The Gut-Brain Axis in Neurodegeneration: Impact of *Helicobacter pylori* Secretome and Exosomal Signaling

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree

of

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**The Gut-Brain Axis in Neurodegeneration: Impact of *Helicobacter pylori* Secretome and Exosomal Signaling**" in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2024 to May 2025 under the supervision of Dr. Hem Chandra Jha, Associate Professor, Indian Institute of Technology Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Srija Mukherjee 22.05.2025

Signature of the student with date

(SRIJA MUKHERJEE)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

22/5/2025

Signature of MSc thesis supervisor

(DR. HEM CHANDRA JHA)

SRIJA MUKHERJEE has successfully given her M.Sc. Oral Examination held on **06-05-2025**

Signature of Supervisor of MSc thesis

Date: 22/5/2025

P.V. Kodgire

Convener, DPGC

Date: 22/05/2025

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DEDICATION

This thesis is a tribute to those whose love and encouragement have been my constant companions- thank you for believing in me, inspiring me, and lifting me higher than I ever thought.

ABSTRACT

Chronic *Helicobacter pylori* (*H. pylori*) infection is increasingly linked to neurodegenerative disorders, though the exact mechanisms still remain unclear. This study investigates how *H. pylori* modify exosome cargo of infected gastric cells to drive neuroinflammation and oxidative stress in a neural triculture model. Conditioned media from *H. pylori*-infected gastric cells induced significant upregulation of proinflammatory cytokines (IL-6, TNF-α, IL-1β, IL-10), chemokines (CXCL1, CXCL11), and the inflammasome component NLRP3 in neural cells, alongside oxidative stress marked by elevated ROS production. Neurodegenerative markers, including amyloid precursor protein (APP), apolipoprotein E4 (APOE4), presenilin 1 (PSEN1), and glial fibrillary acidic protein (GFAP), were profoundly elevated, suggesting direct involvement in pathogenic pathways. Exosomes isolated from infected gastric cells exhibited biophysical alterations, including increased surface roughness (observed via AFM) and aggregation propensity (SEM), likely due to oxidative stress or aberrant protein/lipid composition. These exosomes carried post-translationally modified CD63, a tetraspanin linked to immune evasion, and activated inflammatory signaling pathways in neural cells, notably enhancing phosphorylation of p38 MAPK and NF-κB while suppressing p44/42 ERK (1/2). These findings highlight *H. pylori*'s capacity to hijack host exosomal machinery, facilitating gut-brain axis disruption through oxidative and inflammatory pathways. The study underscores exosomes as critical mediators in *H. pylori*-associated neurodegeneration, linking bacterial persistence to chronic neuroinflammation and protein aggregation seen in conditions like Alzheimer's disease.

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ACRONYMS

AGS	Adenocarcinoma gastric cell
IMR-32	Institute for Medical Research- 32
U87-MG	Uppsala 87 Malignant Glioma
CHME-3	Human Microglia Clone 3
BHI	Brain heart infusion
CagA	Cytotoxin associated gene
Vac A	Vacuolating cytotoxin A
CM	Conditioned media
FBS	Foetal bovine serum
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
qRT-PCR	Quantitative RT-PCR
TNFα	Tumor necrosis factor-α
IL-6	Interleukin-6
IL 1β	Interleukin-1 beta
APP	Amyloid precursor protein
APOE4	Apolipoprotein E
GFAP	Glial Fibrillary Acidic Protein
MBP	Myelin Basic Protein
BACE1	Beta-secretase
PSEN1	Presenilin-1
ACHE	Acetylcholinesterase
BUCHE	Butyrylcholinesterase
COX-2	Cyclooxygenase-2
ALDO-C	Aldolase C

CHAPTER 1: Introduction

1.1 The microbiota-gut-brain axis

The microbiota-gut-brain axis (MGB), a bidirectional communication that links the gastrointestinal tract's microbial communities with the central nervous system (CNS) via neural, endocrine and immune pathways. The axis comprises gut microbiota, the enteric nervous system (ENS), vagus nerve, and systemic mediators like neurotransmitters such as serotonin and dopamine along with metabolites such as short-chain fatty acids (SCFAs) [1]. Gut microbes influence CNS function by modulating blood-brain barrier (BBB) integrity, neuroinflammation, and synaptic plasticity while the brain regulates gut motility and secretion through autonomic pathways [2]. The vagus nerve serves as a critical neural conduit, transmitting gut-derived signals to the brain and vice versa [3]. Dysregulation of the MGB axis is implicated in neurodegenerative and neuropsychiatric disorders, including Alzheimer's disease (AD), Parkinson's disease (PD) and anxiety [4]. Dysbiosis disrupts immune homeostasis, elevating systemic cytokines like IL-6, TNF-α known to compromise BBB integrity and promote amyloid-β deposition in AD. Experimental models show that germfree animals exhibit impaired neurogenesis and cognitive deficits which can be ameliorated by specific probiotics or microbiota-derived metabolites that restore BBB integrity and modulate glial cell function [5]. Clinical and preclinical evidence suggests that interventions such as dietary modification, probiotics, prebiotics, and fecal microbiota transplantation (FMT) can positively influence the MGBA, thereby offering promising therapeutic avenues for neurological disease management by targeting the gut microbiome [6].

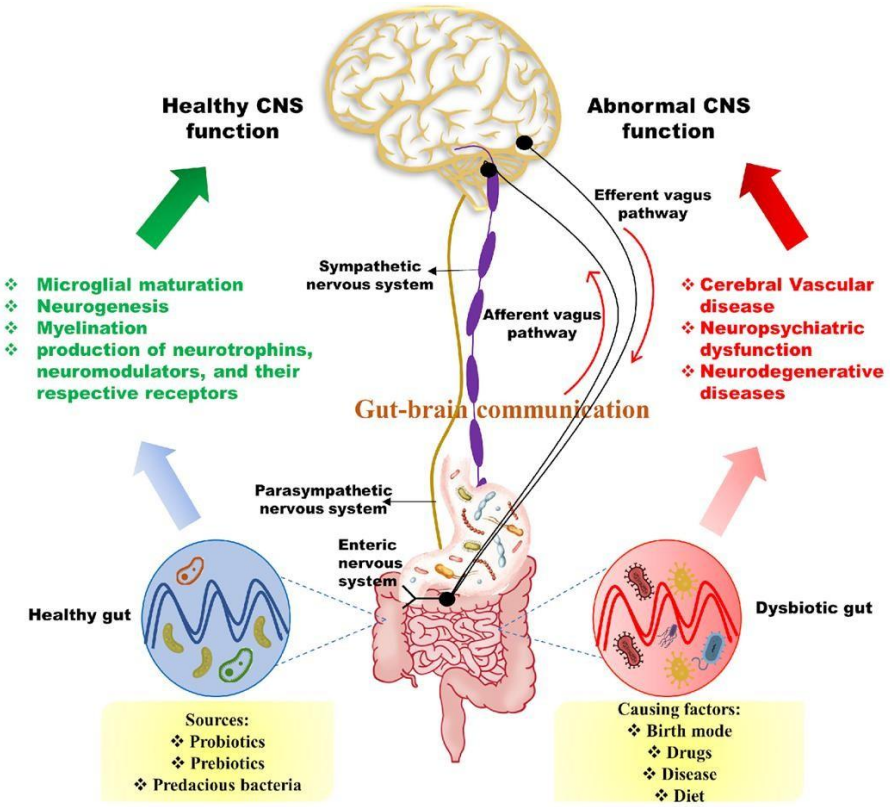


Figure 1: Schematic diagram showing communication between the gut and brain.
(**Source:** Kandpal M., Jha H.C. et al. Metabolites, 2022)

1.2 Pathogen of interest: *Helicobacter pylori*

Helicobacter pylori (H. pylori), a gram-negative and communicable pathogen, is responsible for infecting approximately 4.4 billion people worldwide, or roughly 70% of the population [7-8]. Typically acquired in childhood, the infection persists throughout one's lifetime, leading to progressive chronic gastric inflammation that can result in clinical complications in 1-10% of those infected, such as peptic ulcer disease, gastric atrophy, gastric intestinal metaplasia, and eventually, gastric cancer or mucosa-associated lymphoid tissue (MALT) lymphoma [9]. The pathogenesis of *H. pylori* is a multifactorial process involving dynamic interactions between bacterial virulence factors, host immune responses, and the gastric microenvironment [10]. Colonization begins with the bacterium's ability to survive the acidic gastric milieu, primarily through the production of urease, which hydrolyses urea to

ammonia, thereby neutralizing gastric acid and creating a more hospitable local pH, whereas motility is conferred by flagella which helps the bacteria in traversing the gastric mucus layer and establishing infection at the epithelial surface [10-11]. Adhesion to gastric epithelial cells is mediated by a suite of outer membrane proteins and adhesins, including BabA and SabA, which facilitate intimate bacterial attachment and promote the delivery of virulence factors into host cells. The cytotoxin-associated gene A (CagA) protein is delivered into host cells via a type IV secretion system (T4SS), where it undergoes phosphorylation and interacts with multiple host-signaling pathways, leading to disruption of cell polarity, cytoskeletal rearrangements, and altered cell proliferation and migration. Another critical virulence factor is the vacuolating cytotoxin A (VacA), which forms pores in host cell membranes, induces vacuolation, disrupts mitochondrial function, and impairs immune cell activity by inhibiting T and B cell proliferation and promoting apoptosis [12]. *H. pylori* can alter host gene expression, including epigenetic modifications and microRNA deregulation, which play roles in carcinogenesis and immune dysregulation [13]. As there is no effective vaccine, managing chronic *H. pylori* infection has become the primary approach for controlling the spread of the bacterium in the population, resolving gastric lesions in infected individuals, and preventing the development of subsequent gastric cancer. Furthermore, a significant paradigm shift has occurred since the Kyoto *H. pylori* conference in 2015, which recommended that all *H. pylori* infections be eradicated, unless there are valid reasons to avoid doing so, such as co-morbidities, high rates of reinfection in a particular region, or competing health priorities of society.

