chronic colitis and colon cancer\(^4\). *H. saguini* is a urease negative, fusiform organism, phylogenetically classified as an enterohepatic *Helicobacter* species (EHS).

EHS colonize intestinal sites including the small and large bowel as well as the liver and biliary tract\(^3\),\(^5\)-\(^9\). Infection by EHS is proposed to potentiate the risk of developing IBD in animal models and humans, analogous to the accepted causative relationship between *H. pylori* infection and the occurrence of peptic ulcers and cancer. *H. hepaticus* and *H. bilis* infection are well-established mouse models of pathogen-induced IBD and intestinal carcinogenesis\(^5\),\(^10\)-\(^12\). Experimental infection by *H. cinaedi* and *H. fennelliae*, both species originally isolated from homosexual men with proctitis, elicit diarrhea and gastrointestinal inflammation in pigtail macaques\(^3\),\(^5\),\(^13\). A current meta-analysis has also established a significant association between EHS infection and IBD status in human patients; however, a specific *Helicobacter* spp. that is linked to human IBD remains to be elucidated\(^14\).

Despite such, establishing an etiological relationship between *Helicobacter* infection and IBD in CTTs has been impossible due to the pervasive predisposition of spontaneous colitis and...
protected status of these primates. Recently though, we demonstrated mono-associated infection
of *H. saguini* in a gnotobiotic C57BL/6 IL-10 knockout mouse model recapitulated IBD and pro-
carcinogenic changes in the large intestine. This suggests *H. saguini* infection can influence
the onset and progression of chronic colitis and colon cancer in CTTs.

Though a substantial body of evidence supports a causative association between EHS
infection and IBD, the mechanisms by which these pathogens elicit disease remains incompletely
defined. Recently, gamma-glutamyltranspeptidase (GGT) expression by the gastrointestinal
pathogens *H. pylori*, *H. suis*, *H. bilis*, and *Campylobacter jejuni* has gained growing appreciation
as a critical virulence factor for infectious colonization and inflammatory disease pathogenesis
10,11,16-19.

GGT is a constitutively expressed periplasmic enzyme hypothesized to promote bacterial
survival by enzymatically cleaving extracellular glutamine and glutathione (GSH) into glutamate
for import into the cell as a precursor for numerous metabolic pathways including *de novo* amino
acids synthesis. In vitro and in vivo studies have also indicated GGTs from *H. pylori*, *H.
suis*, *H. bilis*, and *C. jejuni* are uniquely endowed with virulence properties including inhibition
of intestinal epithelial and lymphocyte proliferation, apoptosis, induction of pro-
inflammatory gene expression, generation of reactive oxygen species, oxidative DNA
damage and DNA strand breakage, host colonization persistence, and promotion of
inflammatory-mediated intestinal pathologies.

Biochemical characterizations indicate *H. saguini* has GGT activity. Furthermore, the
draft genome of *H. saguini* contains an annotated ggt gene sequence highly homologous to that
of *H. bilis*. Thus, given the suggestive association between IBD in CTTs and *H. saguini*
infection and the exploitation of virulent GGTs by pathogenic *Helicobacter* spp. and
Campylobacter spp., we hypothesize that *H. sanguini* expresses a functionally virulent *ggt* gene with demonstrate virulence characteristics.

**Methods**

**Bacterial and Cell Line Culture**

*H. sanguini* MIT 97-6194-5 and *H. bilis* (strain ATCC 43879) were cultured on trypticase soy agar plates with 5% sheep blood agar plates in microaerobic conditions (10% CO\(_2\), 10% H\(_2\), 80% N\(_2\)) at 37°C and collected after 48H, which corresponds to the mid-exponential phase of the bacteria growth. Gram staining was used to ensure the purity of the bacteria preparation. HeLa S3 cells (ATCC CCL2.2), AGS stomach cancer epithelial cells (ATCC CRL-1739), HT-29 colon cancer epithelial cells (ATCC HTB-38), and Jurkat T cells (ATCC TIB-152) were grown and maintained in Eagle's Minimum Essential Medium (ATCC. Manassas, VA), Dulbecco’s Modified Eagle's Medium (ATCC), or RPMI-1640 Medium (ATCC) containing 10% Fetal Calf Serum (Sigma-Aldrich. St. Louis, MO) and 1% Antibiotic-Antimycotic (Gibco/Thermo Fisher Scientific. Grand Island, NY) at 37°C with 5% CO\(_2\).

**Whole Genome Sequence Analysis**

Genomic DNA from *H. sanguini* MIT 97-6194-5 was sequenced using Illumina MiSeq sequencing technology as previously described\(^{33}\). The 250-bp paired-end sequencing reads generated by MiSeq were assembled into contigs using Velvet\(^{34}\). The genome was annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline\(^{35}\) and RAST (Rapid Annotation using Subsystem Technology)\(^{36}\). The draft genome was submitted to NCBI’s GenBank under the accession number JRMP00000000.1.

