IJAE
ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY

Firenze University Press
www.fupress.com/ijae

OPEN ACCESS

Citation: Mariangela Mazzone, Maria Carmela Di Marcantonio, Valentina Puca, Beatrice Marinacci, Giulia Nannini, Simone Guarnieri, Amedeo Amedei, Gabriella Mincione, Raffaella Muraro (2022) Effects of *Fusobacterium nucleatum* on migration and cytokines production of ags gastric adenocarcinoma cell line. *Italian Journal of Anatomy and Embryology* 126(2): 59-63. doi: 10.36253/ijae-13798

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Effects of *Fusobacterium nucleatum* on migration and cytokines production of ags gastric adenocarcinoma cell line

Mariangela Mazzone[1,*], Maria Carmela Di Marcantonio[1], Valentina Puca[2], Beatrice Marinacci[2], Giulia Nannini[3], Simone Guarnieri[4], Amedeo Amedei[3], Gabriella Mincione[1], Raffaella Muraro[1]

[1] *Department of Innovative Technologies in Medicine & Dentistry, University "G. d'Annunzio Chieti-Pescara, 66100, Chieti, Italy*
[2] *Department of Pharmacy, University "G. d'Annunzio" Chieti-Pescara, 66100 Chieti, Italy*
[3] *Department of Experimental and Clinical Medicine, University of Florence, Florence 50134, Italy*
[4] *Department of Neuroscience, Center of Advanced Studies and Technology, Imaging and Clinical Sciences, University University "G. d'Annunzio" Chieti-Pescara, 66100 Chieti, Italy*
*Corresponding author. E-mail: mariangela.mazzone@unich.it

Abstract. Gastric cancer (GC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer death worldwide. *Helicobacter pylori* (*Hp*) infection is an important risk factor for GC. However, the etiology of the tumor is multifactorial, since only 1-3% of infected patients develop cancer. Therefore, attention should be focused on the role of microbiota in gastric tumorigenesis since in some studies an alteration of the microbiota in GC has been shown. *Fusobacterium nucleatum* (*Fn*) has been found in biopsies of patients with GC. However, since its role is not clearly established, this study investigated the effects of *Fn* infection on the human gastric adenocarcinoma cell line AGS. Our results showed that *Fn* co-localized at level of the plasma membrane demonstrating the ability of *Fn* to adhere to AGS cells. In addition, increases in incubation times were associated with its intra-cellular localization with loss of the classic curved rod shape. Interestingly, *Fn* determined a greater capacity of cell migration compared to untreated AGS cells. Moreover, IL-4 expression significantly increased in *Fn* infected GC cells. Since cancer cell migration is an integral component of the metastatic process, additional studies are needed to better understand the mechanisms underlying the *Fn*/host interaction.

Keywords: *Fusobacterium nucleatum*, gastric cancer, IL-4 production.

INTRODUCTION

Gastric cancer (GC) is the third leading cause of cancer deaths worldwide (Ferlay et al., 2021) since most patients are diagnosed with an advanced stage of disease. Thus, great efforts are made on tumor prevention and early diagnosis. *Helicobacter pylori* (*Hp*) infection is the major risk factor for the onset