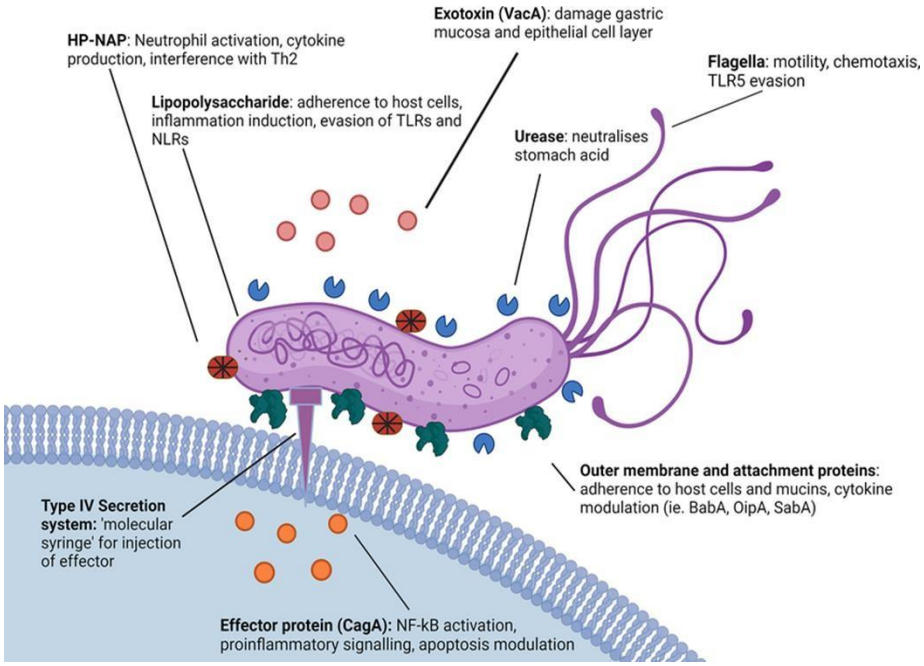


Figure 2: Role of H. pylori virulence factors in innate immune evasion and pathogenesis.
(**Source:** Daniel.S, et al.Frontiers in Immunology, 2022)

1.3 *H. pylori* pathogenesis in neurodegenerative disorders

H. pylori infection exacerbates neurodegeneration through multiple gut-brain axis mechanisms. Chronic infection induces systemic inflammation via elevated pro-inflammatory cytokines, thereby compromising blood-brain barrier integrity and facilitating neurotoxic molecule infiltration, including amyloid-β (Aβ) and bacterial toxins [14-15]. Clinical studies associate *H. pylori* with a 36% increased risk of all-cause dementia, cortical thinning in AD-vulnerable brain regions, and worsened motor symptoms in Parkinson's disease [16]. Research shows that eradication therapy improves cognitive function in AD and motor deficits in PD, suggesting a reversible component to infection-driven neurodegeneration. The bacteria's disruption of gut microbiota diversity further impairs neurotransmitter production such as dopamine and serotonin [17]. *H. pylori* outer membrane vesicles (OMVs) directly promote Aβ aggregation through lipid components like LPC 18:0, accelerating plaque formation and synaptic impairment in Alzheimer's disease (AD) models [18]. The bacterial proteins show molecular

mimicry with neuronal antigens, triggering autoimmune responses, exacerbating neuroinflammation and tau hyperphosphorylation. Additionally, this infection reduces vitamin B12 absorption, elevating homocysteine levels linked to endothelial damage and neuronal death [19].

1.4. Role of extracellular vesicles in neurological modalities

Extracellular vesicles (EVs) are heterogeneous, membrane-bound particles secreted by all cell types, playing critical roles in intercellular communication and disease pathogenesis. They are broadly classified into exosomes (30–150 nm), microvesicles (100–1,000 nm), and apoptotic bodies (50–5,000 nm), distinguished by their biogenesis pathways [20-21]. They are increasingly recognized as pivotal mediators in neurobiology, facilitating intercellular communication within the central nervous system and influencing both physiological and pathological processes. In normal brain function, EVs contribute to neural development, synaptic plasticity, and metabolic support by transferring signaling molecules and genetic material between cells, thus regulating neural circuit formation and maintenance. Conversely, in neurodegenerative diseases, EVs have been implicated in the propagation of pathological proteins such as amyloid-beta and tau, thereby facilitating the spread of neurotoxic aggregates and exacerbating disease progression. Moreover, EVs can modulate neuroinflammation by transporting cytokines and other immune mediators between glial cells, influencing the brain's immune environment. The ability of EVs to cross the blood-brain barrier further highlights their potential as biomarkers for neurological disorders and as vehicles for targeted drug delivery [22]. Host-derived extracellular vesicles (EVs), particularly exosomes, are co-opted by pathogens to enhance infection and immune evasion through diverse mechanisms. Intravacuolar pathogens such as *H. pylori* and *Mycobacterium tuberculosis (M. tuberculosis)* hijack host EV biogenesis pathways to traffic virulence factors: *H. pylori* secrete the oncoprotein CagA into

exosomes, which are systemically disseminated to induce cytoskeletal changes (e.g., hummingbird phenotype) and pro-inflammatory signaling in distal cells [23]. Similarly, *M. tuberculosis* exploits exosomes to deliver immunomodulatory molecules (e.g., bacterial lipids, miRNAs) that suppress antimicrobial responses while promoting cytokine release [24-25]. These EVs act as decoys, shielding pathogens from neutralizing antibodies and facilitating systemic spread. Bacterial outer membrane vesicles (OMVs) further engage host pattern recognition receptors (TLR2/4, NOD1/2) to trigger divergent immune outcomes, ranging from tolerance to chronic inflammation [26]. For example, *H. pylori* OMVs remodel hepatocyte-derived exosomes to downregulate E-cadherin, driving fibrogenic pathways in hepatic stellate cells. Collectively, these strategies highlight EVs as critical mediators of pathogen persistence, bridging localized infections to systemic tissue damage through stealthy molecular cargo delivery [27].

CHAPTER 2: Review of past work and problem formulation

2.1 Literature Review

By means of various clinical and cohort studies, infections with *H. pylori* are associated with a wide array of neurological impairments [14]. Scientific evidence supports the contribution of *H. pylori* infection to cognitive dysfunction and mood disorders and other CNS abnormalities [28-29]. Mechanisms for such effects are not clearly established, but it has been proposed that *H. pylori* may modulate brain functions through multiple pathways. These include induction of systemic inflammation, modulation of immune responses, gut-brain signaling disruption as well as changes in the production of neuroactive compounds and neurotransmitters [30]. Extracellular vesicles budding out from host membrane package both host and pathogenic material and can facilitate the transport of bacterial virulence factors, allowing them to move other parts of the body [23]. Exosomes are type of extracellular vesicles ranging from 50 nm to 150 nm in size and evidence suggests that they are capable of crossing the blood brain barrier [31]. Research suggests that *H. pylori* also have the potential to package its virulent factors inside host derived exosomes and cause extra-gastric diseases. Infection with *H. pylori* triggers several inflammatory pathways which might be responsible for neurodegeneration [30]. The presence of this bacterium initiates the release of pro-inflammatory cytokines, for example, interleukins and tumour necrosis factor alpha that initiates and promotes inflammation. Although this immune reaction starts in the gastrointestinal tract, it may become systemic by entering the brain and causing neuroinflammatory pathways. More specifically, *H. pylori* - induced inflammation could activate microglia, the main immune cells in the brain. Such chronic infection through the persistent activation of the inflammatory pathways can cause persistent neuroinflammation and drive the disease's progression and onset of conditions such as Alzheimer's and Parkinson's [31].

2.2 Problem Formulation

H. pylori infection may contribute to the development of neurodegenerative diseases through exosome-mediated communication, which facilitates the transfer of bacterial components from the gut to the brain. During *H. pylori* infection, the host cells release exosomes containing bacterial proteins, lipopolysaccharides (LPS), microRNAs, and inflammatory mediators as part of the immune response. These exosomes can enter the bloodstream and cross the blood-brain barrier (BBB), either by exploiting a compromised barrier or through direct interactions with brain cells, particularly microglia. Once in the brain, the exosomal cargo triggers the activation of microglia and other immune cells, leading to neuroinflammation, a key factor in the development of neurodegenerative diseases like Alzheimer's, Parkinson's, and multiple sclerosis. This chronic inflammation can induce oxidative stress, disrupt neuronal function, and interfere with the processing of amyloid precursor protein (APP), promoting the accumulation of amyloid-beta plaques, which are characteristic of Alzheimer's disease. In this way, exosomes carrying *H. pylori*-derived material act as a conduit between the gut and the brain, linking chronic infection to neurodegeneration and highlighting the potential role of the gut-brain axis in disease progression.

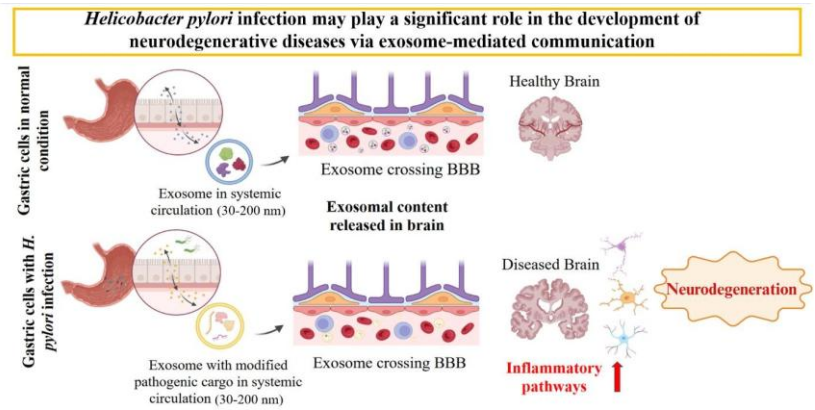


Figure 3: Extracellular vesicle mediated communication between gut and brain.

CHAPTER 3: Aim and objectives

3.1 Aim

The project aims to explore the potential link between *H. pylori* infection and neurodegenerative diseases by investigating the effects of the *H. pylori* secretome and exosomes on neural function. Specifically, the goal is to understand how bacterial secretions and exosome-mediated communication between the gut and the brain contribute to neuroinflammation, oxidative stress, and neuronal dysfunction, which are implicated in the development of neurodegenerative disorders like Alzheimer's and Parkinson's disease. By uncovering these mechanisms, the study seeks to highlight the role of the gut-brain axis in neurodegeneration and identify potential therapeutic targets to mitigate the impact of chronic infections on brain health.

3.2 Objectives

1. **To examine the effects of *H. pylori* secretome on neural function and its potential involvement in neurodegenerative disorders**- This objective focuses on investigating how the bacterial secretions of *H. pylori*, such as VacA and CagA, influence neural function and contribute to neurodegenerative diseases. These secretions can enter the bloodstream and potentially affect the brain by triggering immune responses and promoting neuroinflammation. This neuroinflammatory process may disrupt normal neuronal activity and contribute to the pathogenesis of neurodegenerative conditions like Alzheimer's, Parkinson's, and other cognitive disorders. Understanding the mechanisms by which *H. pylori* secretions interact with the immune system and the brain could reveal how chronic infections impact brain health and accelerate neurodegeneration.

2. **To assess the role of *H. pylori*-derived exosomes in neurodegenerative pathology**- This objective focuses on exploring how *H. pylori*-derived exosomes, which carry bacterial proteins, LPS, microRNAs, and inflammatory mediators, influence the brain's immune response and contribute to neurodegenerative processes. Exosomes can cross the blood-brain barrier and deliver their cargo to neurons and glial cells, triggering the activation of microglia and other immune cells. This can lead to chronic neuroinflammation, oxidative stress, and the accumulation of neurotoxic proteins such as amyloid-beta, which are associated with diseases like Alzheimer's. By studying the role of these exosomes in brain inflammation and damage, this objective aims to deepen our understanding of how *H. pylori* infection via exosome-mediated pathways may play a critical role in the progression of neurodegenerative disorders.