**Construction of an Isogenic GGT-Knockout Mutant (HS\(\Delta\)GGT)**
Approximately 500-bp fragments upstream and downstream of the \textit{H. saguini} ggt gene were PCR amplified from wild-type \textit{H. saguini} MIT 97-6194-5 genomic DNA using the primer pairs FS105/RS105 and FS106/RS106 (Table 1) for upstream and downstream amplifications, respectively. Upstream and downstream products were spliced together by overlap extension PCR using the primer pair FS105/RS106\textsuperscript{37,38} to create a product containing a HincII restriction site. The band containing the spliced PCR product was excised. The spliced product was then cloned into a pCR2.1-TOPO vector and chemically transformed into One Shot TOP10 chemically competent \textit{E. coli} cells according to the manufacture’s protocol (Thermo Fisher Scientific Inc). Plasmids from positive colonies grown on ampicillin-selective LB plates were purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The chloramphenicol acetyltransferase cassette, a chloramphenicol resistance gene lacking its transcription terminator sequence (\textit{catNT}), was amplified using PCR primers ZMG54/ZMG55 (Table 1) as previously described\textsuperscript{39}. Plasmid and chloramphenicol acetyltransferase cassette were digested with HincII enzyme (New England Biolabs. Ipswich, MA) overnight at 37°C. HincII-digested plasmid was treated with Antarctic phosphatase (New England Biolabs) to dephosphorylate 5’ overhangs and then ligated to HincII-digested chloramphenicol acetyltransferase cassette using Blunt/TA ligase (New England Biolabs). Ligated plasmid was chemically transformed into One Shot TOP10 chemically competent \textit{E. coli} cells and grown on chloramphenicol-selective LB plates. Plasmids from positive colonies were purified with the QIAprep Spin Miniprep Kit (Qiagen). Recombinant plasmids were verified by PCR for the upstream and downstream flanking sequence and \textit{catNT} and then transformed into \textit{H. saguini} by electroporation. Mutants were selected as previously described on chloramphenicol-selective blood agar plates under microaerobic conditions\textsuperscript{39-41}. After five passage on chloramphenicol-selective plates, mutants
were confirmed for genetic authenticity by PCR amplification of the upstream-to-downstream flanking sequence (primers FS105/RS106), full HSGGT gene (primers HSGGT-F/HSGGT-R), and catNT (primers ZMG54/ZMG55) (Table 1). Cells were collected in 1 mL of Trizol reagent (Invitrogen) for total RNA extraction following the manufacture protocol. Expression of the upstream gene chemotaxis protein (CheV) and the downstream genes glutamate synthase [NADPH] small chain (GluSyn) and Trk system potassium uptake protein (TrkA) were analyzed by PCR (Table 1). GGT activity of mutant sonicate was tested as described below.

**Preparation of Crude Bacterial Sonicate**

After reaching the mid-exponential phase of growth (about 48H), bacteria were collected from sheep blood agar plates using sterile cotton swabs and transferred to 1 mL of PBS. Samples were centrifuged at 12,000 rpm for 5 minutes at room temperature to pellet bacteria. Supernatant was removed and samples were washed in 1 mL of PBS. Samples were centrifuged at 12,000 rpm for 5 minutes at room temperature to re-pellet bacteria. Pellets were re-suspended in 2 mL of PBS and then sonicated on ice using the following program: amplitude: 35; power: 7 watts; 30 second intervals for a total of 5 minutes with 1 minute breaks between intervals. Sonicate samples were centrifuged at 12,000 rpm for 10 minutes at 4°C to pellet large debris. Supernatant was collected and then filter-sterilized through 0.2 um filters. Crude sonicate samples were stored at -80°C until use.

**Expression and purification of N-terminal 6XHis-tagged recombinant HSGGT and HBGGT**

HSGGT and HBGGT genes, without the signal sequence, were amplified from genomic DNA of *H. saguini* and *H. bilis* (strain ATCC 43879), respectively, with designed PCR primers (HSGGT-F/HSGGT-R for HSGGT and HBGGT-F/HBGGT-R for HBGGT; Table 1) to contain complementary overlapping sequences to the pET-46 expression vector. Purified HSGGT and
HBGGT PCR products were digested with T4 polymerase and cloned into pET-46 expression vectors according the Ek/LIC Vector Kit protocol recommend by manufacture (Novagen, Madison, WI). Vector plasmids were transformed into NovaBlue Giga Singles competent E. coli cells as a non-expression host. Plasmids from positive colonies were purified with the QIAprep Spin Miniprep Kit (Qiagen) and transformed into the BL21(DE3) competent E. coli cells as the expression host. Plasmids from positive colonies were purified with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced with T7 and T7 terminal primers (Table 1) to confirm accuracy of gene sequences.

Expression of the recombinant HSGGT and HBGGT proteins (rHSGGT and rHBGGT) with N-terminal 6x-His tags was induced by treatment with 0.4 mM IPTG for 4H at 37°C. Bacteria were pelleted at 8,000 rpm for 10 minutes at 4°C and washed once with PBS. Pellets were re-suspended in ice-cold binding buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5) containing one 100x Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and sonicated as described above. Supernatant was then filter-sterilized through 0.2 μm filters and loaded on HisTrap column (GE Healthcare Biosciences, Westborough, MA) at 1 mL/min at 4°C. Columns were washed with binding buffer prior to eluting bound proteins with an imidazole gradient of 50, 100, 250, and 500 mM in elution buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.5). Eluted fractions were collected and tested for purity by SDS-PAGE. Fractions containing the expected GGT subunit proteins were buffer exchanged and concentrated into PBS using 3-kDa Amicon Ultra 2mL Centrifugal Filters (EMD Millipore, Billerica, MA).

To track autocatalytic maturation of GGT, 15 μg protein aliquots were incubated at 37°C for 0, 1, 4, 8, or 24H. At the indicated time points, aliquots were immediately boiled in reducing buffer for 5 minutes and then ran on a SDS-PAGE to visualize degree of maturation.
Fractions were tested for GGT enzymatic activity as described below. Fractions were then aliquoted and stored at -80°C until further use.

**Enzyme Assay for Gamma-glutamyltranspeptidase Activity**

GGT transpeptidase enzymatic activity of sonicated *Helicobacter* samples and the recombinant proteins rHSGGT and rHBGGT was determined by measuring the cleavage of L-γ-glutamyl-p nitroanilide (GpNA; Sigma-Aldrich) into 4-p-nitroaniline (pNA). 200 μL reactions containing 5, 1, or 0.5 μg of protein, 20 mM of glycylglycine (Sigma-Aldrich), and 0-2000 uM of GpNA in Tris-HCl buffer (pH 8) were incubated at 37°C for 30 minutes. Recombinant proteins were pre-incubated for 24H at 37°C prior to assaying to increase enzyme maturation. Reactions were performed in duplicate. Enzymatic cleavage into 4-p-nitroaniline (pNA) was quantified by reading optical density at 405 nm for 30 minutes in 30 second intervals with SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices. Sunnyvale, CA). Optical densities were corrected against PBS controls to account for spontaneous autolysis of GpNA into pNA. Replicates were averaged together and concentrations of pNA were calculated according to Beer-Lambert Law using the reported extinction coefficient of 8800 M⁻¹cm⁻¹. Enzyme activity was calculated as mUnits/mg protein, in which one unit was defined as the quantity of enzyme that catalyzes the formation of one umole of pNA per minute. Enzyme kinetics (K_M and V_max) were determined by fitting the data to the Michaelis-Menten equation using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA). The transpeptidase reactions were repeated using samples pre-incubated with 1 mM of acivicin (Cayman Chemical. Ann Arbor, MI) for 30 minutes at 37°C prior to addition to substrates.