and evolution of GC, but effectiveness of its eradication by drug therapies is still discussed (Piscione et al., 2021). Actually, it is not clear whether the presence of *Hp* or the imbalanced gastric microbiota generates the conditions for *Hp* or other pathogens colonization. *Fusobacterium nucleatum* (*Fn*), involved in periodontal disease, has been detected also in human tumor samples (Han et al., 2015). Indeed, several studies showed the presence of *Fn* in colorectal adenomas (Gethings-Behncke et al., 2020) and in primary colorectal cancer as compared to healthy tissue (Kim et al., 2020). These data suggest that *Fn* plays a role in the initiation and progression of colorectal tumorigenesis (Abed et al., 2017). *Fn* has also been isolated in esophageal (Yamamura et al., 2017) and gastric cancer (Hsieh et al., 2018). In the intestine *Fn* alters the cellular pathways through FadA, an adhesin that stimulates the proliferation of host cells and regulates inflammatory response (Sahan et al., 2018). Elevated levels of *Fn* were detected in biopsies of patients suffering from GC, associated with low levels of *Hp* (Hsieh et al., 2018). *Hp* colonizes the gastric mucosa through its virulence factors and, by urease enzyme, it raises the gastric pH, making the stomach a more permissive environment towards other pathogens such as *Fn* (Servetas et al., 2016; Hsieh et al., 2018). Periodontal disease has a link with GC (Zhou et al., 2018) and *Fn* could play a key role in making periodontal disease a worsening factor for the development of GC (Flemer et al., 2018; Toma et al., 2018). Therefore, the aim of this study was to evaluate the effects of *Fn* infection on a model of human gastric adenocarcinoma cell line AGS in order to verify whether it affects metastatic behavior.

MATERIALS AND METHODS

Bacterial culture

Fusobacterium nucleatum (ATCC 25586) was cultured as previously reported (Maccelli et al., 2020). Bacteria were harvested by centrifugation at 6000 × g for 10min at 4°C. The bacterial pellets were washed twice, resuspended with phosphate-buffered saline and quantified using a microplate reader.

Cell line culture

Human gastric adenocarcinoma AGS cell line (Cell Lines Services, Epplheim, Germany) was cultured as previously described (Savino et al., 2022). AGS cells were infected with *Fn* at multiplicity of infection (MOI) of 100-2000. Supernatants were collected after 24h post-infection and used in Luminex Assay.

Confocal microscopy

AGS cells were grown on glass coverslips and infected with *Fn* at different MOI. At the established time they were fixed (2% paraformaldehyde for 10min), treated for 60min with 10% Goat Serum and incubated with primary antibody anti-*Fn* ANT0084 (DIATHEVA, Italy) at 1:100 dilution. Secondary antibody (Alexa Fluor 488 conjugated, Thermo Fisher Scientific, USA) was used at 1:200 dilution. Then, the cells were treated with PHK26 (SIGMA ALDRICH, USA) at 1:1000 dilution to stain AGS cells membrane. Images were obtained using Zeiss LSM800 confocal microscopy and acquired by Zen Blue software (Carl Zeiss, Germany).

Cell migration assay

Cells were infected with *Fn* at a MOI 500-1500 and wound healing scratch assays were performed using noninfected AGS cells as control. Cells were cultured to 95% confluence in 24-well plates and after 24h, a scratch wound was made followed by *Fn* infection. Cellular migration was recorded after 24h and 48h.

Detection and quantification of cytokines with Luminex assay

Expression levels of IFN-γ, IL-1b, IL-4, IL-6, IL-10, IL-17A were determined in AGS cell culture supernatants using Human kits (Milliplex, Merck Millipore, USA) and Luminex MAGPIX detection system following the manufacturer's instructions. The levels of cytokines (pg/ml) were estimated using a 5-parameter polynomial curve (Bio-Plex Manager Software, Bio-Rad, USA).

Statistical analysis

The data are reported as the representative values of three independent experiments.

RESULTS

Localization of Fn *after infection of AGS cells by confocal microscopy*

Fn localization and its effects on AGS cells morphology were evaluated using a confocal microscope. In Figure 1 orange fluorescence locates AGS cell membrane, while green fluorescence identifies *Fn* as detected using a specific antibody. After 24h, polygonal AGS cells were