CHAPTER 4: Materials and methods

4.1. Bacterial culture

The three strains of *Helicobacter pylori* used in this study were HB10 (human biopsy sample 10), HJ9 (human gastric juice sample 9) and HB1 (human biopsy sample1), which were isolated from gastric cancer patients. The bacterial strains were revived from glycerol stock on 1X complete Brain Heart Infusion (cBHI) agar plates supplemented with cefsulodin, vancomycin, trimethoprim, and amphotericin B and incubated in a microaerophilic chamber containing specific growth conditions such as 5% oxygen, 10% carbon dioxide, and 85% nitrogen at 37 °C. After 48 hours colonies appeared on the plate, single colonies were picked up using sterile toothpicks and inoculated in 1X cBHI broth and further incubated under microaerophilic conditions [32]. Subsequently, 200 µl of grown culture was placed in duplicate in 96 well flat-bottom plates, and optical density (OD) was recorded at 600 nm. An optical density of 0.3 at 600 nm represents 500 million CFU/ mL. The final OD value was normalized with media as a negative control. The number of bacterial cells per ml (CFU/mL) of culture was evaluated according to the final OD, and the required volume of the bacterial culture for infection was then calculated. For infection studies with different bacterial isolates, MOI 100 was used [33].

4.2. Mammalian cell culture

The following cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India- AGS, IMR-32, U87-MG, and CHME3. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Himedia, Mumbai, India) supplemented with 10% fetal bovine serum (FBS; Sigma, South America origin), 1% penicillin-streptomycin (Himedia, Mumbai, India) and grown in at 37°C with 5% carbon dioxide. The AGS cells were grown to approximately 70% confluency before they were exposed to *H. pylori* at MOI of 100. For exosome isolation the AGS cells were grown in FBS depleted media.

After 24 hours, conditioned media was collected and filtered through a 0.45 μm filter [34]. For the triculture model, IMR-32, U87-MG and CHME3 were used in a ratio of 5:2:1 (neurons: astrocytes: microglia). After 24 hours of seeding when the cells reached around 60-70% confluency, they were exposed to AGS-infected conditioned media as well as exosomes respectively and incubated for another 24 hours.

4.3. Exosome Isolation

The conditioned media collected from *H. pylori* infected AGS cells grown in FBS depleted media was filtered using a 0.45micron filter followed by successive centrifugations at 300g for 5 minutes, 1200g for 20 minutes and 10,000g for 30 minutes at 4° C. Following this the supernatant was ultracentrifuged at 100,000 g for 3 hours at 4° C [35] [36]. Approximately 3 ml of the supernatant was left in the tube without disturbing the exosome pellet and to it the Total exosome isolation reagent from cell culture media (Invitrogen) (Catalog number 4478359) was added and left overnight at 4° C according to the manufacturer's protocol. The next day it was centrifuged at 10,000g at 4° C for 1 hour. The supernatant was discarded and the resulting exosome pellet was dissolved in PBS and stored at -80° C till further use.

4.4. Dynamic Light Scattering for exosome size estimation

The exosome pellet dissolved in PBS was taken and diluted 200 times using PBS and measurements were carried out using Zetasizer Nano series (Anton Paar). These experiments were carried out in PBS buffer at 25°C with 10 acquisitions of 10 s and repeated three times [37].

4.5. Scanning Electron Microscopy

Exosome preparations were diluted in miliQ water and drop casted on clean glass slide and fixed using 2.5 % glutaraldehyde [38]. They were then dehydrated using 40%, 60%, 80% and 98% ethanol and dried properly for 24 hours before further processing. The samples were

coated with 10 nm gold coating and imaged in FE-SEM Supra 55 (Carl Zeiss, Germany).

4.6. Atomic Force Microscopy

Exosome preparations were diluted in miliQ water and drop casted on clean glass slide and fixed using 2.5 % glutaraldehyde. They were then dehydrated using 40%, 60%, 80% and 98% ethanol and dried properly for 24 hours before further processing. The samples were then imaged using Park Systems NX10 Atomic Force Microscopy.

4.7. q-RT-PCR

RNA was extracted from the cell pellet exposed to secretome and exosomes respectively using the TRIzol reagent, and complementary DNA (cDNA) synthesis was done for further analysis. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of inflammatory genes (IL-6, TNF-α, IL-1β, IL-10, NLRP3, CCR1, CCR3, CCR5, CCR8, CXCL1, CXCL11, ALDO C, COX-2, CCK, ACHE, BUCHE) and disease-related genes (APP, APOE4, GFAP, PS-1, BACE-1, PARK 2, PARK 7, LRRK2, NFκB, p38α and p38β) normalized with GAPDH was performed.

4.8. Western Blotting

The cell pellets and exosomes were lysed respectively in RIPA lysis buffer and vortexed for 1 hour with cycles of 5-minute ice cooling. The lysates were spun at 14,000 rpm for 20 minutes and the protein-containing supernatant was transferred to a new microcentrifuge tube. Protein concentration was determined by Bradford assay for cellular proteins and BCA assay was performed to determine exosome concentration. Equal units of protein were mixed with 4X loading dye and placed at 95°C for denaturation of the protein. The proteins were resolved on SDS-PAGE gels, blotted onto nitrocellulose membranes. The latter was blocked by 4.5% BSA. These membranes were incubated overnight at 4°C using primary antibodies.

Antibodies used were APP, APOE4, GFAP, MBP (disease markers), signaling pathway markers such as p-NF-κB, t-NF-κB, p-p-38 MAPK, p-38MAPK, p-p44/42 MAPK (ERK1/2), p44/42 MAPK (ERK1/2) and CD63 (exosomal surface marker) along with GAPDH used as loading control. Next day it was incubated at room temperature for 1 hour with HRP tagged secondary antibody and was visualized using ECL as substrate.

4.9. DCFDA staining

Triculture cells were incubated with 2',7' Dichlorodihydrofluorescein diacetate (DCFDA) to determine intracellular reactive oxygen species levels. Briefly, cells were treated with 10 µM DCFDA and incubated at 37°C for 30 minutes. During this time, DCFDA diffuses into the cells where it is hydrolyzed by intracellular esterases to yield dichlorofluorescein (DCF), a fluorescent product. After incubation, cells were washed twice in PBS to remove excess dye. The fluorescence was measured using a fluorescence microscope with an excitation of 485 nm and emission at 530 nm.

4.10. Cell viability assay

Cell viability of neural triculture cells upon exposure of exosomes was monitored using MTT assay. $0.8*10^6$ cells were seeded per well of 96 well plate maintaining the ratio of IMR-32, U87MG and CHME3 as 5:2:1. After 24 hours the cells were incubated with three concentration of exosomes-2.5 µg/ml, 5 µg/ml and 10 µg/ml for 24 hours. Post-incubation MTT reagent was added followed by addition of DMSO. The absorbance was recorded at 570 nm and 590 nm using a multiplate reader.

4.11. Immunofluorescence

The exosomes dissolved in PBS were drop casted on coverslip and allowed to dry for 24 hours before fixing them using 4% paraformaldehyde [39]. They were then blocked using 1% BSA and

incubated with primary antibodies for CD63 and CD9 overnight in a moist box. Next day it was incubated for 1 hour with Alexa fluor 555 and 488 and visualized using confocal microscope [40].

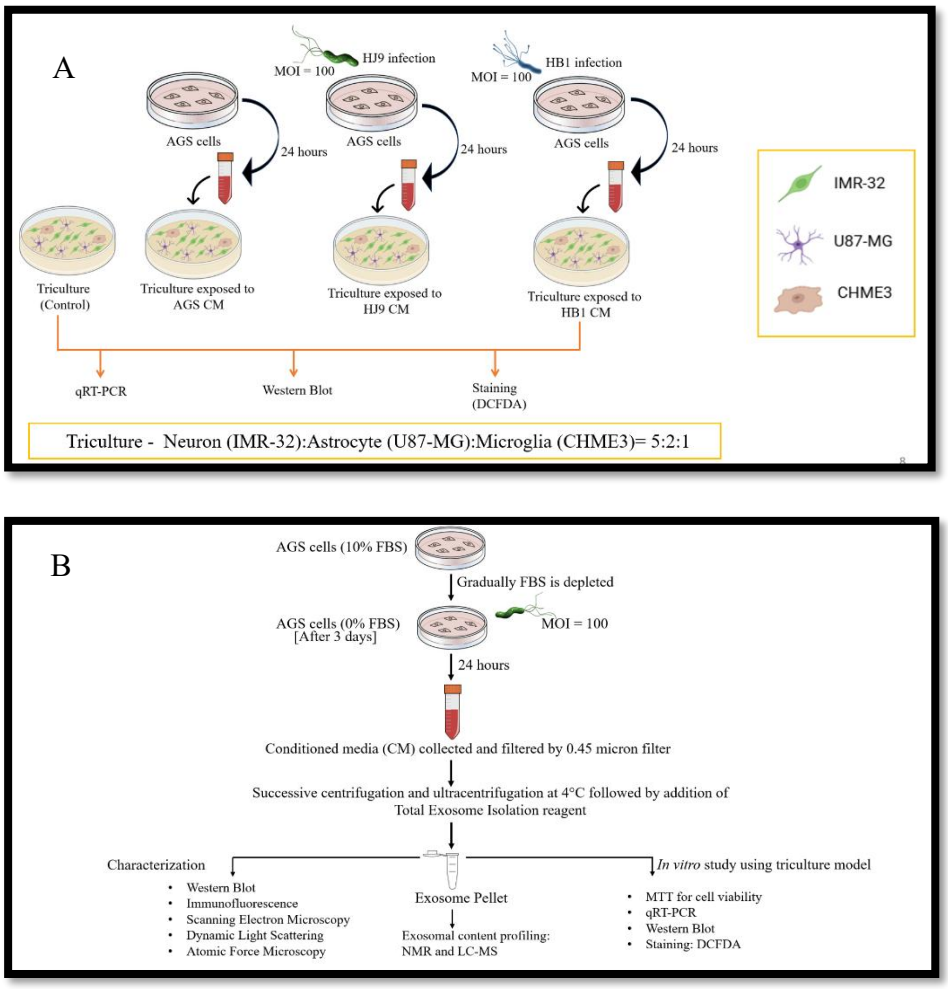


Figure 4: Experimental Methodology.

A) Experimental setup followed for objective 1-Gastric adenocarcinoma cells were infected with different *H. pylori* strains and the collected conditioned media was exposed to neural triculture.

B) Experimental setup followed for objective 2- Gastric adenocarcinoma cells were infected with different *H. pylori* strains and from the collected conditioned media exosomes were isolated and exposed to neural triculture.

CHAPTER 5: Results

5.1. Exacerbation of the inflammatory response in brain cells upon exposure to *H. pylori*-derived secretome in a triculture model

Several studies have indicated that neuroinflammation plays a key role in the progression of neurological disorders, with pro-inflammatory molecules often co-localized with neurodegenerative pathologies. To investigate the potential involvement of *H. pylori* in triggering neuroinflammation, we exposed tricultured cells to conditioned media from *H. pylori*-infected gastric cells and analyzed the transcript expression of various cytokines, chemokines and their receptors.

In tricultured cells upon exposed with HP-CM, we have observed the significantly elevated expression of chemokines: *CXCL1, CXCL11* ($p < 0.01$, 0.01), chemokine receptor: *CXCR1, CXCR3, CXCR5, and CXCR8* ($p < 0.05$) (Fig.5A), pro-inflammatory cytokines: *IL6, TNFα, IL10, IL-1β*, and *NLRP3* ($p < 0.05$, 0.01) (Fig.5B) inflammatory markers: *p38α, p38β, ALDOC, CCK, COX-2* (Fig.5C) and neurotransmitters: *ACHE* and *BUCHE* ($p < 0.05$, 0.01) compared to control (Fig.5D).