**MTT Assay for Quantification of Cell Proliferation**
5,000 HeLa, HT-29, or AGS cells or 10,000 T cells were plated in 100 μL of media in 96-well and incubated for 24 hour at 37°C with 5% CO₂. Cells were treated with 0.5, 0.25, 0.125, 0.0625, and 0 (i.e., PBS control) mUnits doses of crude HS, HSΔGGT, and HB sonicates and rHSGGT and rHBGGT protein in triplet for 24, 48, and 72H. Following incubation, cell proliferation was assessed using the MTT assay (Vybrant/Thermo Fisher Scientific). Media was aspirated from the wells and replaced with 12 mM of MTT reagent dissolved in non-phenol red MEM media (Gibco) containing 10% Fetal Calf Serum (Sigma-Aldrich). Cells were incubated at 37°C with 5% CO₂ for 4 hours. Following incubation with the MTT regent, media was aspirated and replaced with 100 μL of 20 mM HCl and 0.1% Nondent P-40 (NP-40) in isopropanol solution. The solution was gently pipetted to dissolve formazan crystal. Plates were incubated at 37°C with 5% CO₂ for 10 minutes. Optical density was read at 540 nm. Optical densities were blank-corrected and replicates were averaged together. Viability was expressed as a percentage compared to the PBS control.

Pro-Inflammatory Cytokine Expression

50,000 HT-29 cells were plated in 1 mL of media in 24-well plates. Cells were incubated for 24H at 37°C with 5% CO₂ to allow cells to adhere to the plates. Cells were treated with 5.0 mUnits dose of rHSGGT, rHBGGT, or PBS for 4H hours at 37°C with 5% CO₂ in duplicate. Cells were collected in 1 mL of Trizol reagent (Invitrogen) for total RNA extraction following the manufacture protocol. RNA was extracted using Trizol reagent. Total RNA (5 μg) was converted into cDNA using a High Capacity cDNA Archive kit following the manufacturer protocol (Applied Biosystems. Foster City, CA). cDNA levels for TNF-α (HS99999043_M1), IL-1β (HS01555410_M1), IL-6 (HS00985639_M1), IL-8 (HS00174103_M1), CXCL-1 (HS00236937_M1), and GAPDH (REF4332649) mRNA were measured by quantitative PCR.
using commercial primers and probes for each cytokine. Briefly, duplicate 20 µL reactions contained 4 µL of cDNA, 1 µL of a commercial 20x primer-probe solution, 10 µL of 2x master mix (Applied Biosystems), and 5 µL of double-distilled H₂O. Relative expression of mRNA was calculated using the comparative Cₜ method with RNA input standardized between samples by expression levels of the endogenous reference gene, GAPDH. Mean fold changes from three separate experiments were plotted between treated and PBS control cells.

Statistical Analysis

Data are presented as mean ± standard deviation. Statistical analysis were performed by Student’s T-test or one-way ANOVA with a Tukey post-hoc test using GraphPad Prism 5.0 (GraphPad Software, Inc.). Results were considered significant at p-value<0.05. All graphs were generated using GraphPad Prism 5.0 (GraphPad Software, Inc.).

Results

_H. saguini GGT (HSGGT) is Homologous to GGTs with Virulence Properties_

Previous biochemical characterization indicating _H. saguini_ is positive for GGT activity was substantiated by the presence of a partial and fragmented _ggt_ gene in the annotated draft genome of the organism. This putative _H. saguini ggt_ gene (HSGGT) was confirmed and completed by PCR of genomic DNA and sequencing of the products. HSGGT shows considerable homology with representative bacterial and human _ggt_ genes, supporting the assignment made by the genomic annotation (Table 2). The greatest similarity of HSGGT is to the _ggt_ genes from _H. bilis_ (HBGGT), _H. pylori_ (HPGGT), _H. suis_ (HSuGGT), and _C. jejuni_ (CPGGT) (sequence homology ranging from 81-86% and sequence identity ranging from 66-75%; Table 2). Of these _ggt_ genes with virulence properties, HSGGT is most homologous to those from the intestinal pathogens _H. bilis_ and _C. jejuni_ compared to the gastric pathogens _H.
H. *saguini* GGT

252 *pylori* and *H. suis*, possibly representing structural and functional conservation in accordance to gastrointestinal colonization niches. Multi-sequence alignments show the HSGGT protein sequence preserves all residues necessary for enzymatic function and putative virulence, including a signal sequence for periplasmic secretion, the threonine dyad for autocatalytic maturation and transpeptidase activity, and the lid loop for substrate binding (Figure 1).

257 **HSGGT Predicted Protein Structure is Most Similar to the Solved Crystal Structure of HPGGT**

Protein structure prediction by Phyre2 also indicates with 100% confidence and 71% identity that the possible three-dimensional structure of HSGGT would be most similar to the solved crystal structure of HPGGT (PDB entry nqoB\(^42\)) (Figure 2). Together, this homology data suggests HSGGT has enzymatic and virulent functionality.