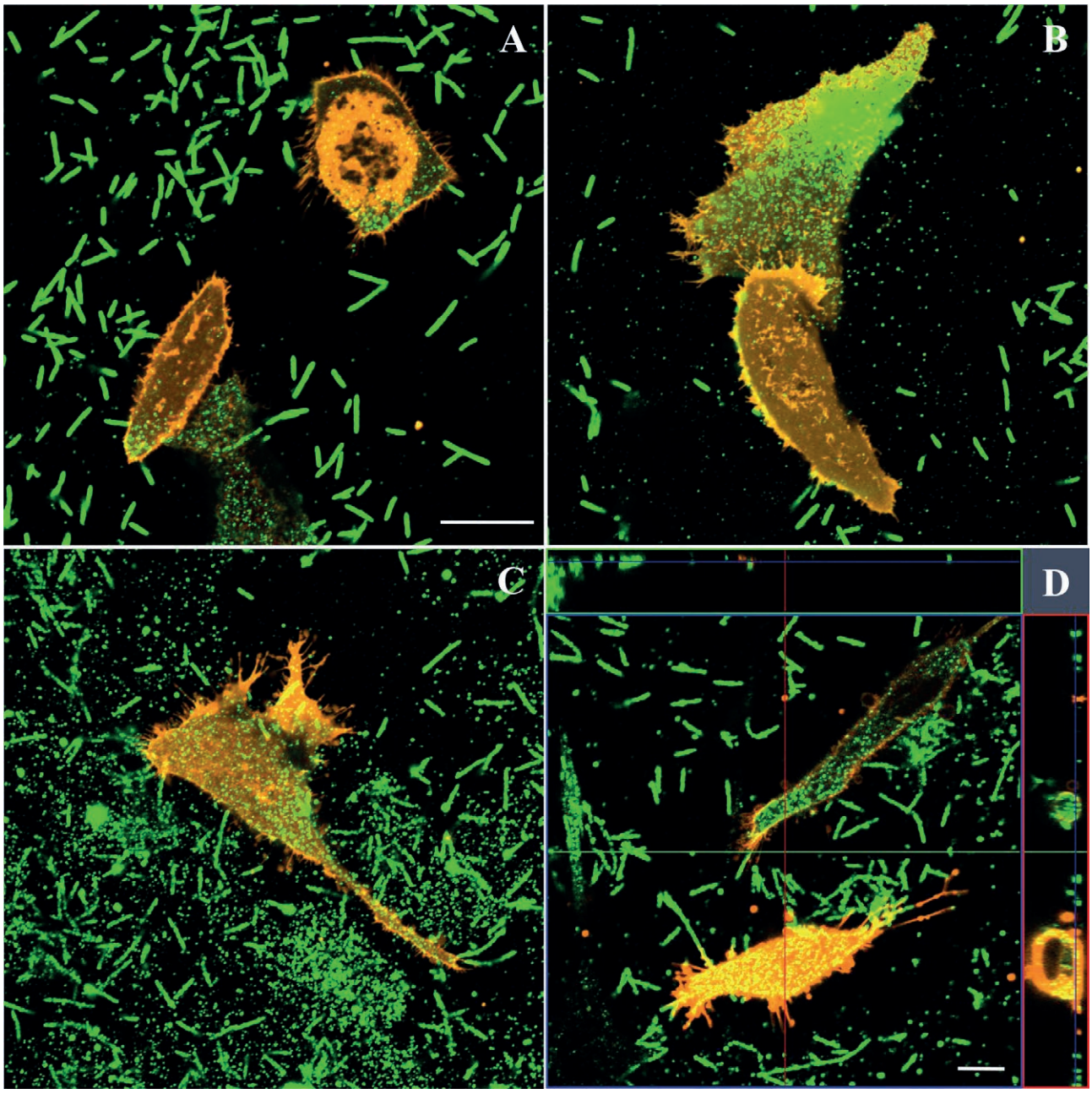

Figure 1. Localization of *Fn* after infection of AGS cells by confocal microscopy. Representative images of co-colture of *Fn* (MOI 100) and AGS cells acquired using confocal microscopy (green and orange fluorescence respectively). Images were captured near the cells contact to coverslip side (A to C) or multiple focal plane (Z stack) to obtain XZ, YZ projection (D). Bar=10μm

surrounded by rod shaped bacteria. Interestingly, signals deriving from bacteria in intracellular compartment appeared with a cytoplasmic spotted pattern, (Figure 1A-C). In addition, some AGS cells showed a yellow fluorescence signal, deriving from *Fn* co-localization with AGS cell membrane (Figure 1 B and D).