Further, investigation of selected inflammatory marker, pNF-κB and tNF-κB through western blot analysis demonstrated a significantly enhanced expression of pNF-κB and tNF-κB in HJ9 and HB1 exposed cells ($p < 0.05$, 0.01) compared to control (Fig.6).

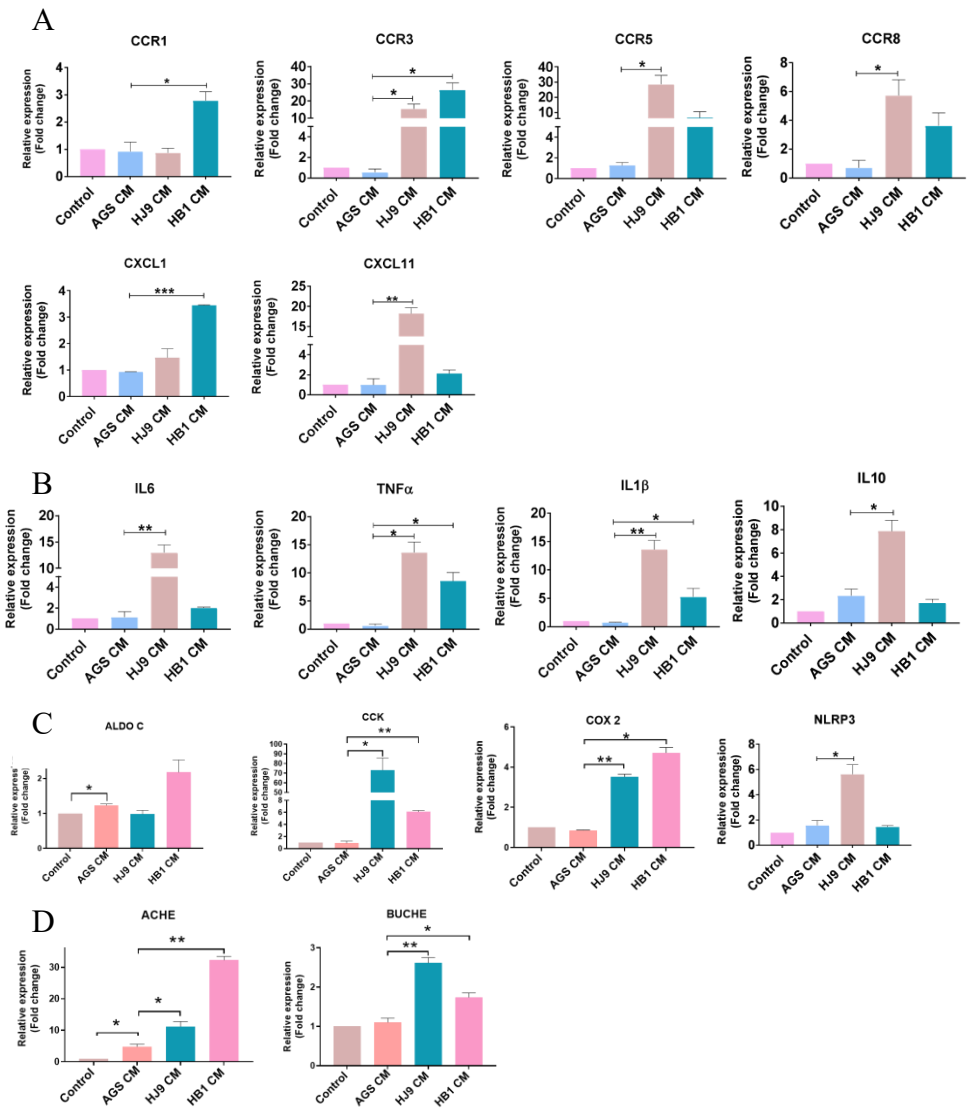


Figure 5: Deregulation of inflammatory markers (cytokines) in triculture upon exposure of *H. pylori* conditioned media. Significantly elevated expression of A) chemokines: *CXCL1, CXCL11* ($p < 0.01$, 0.01), chemokine receptor: *CXCR1, CXCR3, CXCR5, and CXCR8* ($p < 0.05$), B) pro-inflammatory cytokines: *IL6, TNFα, IL10, IL-1β*, and *NLRP3* ($p < 0.05$, 0.01), C) inflammatory markers: *p38α, p38β, ALDOC, CCK, COX-2* and D) neurotransmitters: *ACHE* and *BUCHE* ($p < 0.05$, 0.01) compared to control.

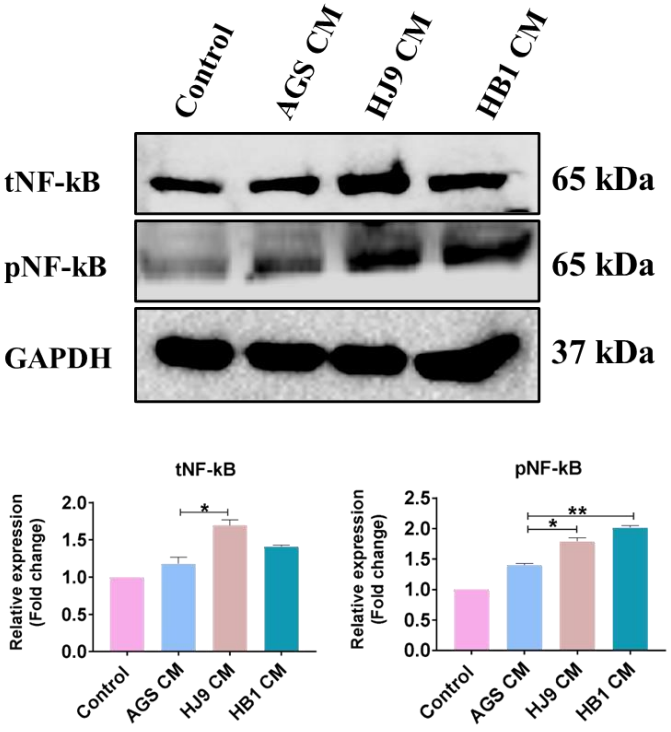


Figure 6:NF-kB pathway activation.
Expression of NF-kB (phospho and total) to assess inflammation demonstrated a significantly enhanced expression of pNF-KB and tNF-kB in HJ9 and HB1 exposed cells ($p < 0.05, 0.01$) compared to control**.**

5.2. *H. pylori*-derived secretome triggers the onset of oxidative stress within the neural compartment

H. pylori infection is well-documented for eliciting oxidative stress in gastric epithelial cells. To investigate whether this effect extends to neural cells, we quantified reactive oxygen species (ROS) levels in tricultured cells exposed to H. pylori-conditioned media (HPCM). ROS generation was assessed using the redoxsensitive probe 2′-7′-Dichlorodihydrofluorescein di-acetate (H2-DCFDA). Our results revealed a statistically significant increase in ROS production in HPCM-exposed cells compared to control ($p < 0.01, 0.05$). Furthermore, tricultured cells exposed to HJ9-conditioned media exhibited a more pronounced oxidative stress response ($p < 0.01$) (Fig.7). These findings provide evidence that the *H. pylori* secretome

induces oxidative stress in neural cells, suggesting a potential mechanism for its role in neuroinflammation.

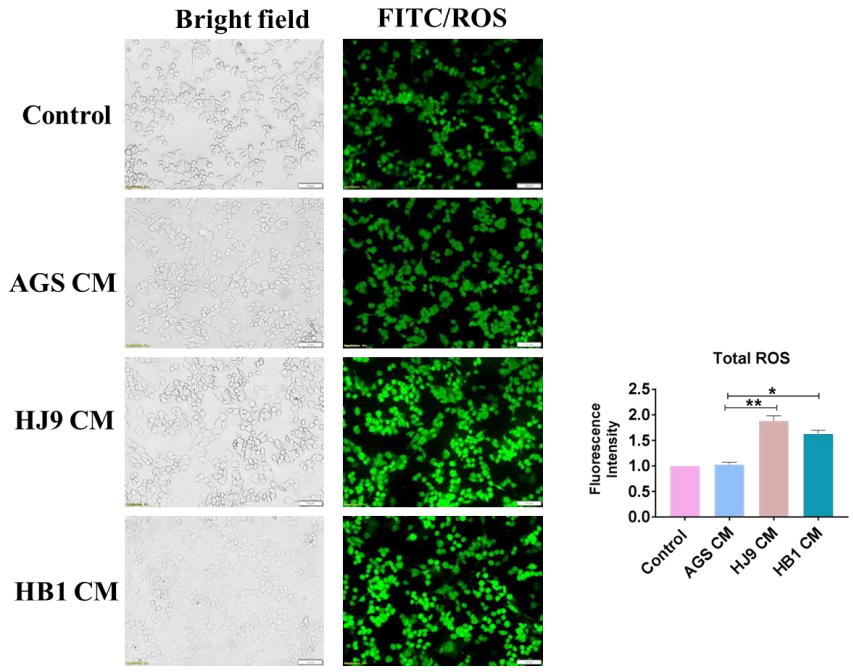


Figure 7: Total intracellular ROS estimation through DCFDA staining.
Statistically significant increase in ROS production in HPCM-exposed cells was observed as compared to control ($p < 0.01, 0.05$). Furthermore, tricultured cells exposed to HJ9-conditioned media exhibited a more pronounced oxidative stress response ($p < 0.01$).

5.3. *H. pylori* secretome provokes the neurodegenerative disease markers

To further verify the plausible association of *H. pylori* infection in neurological complications, we assessed the several neurodegenerative markers in exposed tricultured cells at transcript and protein level. We observed the significantly augmented expression of APP, APOE4, GFAP, and PSEN1 in HJ9 CM exposed tricultured cells ($p < 0.05, 0.01$) (Fig.8A). In contrast, we observed elevated expression of APOE4, and GFAP in HB1 CM exposed tricultured cells. At transcript level we observed elevated expression of PARK 2 and PARK 7 genes in case of HB1 CM ($p < 0.05, 0.01$) (Fig.8B).

Furthermore, at protein level, we observed the enhanced expression of APP and APOE4 ($p < 0.05, 0.01$) in the experimental panel compared

to control. The protein expression of GFAP were found to be upregulated in HPCM exposed tricultured cells ($p < 0.05$) (Fig.8C).