262 **Construction of Isogenic *H. saguini* GGT-Knockout Mutant (HS\(\Delta\)GGT)**

To test the virulence potential of HSGGT, an isogenic GGT-knockout mutant of *H. saguini* (HS\(\Delta\)GGT) was created for comparison against the wild-type strain. The *ggt* gene in *H. saguini* was successfully replaced with *catNT*, a chloramphenicol resistance gene (Figure 3a, b, c). Flanking upstream and downstream gene expression at the site of mutagenesis were identical for the wild-type and mutant strains (Figure 3d). Likewise, mutant bacteria grew normally *in vitro*, consistent with other GGT-knockout *Helicobacter* spp. mutants\(^10,32\). Sonicated HBGGT and wild-type HSGGT cleaved the glutamate substrate analog GpNA into pNA at comparable levels and was completely abolished for both by the GGT-specific inhibitor acivicin (Figure 4). Conversely, GGT activity was undetectable in sonicated HS\(\Delta\)GGT (Figure 4a).

272 **Purification of Functional rHBGGT and rHSGGT Proteins**

Recombinant His-tagged *H. bilis* GGT (rHBGGT) and *H. saguini* GGT (rHSGGT) proteins, without the signal peptide, were expressed in a BL21(DE3) *E. coli* host and purified
from crude sonicates by Ni-affinity chromatography. Fractions were separated by SDS-PAGE, resulting in prominent bands at ~60-kDa, ~40-kDa, and ~20-kDa (Figure 5), consistent with the purification of recombinant GGT (rGGT) proteins as previously described\textsuperscript{10,17,29}. Following buffer exchange and concentration into PBS, fraction #3 for both rHBGGT and rHSGGT visually appeared to be the most purified fraction. Thus, subsequent experiments only used recombinant proteins collected from this fraction. Further means to remove non-specific proteins were not undertaken due to risk of losing excessive product. While rHBGGT showed maturation immediately after purification, rHSGGT matured into detectable 40-kDa and 20-kDa subunits by 24H incubation at 37°C (Figure 5).

Purified rHBGGT and rHSGGT exhibited GGT activity that was completely ablated by pre-treatment with acivicin (Figure 6). The mean binding affinity (Km) of rHBGGT for GpNA from two separate batches was $6.73 \pm 0.31$ uM, consistent to that of $7.7 \pm 1.2$ uM as previously reported\textsuperscript{10}. Three separate batches of rHSGGT had a mean Km of $12.3 \pm 2.35$ uM, which is of similar magnitude to both rHPGGT (reported as $9.8 \pm 1.5$ uM\textsuperscript{10}) and rHBGGT. rHBGGT had about 2-fold higher transpeptidase activity compared to rHSGGT ($V_{\text{max}}$ of 299.6 mUnits/mg protein for rHBGGT versus 145.6 mUnits/mg protein for rHSGGT), which agrees with a previous report showing the magnitude of GGT activity is dependent on the degree of enzyme maturation\textsuperscript{43}.

**HSGGT Inhibits Intestinal Epithelial and Lymphocyte Cellular Proliferation**

The anti-proliferative effects of virulent GGT have been primarily described using AGS cells, thus this cell line served as a comparative control. Both HB and HS sonicates and rGGT proteins significantly inhibited AGS cell proliferation at each time point compared to the PBS control (Figure 7a, c). Importantly, wild-type HS sonicate treatment significantly blocked
proliferation compared to an equivalent total protein dose of HSΔGGT sonicate (Figure 7a).

Interestingly, HSΔGGT sonicate still statistically suppressed cell proliferation at each time point compared to the PBS control (Figure 7a). This finding indicates *H. saguini* expresses anti-proliferative virulence factors besides GGT, though these other potential factors are not readily apparent from the genomic or biochemical characterization of the organism and require further investigation to identify. HB and HS sonicates and rGGT proteins also inhibited AGS cell proliferation in a dose-dependent manner after 72H of treatment (Figure 7b, d).

Both *H. bilis* and *H. saguini* colonize the mucosal epithelium of the large intestine, therefore mandating characterization of HBGGT and HSGGT in colon epithelial cells.

Analogous to the anti-proliferative function in AGS cells, both HB and HS sonicates and rGGT proteins significantly blocked proliferation of the colon epithelial cell line HT-29 (Figure 7e, g).

Wild-type HS sonicate also caused statistically greater inhibition of proliferation compared to HSΔGGT sonicate treatment (Figure 7e). Again, HSΔGGT sonicate still yielded significant anti-proliferative effects compared to the PBS control, suggesting the expression of other virulence factors by *H. saguini* (Figure 7e). Additionally, HB and HS sonicates and rGGT proteins blocked proliferation in a dose-dependent manner after 72H of treatment (Figure 7f, h).

The effects of HBGGT and HSGGT were also assayed with HeLa cells because this cell line is frequently employed in the screening and characterization of bacterial cytotoxins expressed by *Helicobacter* spp., *Campylobacter* spp., and other gastrointestinal species. HB and HS sonicates and rGGT proteins caused time- and dose-dependent anti-proliferative effects in HeLa cells that parallel the effects seen in the AGS and HT-29 cell lines (Figure 7i, j, k, l).

Virulent GGTs from HPGGT, HSuGGT, HBGGT, and CJGGT all share the ability to impair the proliferation of lymphocytes. Likewise, HS sonicate and rGGT proteins significantly
blocked Jurkat T cell proliferation after 48 and 72H of treatment compared to the PBS control (Figure 8a, c). Furthermore, the anti-proliferative effects caused by wild-type HS sonicate treatment were statistically greater versus HSΔGGT sonicate (Figure 8a). Interestingly, although HSΔGGT sonicate treatment inhibited epithelial cell proliferation, it had no significant effects in inducing T cell proliferation (Figure 8a). This suggests other putative anti-proliferative virulence factors expressed by *H. saguini* are selective for cell type. Surprisingly, while rHBGGT blocked T cell proliferation (Figure 8c), HB sonicate exerted a dramatic decline in viability of the T cells over the course of treatment (Figure 8a). This effect may be due to expression of *cdtB*, the gene encoding the active subunit of cytolethal distending toxin (CDT). *H. bilis* harbors *cdtB* in its genome, and CDT activity by other bacterial species induced apoptosis in lymphocytes and other cell types. *H. bilis* may also express other novel cytotoxic virulence factors that affect lymphocyte viability. T cells, therefore, appear to be more sensitive to cytotoxicity by HB sonicate than the anti-proliferative effects of HBGGT. The effects of HB and HS sonicates and rGGT protein treatments on T cells were dose-dependent (Figure 8b, d).