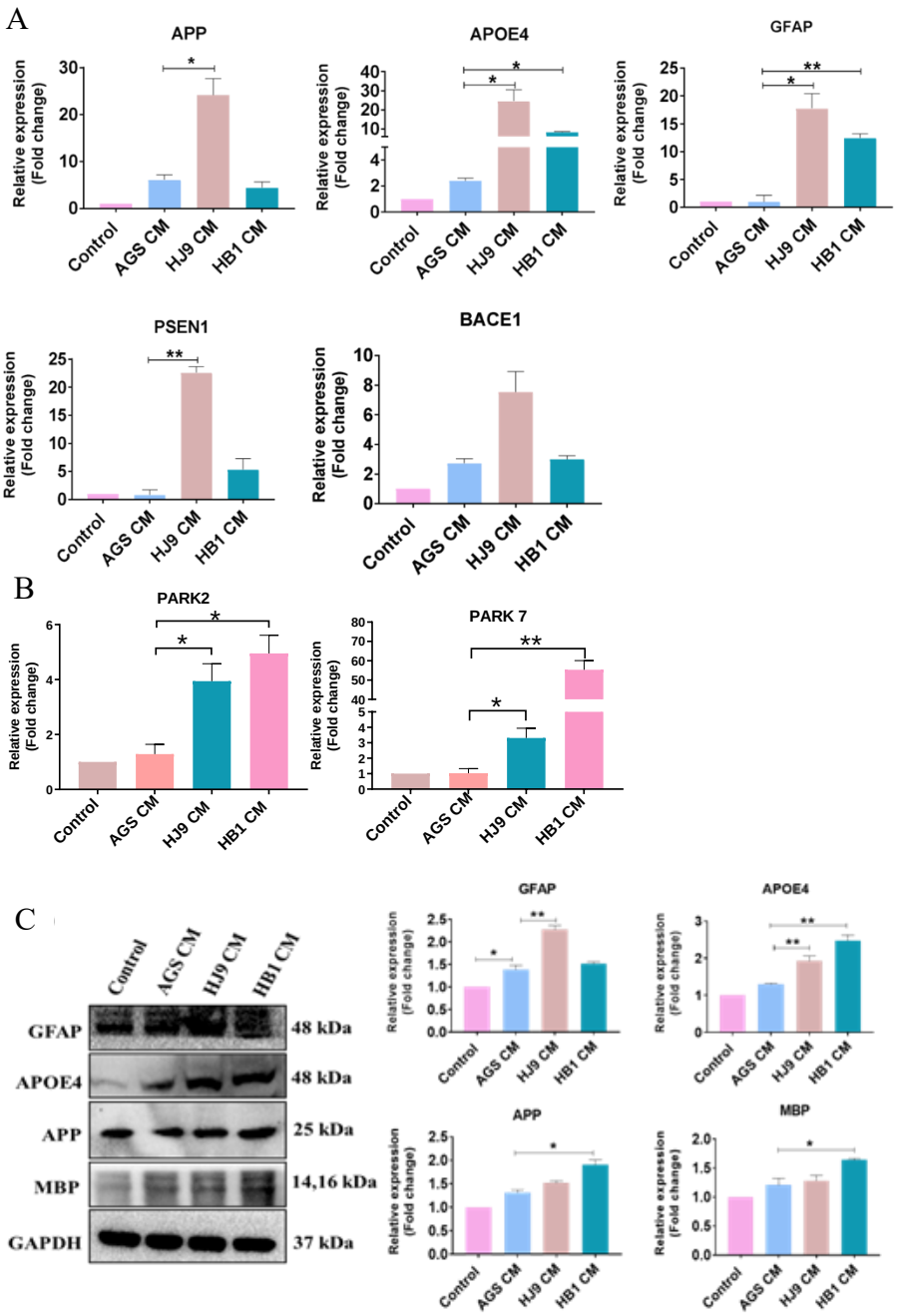


Figure 8:Alteration of neurodegenerative disease markers in triculture upon exposure of H. pylori conditioned media.
At transcript level (A&B) and at protein level (C). Enhanced expression of APP and APOE4 ($p < 0.05, 0.01$) in the experimental panel compared to control was observed. The protein expression of GFAP was found to be upregulated in HPCM exposed tricultured cells ($p < 0.05$). C. Western blot images and Quantification of western blot.

5.4. Estimation of exosome size, morphology and surface topology

The DLS analysis gave us idea about the hydrodynamic diameter of the exosomes. The diameter of the exosomes isolated from AGS CM, HB10 CM, HJ9 CM and HB1 CM were of size 115 nm, 163nm, 165 nm and 204 nm respectively (Fig.9). The SEM images showed that the exosomes had a rounded morphology and are 94 nm, 113nm, 115 nm, 144 nm (Fig.10) in size isolated from AGS CM, HB10 CM, HJ9 CM and HB1 CM respectively. The AFM gave us an idea of the surface topography of the exosomes and the roughness of the exosome surface was found to be 3.49 nm, 6.18 nm, 4.07 nm (Fig.11) in case of AGS CM, HJ9 CM and HB1 CM respectively.

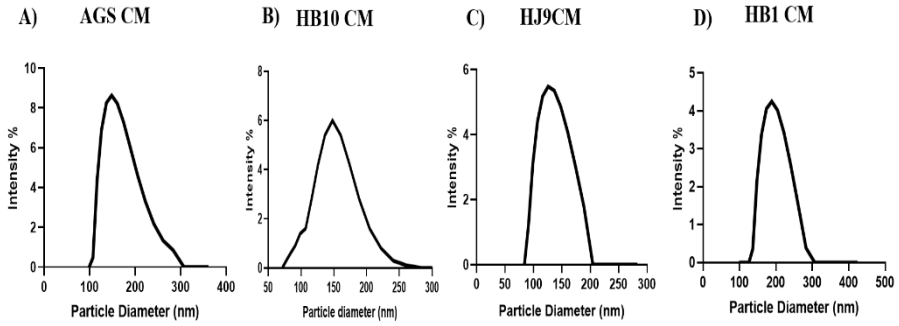


Figure 9:Distribution of exosome size by DLS.
Graph showing the distribution of exosome size isolated from A) AGS CM, B) HB10 CM, C) HJ9 CM D) HB1 CM by DLS analysis**.** The diameter of the exosomes isolated from AGS CM, HB10 CM, HJ9 CM and HB1 CM were of size 115 nm, 163nm, 165 nm and 204 nm respectively.

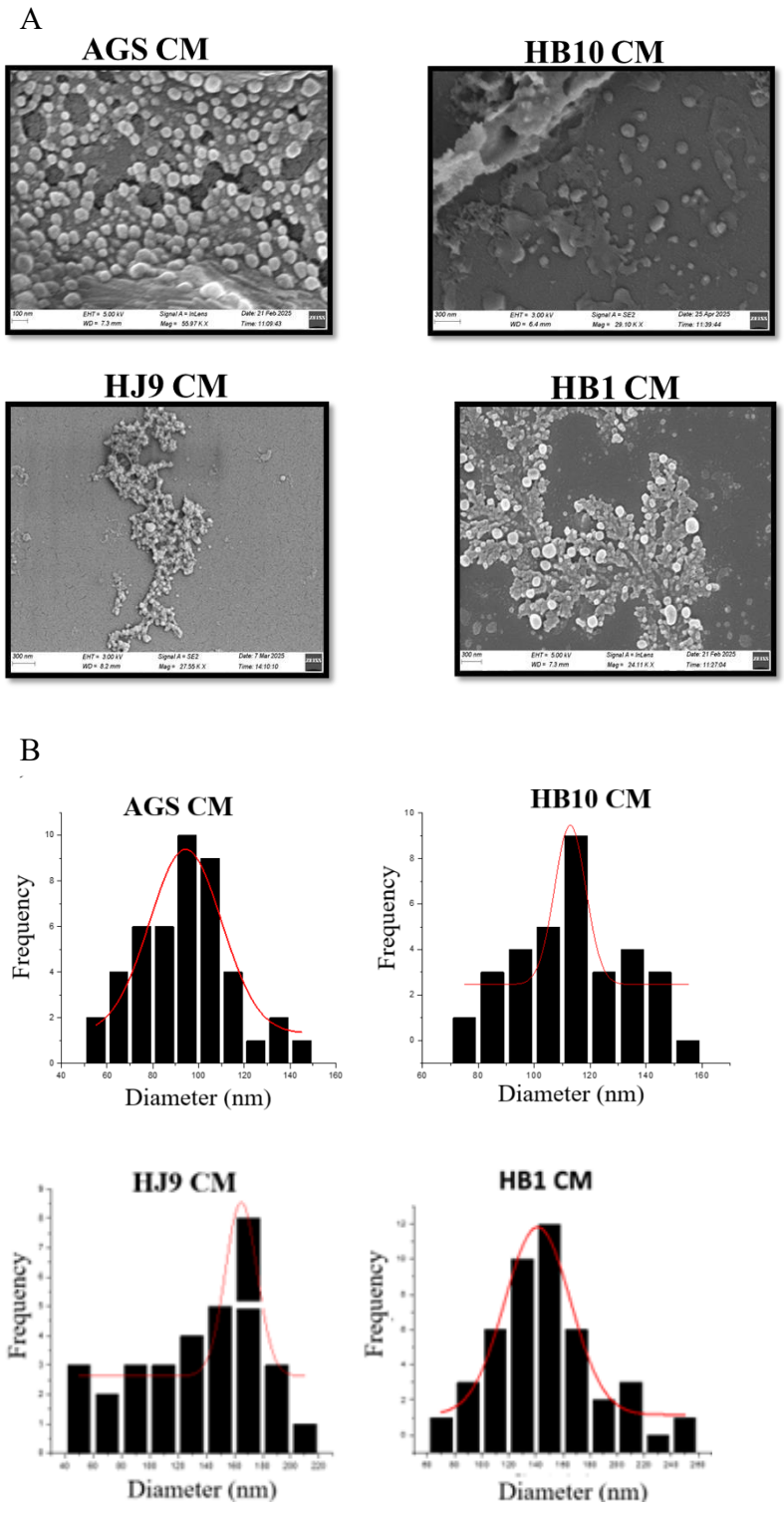
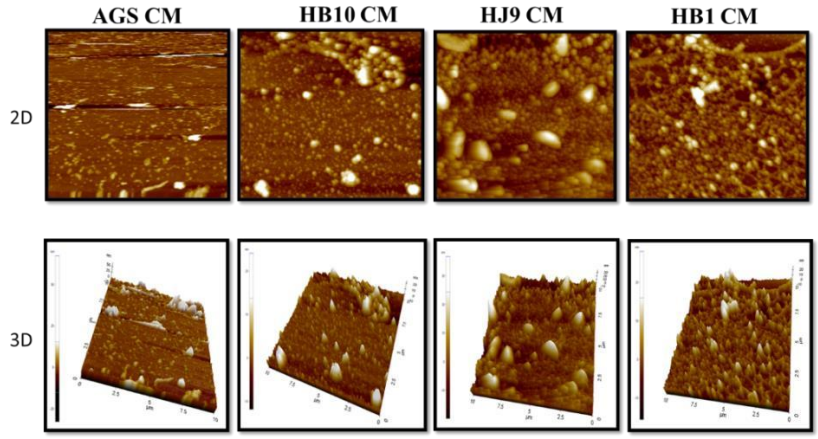


Figure 10:Exosome morphology and size.
A) FE-SEM images of the isolated exosomes.
B) Size distribution graph of exosomes.
The SEM images showed that the exosomes had a rounded morphology and are 94 nm, 113nm, 115 nm, 144 nm in size isolated from AGS CM, HB10 CM, HJ9 CM and HB1 CM respectively.

A



B

Sample	Rq (nm)	Ra (nm)
AGS CM	3.88	2.130
HB10 CM	3.49	2.31
HJ9 CM	6.18	4.51
HB1 CM	4.07	2.992

Figure 11:Exosome surface topography.
A) 2D and 3D AFM images of the isolated exosomes.
B) Table showing roughness parameters of the isolated exosomes.