Megalocytosis (abnormal cell body enlargement), multinucleation, vacuolation, and other morphological abnormalities of epithelial cells were not observed after 72H of *Helicobacter* spp. sonicate and rGGT protein treatment (Figure 9). Thus, suppression of proliferation was attributable to GGT and not virulence factors such as CDT, cytotoxic necrotizing factor (CNF), colibactin, and vacuolating toxin (VacA). Unexpectedly, despite *H. bilis* having the *cdtB* gene, HB sonicate treatment lacked morphological evidence of CDT towards epithelial cells. This contradicts other reports showing HB sonicate has CDT activity; however, it should be noted that only 10-15% of HeLa cells became megalocytic. Thus, *H. bilis* may exhibit weak CDT actions towards epithelial cells, which possibly can be masked by insufficient doses of sonicate.
or by HBGGT activity. Conversely, T cells may be more vulnerable to CDT than HBGGT activity, resulting in cell death instead of suppressed proliferation.

Contrary to reports that have shown HPGGT and HSuGGT can induce apoptosis in AGS cells, observable indicators of cell death, such as cellular rounding, membrane blebbing, and plate detachment, were not evident in the epithelial cell lines tested even after 72H of treatment with Helicobacter sonicate and rGGT protein (Figure 9). Therefore, HBGGT and HSGGT appear to inhibit epithelial proliferation without causing overt cell death.

rHSGGT and rHBGGT Induce Pro-Inflammatory Gene Expression

Virulent GGTs are capable of inducing pro-inflammatory changes in vitro and in vivo. HPGGT and HBGGT both increase activation of NF-kB and IL-8 expression\(^\text{11,31}\), while infection by GGT-knockout H. pylori and H. suis mutants causes significantly less inflammatory-mediated pathologies in the rodent stomach compare to wild-type strains\(^\text{19}\). Therefore, we assessed the pro-inflammatory potential of rHSGGT and rHBGGT towards HT-29 cells. Following 4H of treatment, both rHSGGT and rHBGGT significantly increased expression of the neutrophil chemoattractant IL-8 (Figure 10a) and CXCL-1 (Figure 10b). Additionally, the pivotal inflammatory cytokines TNF-\(\alpha\) (Figure 10c) and IL-\(\beta\) (Figure 10d) were also significantly increased by rHSGGT and rHBGGT treatment. IL-6 expression increased as well, but failed to reach a statistical difference from the controls (Figure 10e). Interestingly, despite being treated with equal doses of GGT activity, rHBGGT caused significantly greater inflammatory cytokine expression for IL-8, CXCL-1, TNF-\(\alpha\), and IL-\(\beta\) compared to rHSGGT. Nevertheless, this data shows HSGGT is capable of promoting pro-inflammatory gene expression profile by colon epithelial cells.

Discussion
Recently, we demonstrated that *H. saguini* infection in a gnotobiotic IL-10 knockout mouse model elicits inflammatory and pro-carcinogenic changes to the large intestine, suggesting an etiological role of *H. saguini* infection in the pathogenesis of IBD in captive CTTs. Although EHS infection is strongly associated with gastrointestinal inflammation and cancer progression, the mechanisms by which EHS colonize their host and cause disease are poorly defined.

CDT is the most thoroughly characterized virulence factor expressed by EHS, including *H. bilis* and *H. hepaticus*. CDT is a DNA nuclease shown to be genotoxic and pro-apoptotic towards the gastrointestinal epithelium and lymphocytes *in vitro* and *in vivo*. Mice infected with an isogenic CDT-knockout mutant of *H. hepaticus* incur significantly less severe colitis and pathology, demonstrating the critical contribution of CDT towards EHS-induced IBD. According to genomic and cell culture screenings though, *H. saguini* does not express CDT.

The annotated draft genome of *H. saguini* does contain virulence factors expressed by *Helicobacter* spp., *Campylobacter* spp., and other gastrointestinal pathogens, including flagella components, flavodoxin *fldA*, the secreted serine protease *htrA*, the type VI secretion component *vgfG*, arginase, and gamma-glutamyltranspeptidase. GGT activity by *Helicobacter* spp. and *Campylobacter* spp. has been shown to promote persistent colonization, pro-inflammatory responses, and DNA damage. Therefore, *H. saguini* may utilize virulent GGT expression to establish or maintain infection within the gastrointestinal tract that subsequently evolves into chronic colitis and potentially colon cancer.

GGT is a threonine N-terminal nucleophilic hydrolase that catalyzes the transpeptidation and hydrolysis of gamma-glutamyl bonds in glutamate, GSH, and similar substrates. Both mammalian tissues and numerous bacterial species express GGT, but, despite considerable
H. saguini GGT

homology amongst each other, these genes have evolved divergent physiological functions. Mammalian GGTs, including from humans, recycle extracellular GSH as a source of cysteine, glutamate, and glycine for intracellular GSH and protein synthesis as well as function in the metabolism of secreted GSH conjugates\textsuperscript{49-51}. Bacterial GGTs are hypothesized to metabolize extracellular glutamine and GSH as sources of glutamate, which is then transported into the cell for use in the Krebs cycle and glutamine synthesis to produce amino acid precursors for later protein synthesis\textsuperscript{22}. This is supported by the presence of sodium/glutamate symporter, glutamate dehydrogenase, and glutamine synthetase genes in the annotated genomes of GGT-positive bacterial species, including \textit{H. saguini}. In this context, bacterial \textit{ggt} genes are proposed to offer growth advantages in order to facilitate and maintain infectious colonization of their host. Furthermore, GGT from \textit{E. coli} K12 strain (ECGGT) may also be involved in osmoadaptation\textsuperscript{52}, while GGT activity by the pathogenic \textit{Helicobacter} spp. and \textit{Campylobacter} spp. demonstrate a multitude of virulent actions.

Why \textit{ggt} gene expression by \textit{Helicobacter} spp. and \textit{Campylobacter} spp. is capable of virulent functions whereas the hGGT and ECGGT homologs are not is unknown, but dichotomies in enzyme activity and structure may be relevant. While bacterial and mammalian GGTs exhibit comparable hydrolysis reaction efficiencies, bacterial GGTs have substantially less active transpeptidase reactions and display differences in substrate specificity compared to mammalian GGT\textsuperscript{42,50,51}. Furthermore, noTable differences are apparent between the crystals structures of HPGGT and hGGT, namely disulfide bond potential, binding pocket conformational flexibility and substrate interactions, lid loop regulation of substrate and solvent access into the active site, and chloride ions integration near the active site\textsuperscript{42,50,53-55}. Likewise, C-
terminal residues that influence HPGGT enzymatic activity are absent in ECGGT and may contribute towards a virulent GGT function\textsuperscript{54}.

Given the greater sequence homology of HSGGT to virulent versus non-virulent GGTs, we hypothesized that GGT activity by \textit{H. saguini} is virulently active. The common \textit{in vitro} effect shared by all virulent GGTs is the ability to inhibit gastrointestinal epithelial and lymphocyte proliferation. We found that HS sonicate and rHSGGT significantly impaired AGS, HT-29, HeLa, and Jurkat T cell proliferation on par with HBGGT. Cellular proliferation by HSGGT and HBGGT was not accompanied by evidence of cell death. This agrees with reports showing HBGGT and CJGGT, the closest homologs of HSGGT, block epithelial cell proliferation without pro-apoptotic activity. Conversely, HPGGT and HS\textit{u}GGGT can induce apoptosis in AGS cells, but this finding may be confounded by methodologies requiring serum starvation\textsuperscript{10}. Furthermore, we showed rHSGGT alone is capable of stimulating significant chemoattractant and cytokine gene expression by colon epithelial cells. This property of HSGGT activity is accordance with the pro-inflammatory nature of HPGGT, HS\textit{u}GGGT, and HBGGT. Accordingly, the data taken together assigns HSGGT a virulent functionality, similar as that of HBGGT and other known virulent GGT homologs.

The anti-proliferative effects of HSGGT towards colon epithelial cells suggests \textit{H. saguini} may compromise intestinal barrier integrity in the host. Impaired barrier function is a defining characteristic of IBD pathogenesis and hypothesized to drive unregulated and sustained pro-inflammatory responses by allowing excessive penetration of luminal bacteria and foreign antigens into the lamina propria\textsuperscript{56-58}. Thus, it follows that HSGGT provides a mechanistic link towards this onset. Likewise, waning barrier integrity by GGT activity may also contribute towards mucosal ulcerations evident in IBD.
While *H. saguini* and other *Helicobacter* spp. directly colonize the mucosal and epithelial surface of the gastrointestinal tract *in vivo*, HSGGT as well as other GGTs with virulence properties can still impair lymphocyte proliferation *in vitro*. Declining barrier integrity may allow *Helicobacter* spp. to invade intestinal tissue where GGT can act to directly inhibit lymphocytes. Alternatively, GGT may also be transported from the intestinal lumen into the lamina propria by outer membrane vesicles to impact lymphocyte proliferation\textsuperscript{23}. By suppressing lymphocytes proliferation, GGT expression may enable *H. saguini* to dampen and evade the host immune systems and consequently facilitate long-term infection.

HSGGT also induces expression of potent pro-inflammatory chemoattractant and cytokines genes, indicating stimulation of an inflammatory response. For example, HSGGT may exert its effects by inducing local inflammation within the gastrointestinal tract and also impair systemic activation of the adaptive immune system. By doing so, HSGGT could enable *H. saguini* to fine-tune immune function in order to survive within the host, while simultaneously allowing pernicious infection to evoke chronic inflammation underpinning IBD and colon cancer pathogenesis.

GGT expression has been shown to be pivotal for infection and disease pathogenesis by *H. pylori*, *H. suis*, and *C. jejuni*. Infection of mouse and avian hosts by *H. pylori* and *C. jejuni*, respectively, indicates GGT is required for colonization persistence, given colonization by GGT-knockout mutants was significantly decreased or undetectable\textsuperscript{16,19,32}. This coincides with clinical findings that *H. pylori* strains with greater GGT activity are associated with the occurrence of peptic ulcer disease in human patients\textsuperscript{31}. In contrast, GGT expression in *H. suis* had no effect on stomach colonization persistence in mice or Mongolian gerbils, but did promote more severe inflammation and pathology compared to infection by a GGT-knockout mutant\textsuperscript{19}. Whether *H.
*H. saguini* GGT activity has similar properties *in vivo* is unconfirmed because analogous animal studies have not been performed, although the anti-proliferative and pro-inflammatory prolife of HBGGT *in vitro* suggests it may.

The putative mechanism of GGT virulence is dependent on enzymatic activity, suggesting depletion of host glutamine and GSH is central. In mammals, glutamine serves as a precursor for nucleotides, other amino acids, glycosylation reactions, and GSH\(^{59}\). Thus, disturbances in glutamine availability by virulent GGT could detrimentally affect normal cellular function and lead to adverse host responses. The anti-proliferative effects of GGT have been attributed to glutamine deprivation\(^{22,24,26,60}\). Conversely, GSH metabolism by GGT may deplete antioxidant reserves making cells susceptible to oxidative stress. Oral glutamine and GSH supplementation can attenuate gastric inflammation and pathology in Mongolian gerbils infected with *H. suis*; this result reinforces the hypothesis that GGT virulence is related to glutamine and GSH access by the host\(^{18}\).