5.5. Evaluation of surface markers in isolated exosomes from *H. pylori* secretome

Exosomes were isolated from *H. pylori*-derived conditioned media using a multi-step ultracentrifugation process. This involved sequential centrifugation steps to remove cellular debris and microvesicles, followed by high-speed ultracentrifugation to pellet the exosomes. We checked the expression of exosomal surface marker (CD63) in isolated exosomes via western blotting (Fig 12) and observed a highly glycosylated band corresponding to molecular weight of 30-60 kDa in

case of infected samples. We performed immunofluorescence for checking the expression of exosome surface markers CD9 and CD63 (Fig 13).

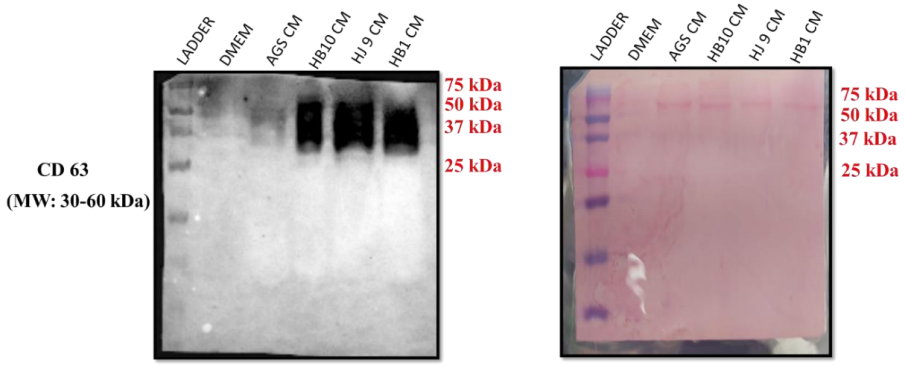
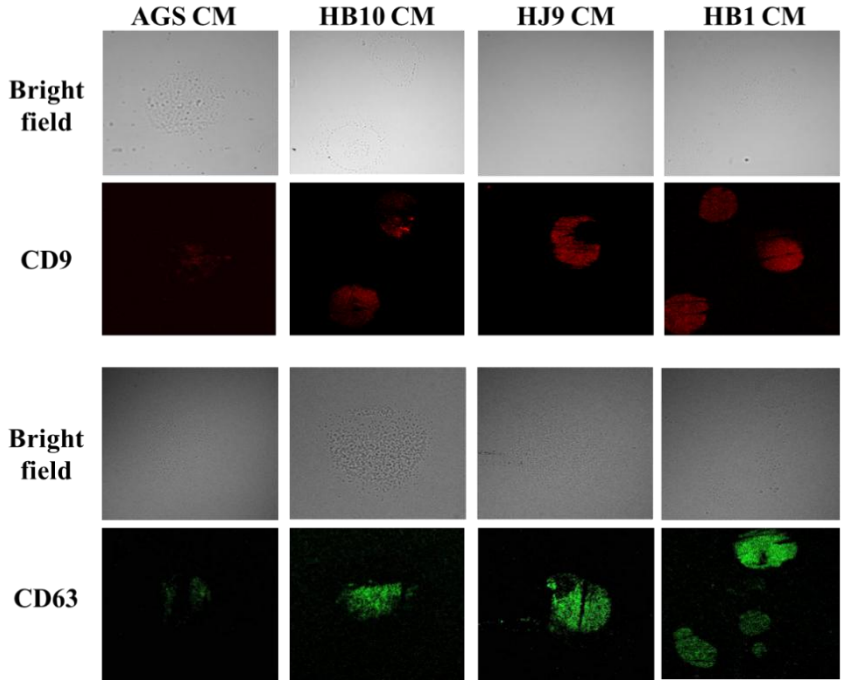


Figure 12: Western blot image of isolated exosomes.
Western blot of isolated exosome from *H. pylori*-infected gastric epithelial cells showing the expression of surface marker CD63 corresponding to molecular weight 30-60 kDa



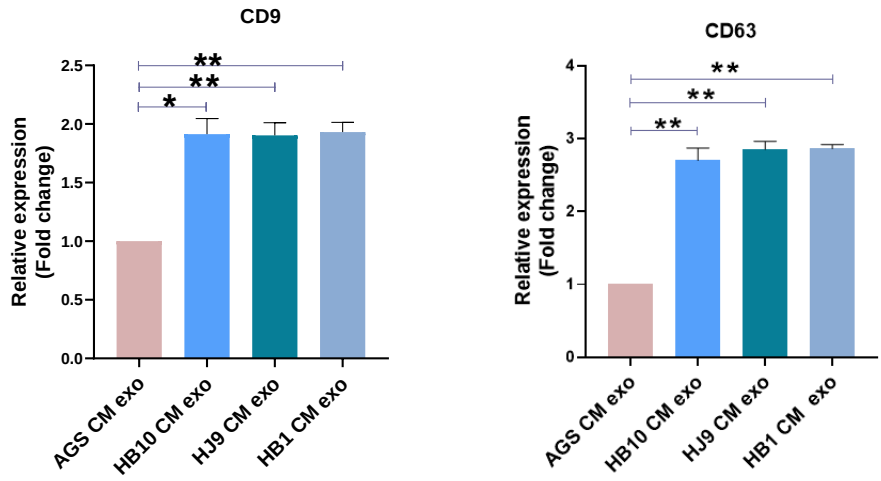


Figure 13:Immunofluorescence image of exosome surface markers. Representative IF image of surface markers (CD9 & CD63) of exosomes isolated from gastric epithelial cells. The expression of both the markers was found to be significant with *p*-values of < 0.05 for HB10 CM exo (CD9) and < 0.01 for HJ9 CM exo and HB1 CM exo (CD9); and *p*-values of < 0.01 for HB10 CM exo, HJ9 CM exo and HB1 CM (CD63)

5.6. Cell viability assay of exosomes

The cell viability assay was performed by exposing the neural triculture system to varying concentrations of exosomes isolated from different conditioned media, namely AGS CM, HB10 CM, HJ9 CM, and HB1CM. These exosomes were applied to the cells to assess their impact on cellular survival and proliferation. The results from the assay provided the IC10 (concentration that causes a 10% reduction in cell viability) values for each exosome preparation. The IC10 values obtained are 27.2 µg/ml, 9.02 µg/ml, 5.44 µg/ml, and 2.54 µg/ml in case of AGS CM, HB1O CM, HJ9 CM, HB1 CM respectively (Fig. 14). The IC10 values represent the lowest concentration of exosomes at which a slight reduction in cell viability is observed, indicating a threshold at which the exosomes begin to influence cell health. For future experiments, the IC10 values will be particularly important as they will be used to select a concentration range that minimizes cytotoxicity,

ensuring that the exosomes' effects on the cells can be studied without causing substantial cell death. By using the IC10 concentration in subsequent assays, we can further explore the subtle, non-lethal effects of exosomes on neural cell behaviour, signaling, and interactions, allowing for a deeper understanding of their biological functions in this context.

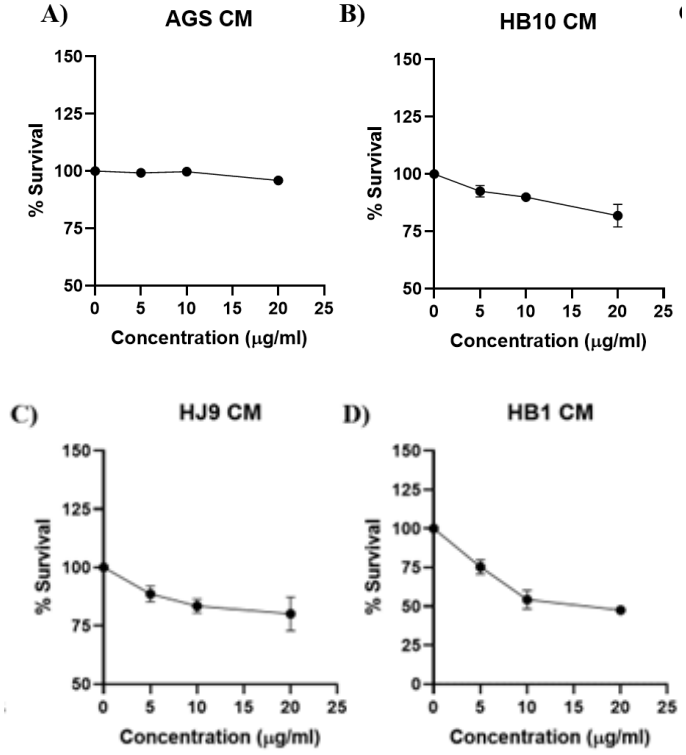


Figure 14:Percentage survivability curve.
The graphs above represent the percentage survivability curve of the triculture cells upon exposure to exosomes isolated from A) AGS CM, B) HB1O CM, C) HJ9 CM, D) HB1 CM. The IC10 values obtained are 27.2 μg/ml, 9.02 μg/ml, 5.44 μg/ml, 2.54 μg/ml in case of AGS CM, HB1O CM, HJ9 CM, HB1 CM respectively

5.7. Inflammatory cascades in triculture model upon exposure of *H. pylori* derived exosomes

Numerous studies have identified neuroinflammation as a pivotal contributor to the progression of neurological disorders, with pro-inflammatory molecules frequently co-localizing with hallmark neurodegenerative features. To investigate whether *Helicobacter*

pylori-derived extracellular vesicles (EVs) play a role in initiating neuroinflammatory responses, we exposed triculture neural cells to exosomes isolated from gastric epithelial cells infected with *H. pylori*. We subsequently evaluated the transcriptional profiles of a panel of proinflammatory markers and key components of associated signaling cascades to delineate the inflammatory responses potentially elicited by *H. pylori*-derived extracellular vesicles.

As a result, we found a significant upregulation of inflammatory markers at transcript level in the experimental set of panels compared to control. We have observed the significantly elevated expression of cytokines: *IL6, IL1β,* and *COX2* ($p < 0.01$, 0.05). Additionally, transcript expression of inflammatory associated signaling pathways such as *NF-kB, p38α,* and *p38β* ($p < 0.01$, 0.05) (Fig.15) were also found to be upregulated in exosomes exposed samples compared to control.