Toxic metabolites and byproducts may also contribute towards the virulence mechanisms of GGT. For example, cell media deprived of glutamine prevented inhibition of lymphocyte by GGT, suggesting transpeptidase metabolites influence cellular inhibition\(^{26}\). Media supplemented with extra GSH enhanced the anti-proliferative effects of HSuGGT, possibly through the formation of unknown pro-oxidant GSH metabolites\(^{23}\). Likewise, hydrogen peroxide generated by GGT is augmented by extra GSH in cell media\(^{29}\). Also, glutamine metabolism releases ammonia as a byproduct, which can elicit toxicity to host cells\(^{22}\). It is also important to consider that although GGT can metabolize glutamine and GSH, other unidentified virulence-specific substrates may be important as well. Though glutamine and GSH metabolism and
oxidative stress appear to be relevant, a precise mechanism of how GGT mediates virulence still needs to be resolved.

In conclusion, we have shown *H. saguini* expresses an enzymatically active GGT homolog with virulence properties. The multi-modal influences of GGT, including functions in persistent colonization, inflammation, and carcinogenesis, suggest GGT expression by *H. saguini* is holistically important in IBD pathogenesis. Future studies will assess the immunomodulatory and carcinogenic potential of HSGGT *in vitro* and *in vivo*. In particular, our *H. saguini* mouse model of IBD will affirm the function of HSGGT *in vivo* and further elucidate connections between *Helicobacter* spp. infection and IBD in CTTs as well as humans. Most importantly, by understanding the biochemical characteristics that differentiate virulent versus non-virulent homologs, GGT represents a novel pharmacological target for antibiotics and vaccines that may offer more efficacious ways to treat and prevent *Helicobacter* spp. and *Campylobacter* spp. infection in veterinary and human patients.

**Table and Figure Descriptions**

**Table 1**

Primers sequences used for construction of isogenic GGT-knockout mutant and recombinant GGT protein.

**Table 2**

*H. saguini*, *H. bilis* ATCC 43879 (GenBank: EEO24771.2), *H. pylori* 26695 (NCBI Reference Sequence: NP_207909.1), *H. suis* (GenBank: ADF28653.1), *C. jejuni* strain 81116 (GenBank: AAV30679.1), *E. coli* str. K-12 (NCBI Reference Sequence: NP_417909.1), and human (NCBI Reference Sequence: NP_005256.2) GGT amino acid homology comparison with Protein Blast 

61.
Figure 1

Multi-sequence alignment of bacterial and human ggt genes generated with Clustal Omega\(^{62}\). Signal sequence predicted with SignalP 4.1 Server using the “Sensitive (reproduce SignalP 3.0's sensitivity)” setting\(^{63}\). Green highlighted residues designate the signal sequence exclusion site. Yellow highlighted residues and brackets designate disulfide bonds. Orange highlighted residues designate the autocatalytic cleavage site. Gray highlighted residues designate the conserved GXXGXXI motif. Purple colored font residues designate the threonine catalytic dyad. Red colored font residues designate the lid loop. Boxed residues indicate positions in which amino acids are conserved between all aligned sequences. Colons (:) underneath the sequence indicate positions in which there are conserved amino acid substitutions between aligned sequences. Dots (.) underneath the sequences indicate positions in which there are semi-conserved amino acid substitutions between aligned sequences.

Figure 2

Phyre2 (Protein Homology/AnalogY Recognition Engine V 2.0;\(^{64}\) prediction of the 3D structure of HSGGT (green) according to its sequence homology with solved protein crystal structures. HSGGT showed the greatest similarity to the crystal structure of HPGGT (PDB entry nqoB\(^ {42}\)) with 100% confidence (measure of the probability the match between HSGGT sequence and template is a true homology) and 71% identity (a measure of the proportion of HSGGT protein residues equivalent to identical template residues in the generated alignment), indicating an accurate prediction. PyMOL computer software\(^ {65}\) was used to superimpose the predicted 3D structure of HSGGT (green) on the crystal structure of HPGGT (yellow). L-glutamate (orange) was aligned in substrate-binding pocket according to the HPGGT crystal
structure. In general, the structure of HSGGT closely aligns with HPGGT; however, a discrepancy was apparent in the position and secondary structure of the lip loop between HSGGT (circled red) and HPGGT (circled purple). The lid loop is proposed to regulate substrate and solvent access to the binding site as well as catalytic efficiency of the active site, so this dissimilarity may represent significant differences in enzymatic or virulent properties between HSGGT and HPGGT.

*Figure 3*

a) The chloramphenicol resistance gene, *catNT*, was detected by PCR in genomic DNA of HSΔGGT, but not in the wild-type strain. Red boxes outline the *catNT* PCR product at ~700 bp.

b) The HSGGT gene was detected by PCR in the genomic DNA of the wild-type strain but not in HSΔGGT. Red boxes outline the HSGGT gene PCR product at ~1700 bp.

c) The upstream-to-downstream flanking region in the genomic DNA of the HSGGT gene was about 1.2kb shorter in HSΔGGT compared to the wild-type strain, consistent with the replacement of the HSGGT gene with *catNT*. Red boxes outline the ~3000 bp and ~1800 bp PCR products for the wild-type strain and HSΔGGT, respectively.

d) The wild-type and mutant strains both expressed mRNA for the upstream gene Chemotaxis protein (*CheV*) and the downstream genes Glutamate synthase [NADPH] small chain (*GluSyn*) and Trk system potassium uptake protein (*TrkA*), indicating flanking gene expression was not affected by the isogenic mutagenesis.

*Figure 4*
a) HB and HS sonicates exhibited comparable levels of GGT activity. No GGT activity was detected by HSΔGGT sonicate, indicating successful removal of the functional ggt gene.

b) GGT activity by HB and HS sonicates was completely inhibited by pre-treatment with the GGT-specific inhibitor acivicin.