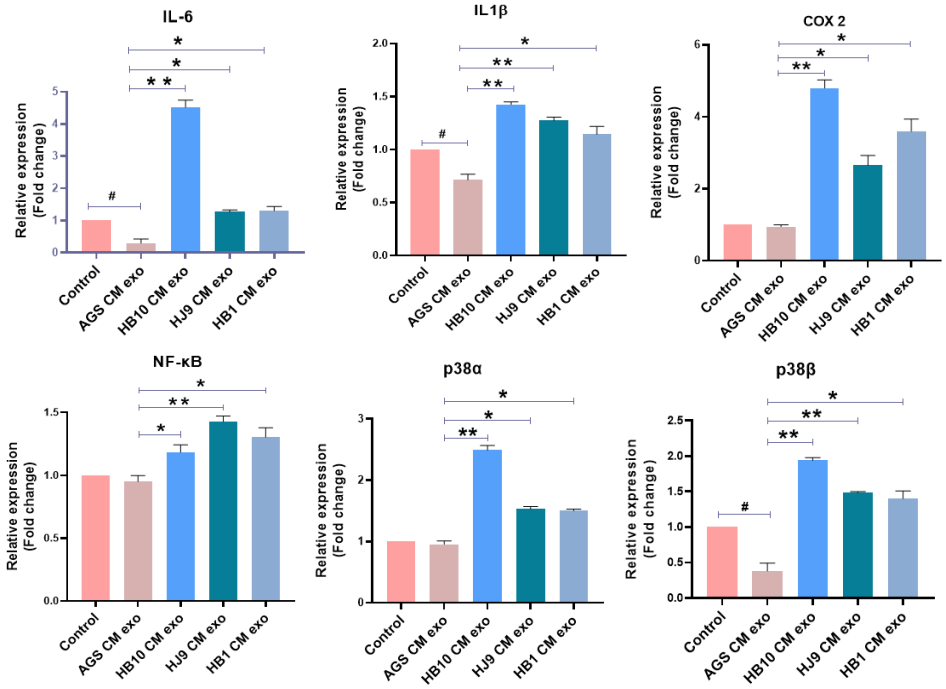


Figure 15:Deregulation of inflammatory markers in triculture upon exposure of *H. pylori* derived exosomes.
Significantly elevated expression of pro-inflammatory cytokines: *IL-6, IL-1β*, COX-2 ($p < 0.01$), in exosome samples isolated from HB1O CM; inflammatory markers: *p38α, p38β, NF-KB* ($p < 0.05$, 0.01) compared to control

5.8. Neurodegenerative signature markers upon exposure of *H. pylori* derived exosomes

Extracellular vesicles are well established mediators of intercellular communication, often encapsulating pathogen-associated virulence factors capable of crossing the blood–brain barrier and potentially contributing to the onset or progression of neurological pathologies. Further, we analyzed the expression of several neurodegenerative signature markers at transcript and protein level. As a result, at transcript level we observed the elevated expression of *APP, APOE4*, *GFAP* ($p < 0.05$, 0.01, 0.001) (Fig. 16) in the experimental panel more significantly in HJ9 and HB1 exosomes exposed samples. At protein level, we found the similar result with the enhanced expression of APOE4, GFP, and MBP (Fig. 17).

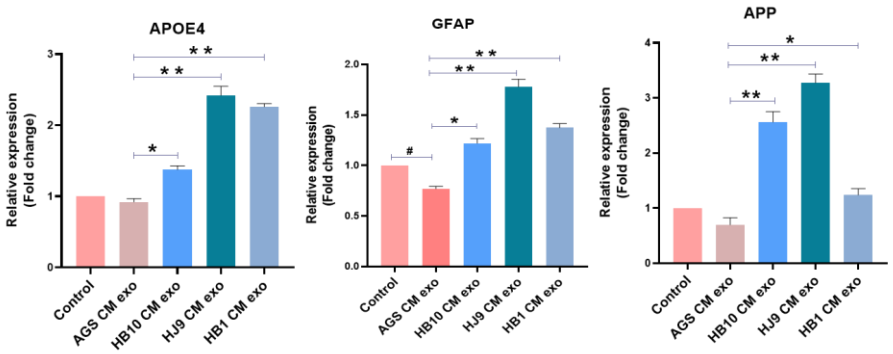


Figure 16:Alteration of neurodegenerative disease markers in triculture upon exposure of exosomes at transcript level.
Elevated expression of *APP, APOE4*, *GFAP* ($p < 0.05$, 0.01, 0.001) was observed in samples exposed to HJ9 and HB1 CM exosomes

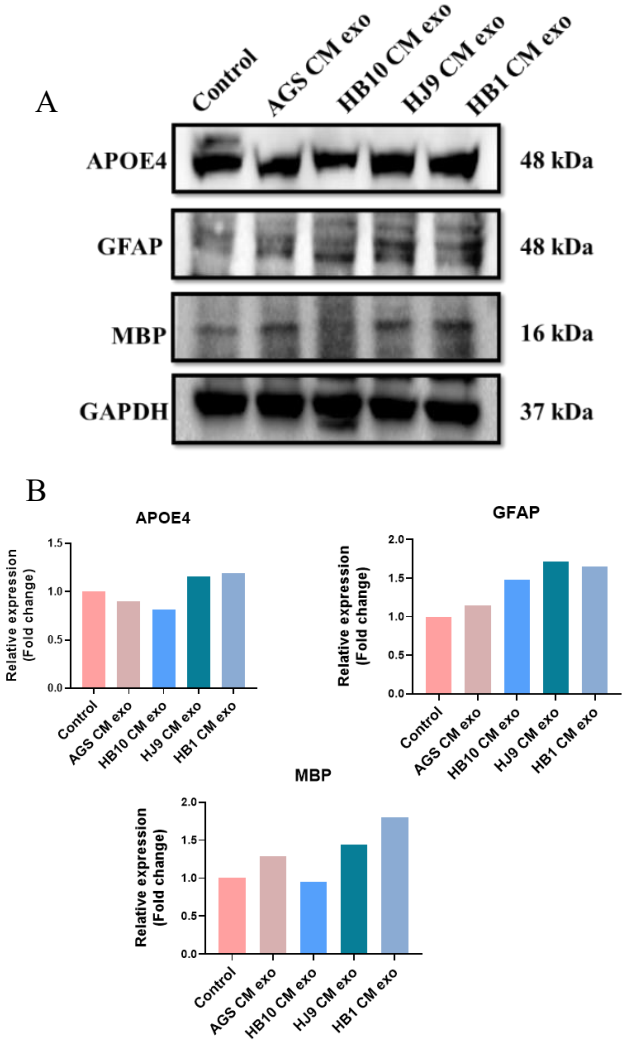


Figure 17: Alteration of neurodegenerative disease markers in triculture upon exposure of exosomes at protein level.
A) Western blot image. Upregulation of APOE4, GFAP and MBP was observed in case of samples exposed to exosomes from infected cells. (B) Quantification of western blot. Slight upregulation of APOE4, GFAP and MBP is observed in the infected samples compared to control

5.9. Instigation of Inflammatory signaling pathway upon exposure of *H. pylori* derived exosomes

Mitogen-activated protein kinases (MAPKs) represent a key family of serine/threonine kinases that mediate the cellular response to a wide range of extracellular stimuli. These kinases function through a conserved cascade of phosphorylation events, ultimately leading to the activation of specific transcription factors and modulation of gene expression. Within this family, the extracellular signal-regulated kinase

(ERK) pathway is particularly associated with the regulation of cell proliferation, differentiation, and inflammatory responses. We further examined the capacity of *H. pylori*-derived exosomes to activate key inflammation associated signaling pathways within a neural triculture model. As a result, we observed the phosphorylation of p38 MAPK ($p < 0.05$, 0.01) (Fig.18) suggesting the activation of MAP kinase pathway in exosome exposed samples. Interestingly, we observed a downregulation of phosphorylated ERK1/2 (p44/42) ($p < 0.05$, 0.01) (Fig.18) in samples exposed to exosomes derived from *H. pylori* strains HB10, HJ9, and HB1, suggesting that these vesicles may facilitate immune evasion mechanisms that promote pathogen persistence within host cells.

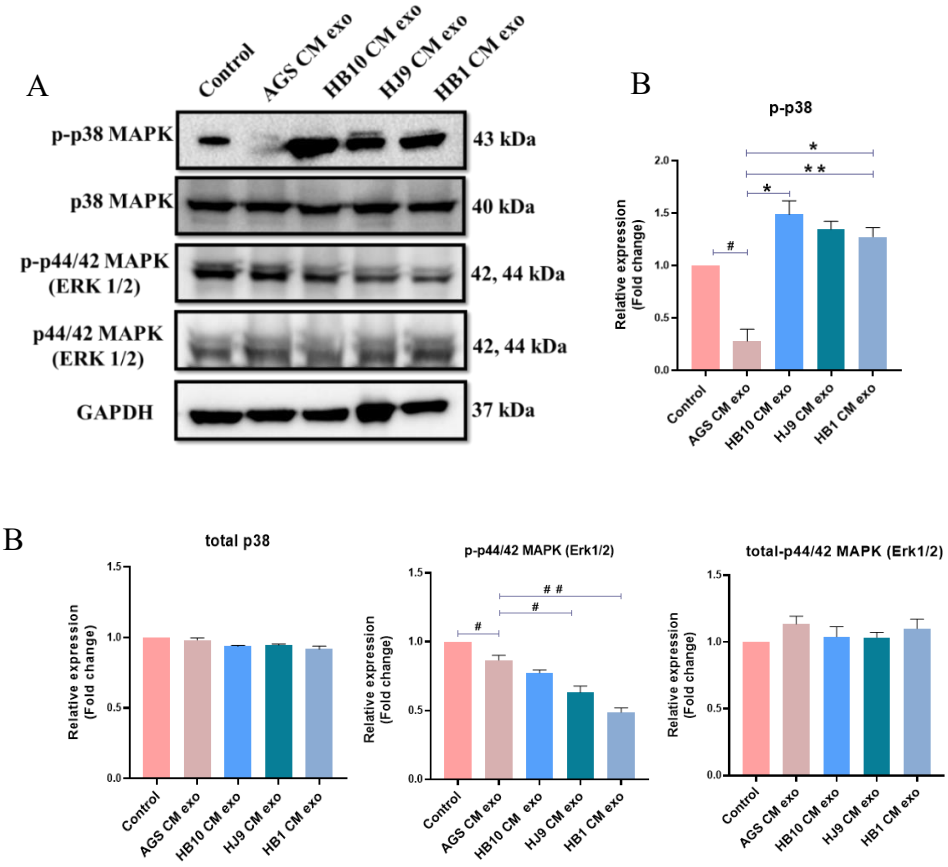
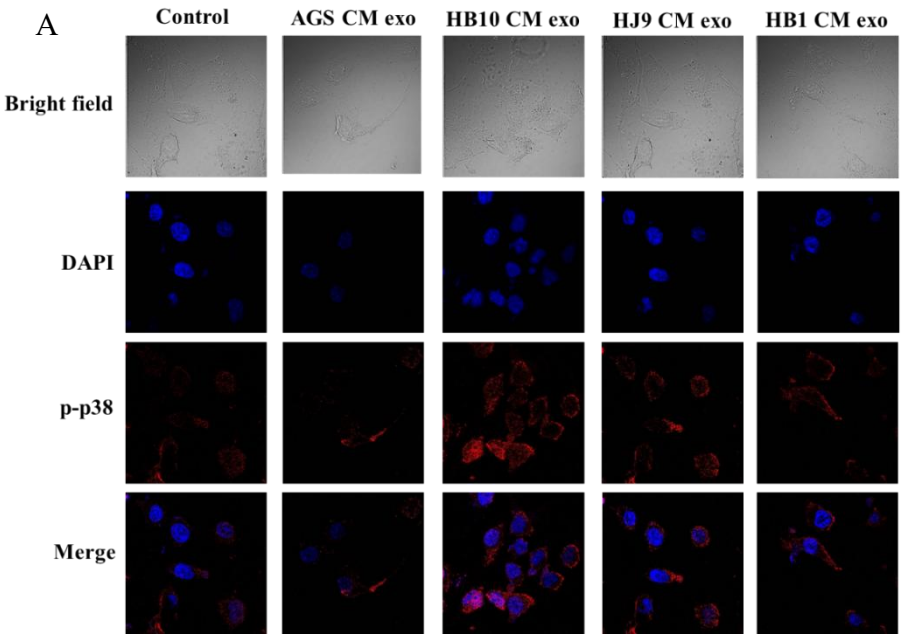


Figure 18:Alteration of MAPK signaling pathway markers in triculture upon exposure of exosomes.
(A) Western blot image, (B) Quantification of western blot. $p < 0.05$ was considered significant in all the cases.



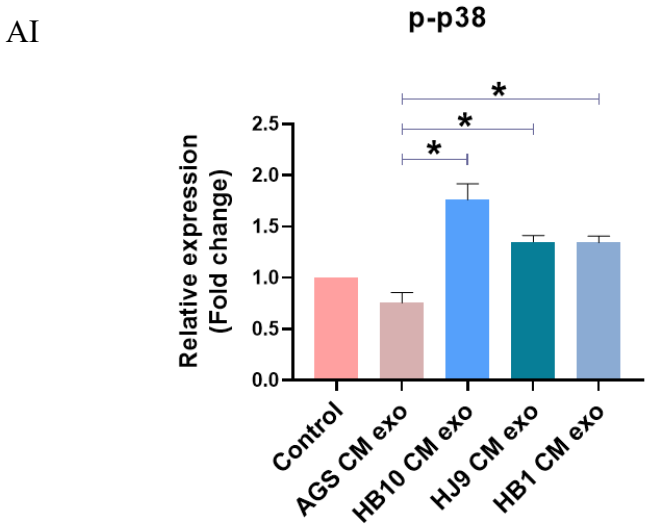
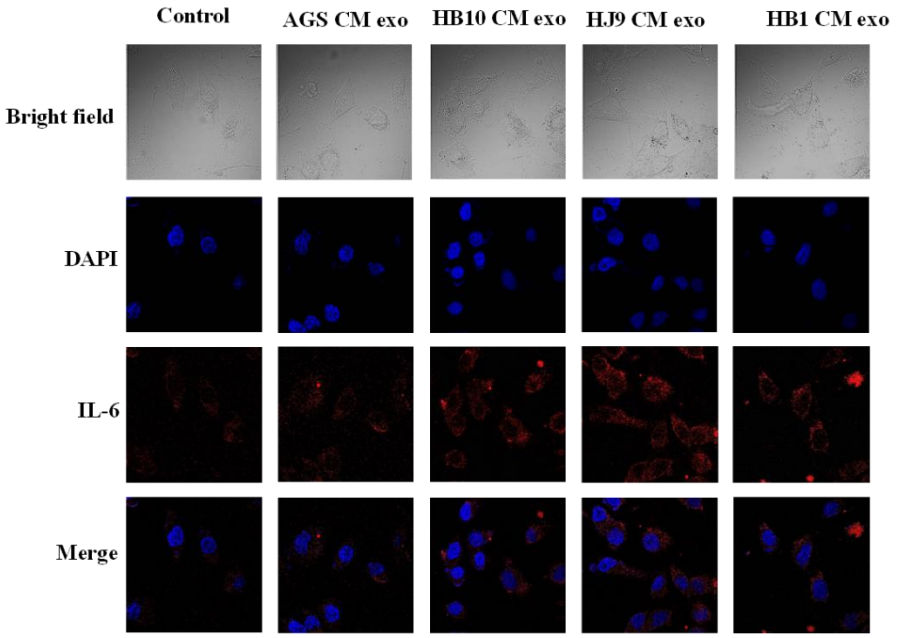


Figure 19:Alteration of p-p38 MAPK signaling pathway marker in triculture upon exposure of exosomes.

(A) IF images, (AI) Quantification of the images. $p < 0.05$ was considered significant in all the cases. p-values of < 0.05, < 0.01 and < 0.001 were represented with *, ** and *** respectively for significant upregulation and #, ##, and ### for significant downregulation.

A



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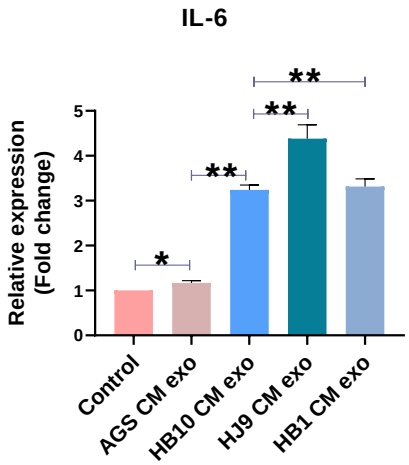

Figure 20:Alteration of IL-6 marker in triculture upon exposure of exosomes.

(A) IF images, (AI) Quantification of the images. $p < 0.05$ was considered significant in all the cases. p-values of < 0.05, < 0.01 and < 0.001 were represented with *, ** and *** respectively for significant upregulation and #, ##, and ### for significant downregulation. Significant upregulation was observed in all infected samples with HJ9 CM resulting in maximum intensity

CHAPTER 6: Discussion

The data demonstrates that conditioned media from *H. pylori*-infected gastric cells (HP-CM) robustly induces an inflammatory response in a triculture brain cell model. The increased expression of these molecules indicates heightened immune cell recruitment and activation, hallmark features of neuroinflammation. Additionally, the elevation of inflammatory markers (p38α, p38β, ALDOC, CCK, COX-2) and neurotransmitter-related enzymes (ACHE, BUCHE) suggests broader impacts on neuronal signaling and metabolic stress [33]. The western blot analysis further supports these findings, with significantly elevated levels of both phosphorylated and total NF-κB in cells exposed to HP-CM, particularly with HJ9 and HB1 strains. This points to activation of the canonical NF-κB pathway, a central regulator of inflammatory gene expression [41]. Collectively, these results indicate that factors secreted by *H. pylori*-infected gastric cells can strongly stimulate inflammatory signaling in brain cells, potentially linking gastric infection to neuroinflammatory processes implicated in neurodegenerative diseases. The observed increase in ROS production in neural tricultures exposed to *H. pylori*-conditioned media suggests that the oxidative stress response, well-documented in gastric epithelial cells, also extends to neural cells [42]. This aligns with findings that *H. pylori* infection triggers chronic oxidative stress through mechanisms involving NADPH oxidase activation, superoxide generation, and downstream formation of toxic ROS species, which can damage cellular components and disrupt mitochondrial function. Notably, the more pronounced oxidative response to the HJ9 strain indicates that specific bacterial factors or virulence determinants (such as CagA) may amplify ROS production, as seen in both gastric and neural contexts. Elevated ROS can activate redox-sensitive inflammatory pathways, including NF-κB and NLRP3 inflammasome signaling, fostering a pro-inflammatory environment that may contribute to neurodegenerative processes [43]. These findings support the hypothesis that *H. pylori*-induced oxidative stress is not limited to the

gut but may play a mechanistic role in neuroinflammation and potentially in the pathogenesis of neurological disorders via the gut-brain axis. Exposure to *H. pylori* HJ9 and HB1 secretomes significantly upregulated neurodegenerative markers in tricultured cells, with strain-specific effects. HJ9-CM elevated APP, APOE4, GFAP, and PSEN1, implicating Alzheimer's disease pathways through amyloid precursor protein processing and ApoE4-driven amyloid aggregation [44]. HB1-CM increased PARK2, PARK7 (Fig.5B), suggesting Parkinson's disease associated mechanisms via mitochondrial dysfunction and proteasomal impairment [45]. GFAP upregulation indicates astrocyte activation, consistent with neuroinflammatory responses triggered by *H. pylori* secretome [33]. The differential expression of APOE4 (elevated in both strains) aligns with its role in exacerbating amyloid pathology and synaptic toxicity, while strain-specific virulence factors (e.g., CagA in HJ9) may drive distinct neurodegenerative pathways. These findings reinforce *H. pylori*'s potential to modulate AD/PD-associated molecular cascades via gut-brain axis interactions.

The analysis of exosomes isolated from *H. pylori*-infected and control conditioned media reveals notable differences in their physical characteristics, suggesting infection-driven modifications. Exosomes from *H. pylori*-infected samples, particularly HB1 and HJ9, displayed significantly larger hydrodynamic diameters (204 nm and 165 nm, respectively) compared to control AGS-derived exosomes (115 nm), as measured by DLS. SEM imaging corroborated these findings, showing increased exosome size in infected samples, though with slightly smaller values due to dehydration during sample preparation. Furthermore, AFM analysis highlighted increased surface roughness in exosomes from infected samples, especially HB1 (6.18 nm), compared to controls (3.49 nm), indicating altered surface topography. These changes in size and surface roughness may reflect strain-specific differences in exosomal cargo or membrane composition, potentially enhancing their capacity to interact with recipient cells and deliver pro-inflammatory or neurodegenerative signals [29]. Collectively, these

findings suggest that *H. pylori* infection not only alters exosome morphology but may also influence their functional role in mediating gut-brain axis communication and contributing to neuroinflammatory processes. These exosomes also elevated neurodegenerative markers (APP, APOE4, GFAP), aligning with Alzheimer's-associated amyloid pathology and astrocyte activation. Strain-specific effects (HJ9/HB1) likely stem from differential EV cargo, as *H. pylori* modifies exosomal content to influence recipient cells. While p38 MAPK activation ($p < 0.05–0.01$) drove inflammation, ERK1/2 suppression ($p < 0.05–0.01$) suggests immune evasion, mirroring mechanisms where OMVs impair host defenses [46]. Structural changes in exosomes (larger size, rougher surfaces) may enhance BBB penetration and cargo delivery, facilitating systemic neuroinflammatory cascades via the gut-brain axis.

CHAPTER 7: Conclusion and future prospects

The comparative analysis of *H. pylori* secretome (soluble factors) and exosome (vesicle-mediated) effects on neural tricultures reveals a multifaceted pathogenic strategy employed by the bacterium to disrupt neural homeostasis, with both overlapping and distinct mechanisms driving neuroinflammation and neurodegeneration. The secretome, comprising diffusible virulence factors, metabolites, and host-derived inflammatory mediators, induces a broad-spectrum inflammatory response characterized by oxidative stress, widespread activation of NF-κB pathway, and upregulation of pro-inflammatory cytokines and chemokines. This aligns with *H. pylori*'s known ability to trigger systemic inflammation via gastric epithelial cell activation, releasing soluble factors that permeate the bloodstream or vagus nerve to reach the central nervous system (CNS). For instance, ROS generation disrupts mitochondrial function, exacerbating neuronal stress and apoptosis, while NF-κB activation perpetuates cytokine release, creating a feedforward loop of neuroinflammation. In contrast, exosomes carrying bacterial and host-derived cargo exhibit a more targeted and nuanced impact. While they also upregulate inflammatory markers and activate p38 MAPK, their suppression of ERK1/2 phosphorylation suggests a sophisticated immune evasion strategy. ERK1/2, critical for cell survival and antimicrobial responses, is often inhibited by pathogens to dampen host defences; and *H. pylori* exosomes may exploit this mechanism to prolong bacterial persistence.

Future research on *H. pylori*-mediated neural dysregulation should prioritize systemic characterization of exosomal cargo using proteomic, lipidomic and transcriptomic profiling to identify strain-specific neuropathogenic determinants. Translational efforts should focus on developing dual-pathway therapeutics targeting exosomal LPC 18:0 via small-molecule inhibitors while engineering blood-brain barrier penetrant nanoparticles to neutralize circulating bacterial vesicles. Multi-omic integration of gastric-and neural-tissue can uncover novel

biomarkers for early intervention, while mechanistic studies on vagus nerve-mediated exosomes transport may redefine our understanding of gut-brain axis pathophysiology in *H. pylori* infections.

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