Figure 5

Partially purified rHBGGT (a) and rHSGGT (b) proteins were separated by SDS-PAGE. Red boxes outline the expected ~60-kDa proenzyme and mature ~40-kDa, and ~20-kDa GGT subunits were eluted in fraction #3 and #4.

15 ug of protein aliquots of rHBGGT (c) and rHSGGT (d) from fractions #3 were incubated at 37°C for 0, 1, 4, 8, or 24H. At the indicated time points, aliquots were denatured by boiling at 90°C in reducing buffer for 10 minutes and then protein were separated by SDS-PAGE. Red boxes outline the expected GGT protein bands and indicate increased autocatalytic maturation of rHSGGT after 24H of incubation.

Figure 6

a) Partially purified rHBGGT and rHSGGT proteins from fraction #3 exhibited GGT activity. Enzyme activity for both rGGTs conformed to the Michaelis–Menten kinetic profile. Binding affinities to the substrate GpNA for rHBGGT and rHSGGT were 6.73±0.31 uM and 12.3±2.35 uM, respectively. Likely due to a higher concentration of mature enzyme (see Figure 5), rHBGGT had a higher transpeptidase V_max of 299.6 mUnits/mg compared to 145.6 mUnits/mg for rHSGGT.

b) GGT activity by rHBGGT and rHSGGT was completely inhibited by pre-treatment with the GGT-specific inhibitor acivicin.
Figure 7
Cellular proliferation of AGS (a, c), HT-29 (e, g), and HeLa (i, k) cells after treatment with 0.5 mUnits of Helicobacter sonicate or rGGT for 24, 48, and 72H. Statistical differences (p-value<0.05) between treatments designated as follows: a, PBS vs. HSΔGGT Sonicate; b, PBS vs. HS Sonicate; c, PBS vs. HB Sonicate; d, HSΔGGT Sonicate vs. HS Sonicate; e, HSΔGGT Sonicate vs. HB Sonicate; f, HS Sonicate vs. HB Sonicate; g, PBS vs. rHSGGT; h, PBS vs. rHBGGT; i, rHSGGT vs. rHBGGT.

Cellular proliferation of AGS (b, d), HT-29 (f, h), and HeLa (j, l) cells after treatment with increasing doses of Helicobacter sonicate or rGGT for 72H.

Figure 8
Cellular proliferation of Jurkat T cells (a, c) after treatment with 0.5 mUnits of Helicobacter sonicate or rGGT for 24, 48, and 72H. Statistical differences (p-value<0.05) between treatments designated as follows: a, PBS vs. HSΔGGT Sonicate; b, PBS vs. HS Sonicate; c, PBS vs. HB Sonicate; d, HSΔGGT Sonicate vs. HS Sonicate; e, HSΔGGT Sonicate vs. HB Sonicate; f, HS Sonicate vs. HB Sonicate; g, PBS vs. rHSGGT; h, PBS vs. rHBGGT; i, rHSGGT vs. rHBGGT.

Cellular proliferation of Jurkat T cells (b, d) after treatment with increasing doses of Helicobacter sonicate or rGGT for 72H.

Figure 9
Representative images at 20x magnification of HT-29, AGS, and HeLa cells after treatment with 0.5 mUnits of Helicobacter sonicate or rGGT for 72H. Aside from qualitative decreases in cell confluence, HBGGT and HSGGT did not induce cytopathogenic or morphological changes characteristics of known virulence factors such as CDT, CNF, and VacA.
IL-8 (a), CXCL-1 (b), TNF-α (c), IL-1β (d), and IL-6 (e) pro-inflammatory gene expression by HT-29 cells after treatment with 0.5 uUnits of rHBGGT and rHSGGT for 4H.

Asterisk (*) designates statistical difference (p-value<0.05) between rHBGGT or rHSGGT treatment and PBS control cells. Hashtag (#) designates statistical difference (p-value<0.05) between rHBGGT and rHSGGT treatments.

References


H. saguini GGT


Virulent Gamma-Glutamyltranspeptidase Expression by *Helicobacter saguini*, an Enterohepatic Helicobacter Species Isolated from Cotton Top Tamarins with Chronic Colitis and Colon Cancer

Tables and Figures with Descriptions
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Table 2. % Sequence Homology (% Sequence Identity) of Bacterial and Human ggt genes

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<th>C. jejuni (CJGGT)</th>
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<th>Human (hGGT)</th>
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Figure 3

A

B

C

D

Mannion; H. saguini GGT
Figure 4

A

B

% GGT Activity

HB Sonicate
HS Sonicate
HSsonicate + Activicin

HB Sonicate + Acivicin

HB Sonicate
HS Sonicate

HS Sonicate + Acivicin

m Units/mg

GpNA (uM)

0 500 1000 1500 2000
Figure 5

A

B

Mannion; H. saguini GGT
Figure 5 (cont.)

C

D

Mannion; H. saguini GGT
Figure 6
Figure 7

A. AGS Time Sonicate

B. AGS Dose Sonicate

C. AGS Time rGGT

D. AGS Dose rGGT

Mannion; H. saguini GGT
Figure 7 (cont.)

E. HT-29 Time Sonicate

F. HT-29 Dose Sonicate

G. HT-29 Time rGGT

H. HT-29 Dose rGGT

Mannion; H. saguini GGT
Figure 7 (cont.)
Figure 8

A. T cell Time Sonicate

B. T cell Dose Sonicate

C. T cell Time rGGT

D. T cell Dose rGGT

Mannion; H. saguini GGT
Figure 9

PBS Control  |  Crude Sonicate  |  Recombinant Protein

HT-29  
(colon epithelial cells)

AGS  
(stomach epithelial cells)

HeLa  
(cervical epithelial cells)

HB Sonicate  |  HS Sonicate  |  HSΔGGT Sonicate  |  rHBGTT  |  rHSGGT
Figure 10

A. IL-8

B. CXCL-1

C. TNF-α

D. IL-1β

E. IL-6

Mannion; H. saguini GGT