Effects of Fn *infection on migration and cytokine expression in AGS cells*

Acquisition of migratory properties is a prerequisite for cancer progression. *Fn* modulates migration and immune cell signaling in colorectal cancer (Casasanta MA et al., 2020).

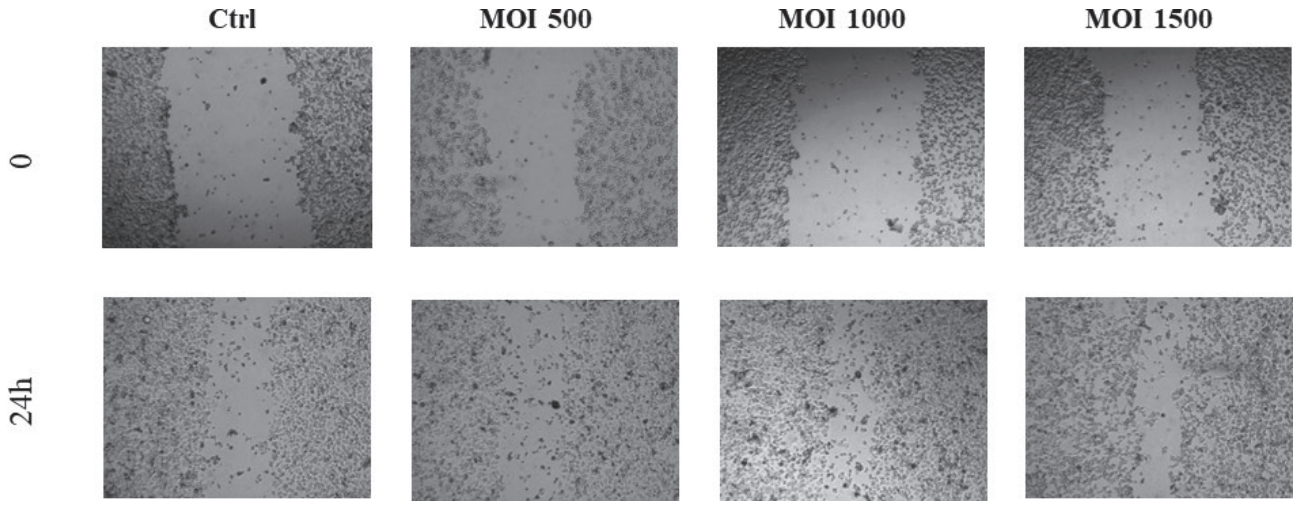

Figure 2. Effects of 24h *Fn* infection at different MOI on migration of AGS cells using a wound healing assay.

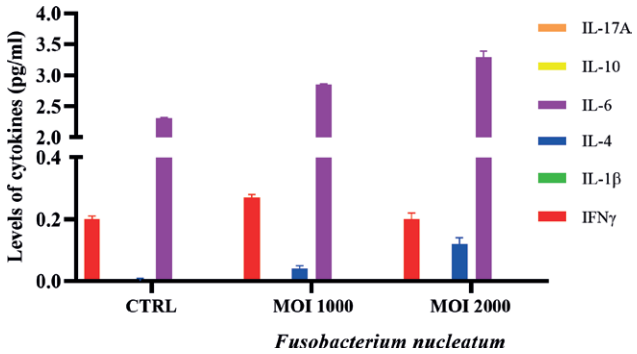

Figure 3. LUMINEX assay to quantify cytokine secretion in cell culture media from AGS cells infected by *Fn*. Infection parameters were MOI 1000 and 2000 *Fn* co-cultured with AGS cells for 24h.

Cell migration of AGS cells was assessed using a wound healing assay after 24h of infection. Our results (Figure 2) demonstrated that *Fn* infection promoted cell migration, as compared to AGS untreated cells.

To find out whether *Fn* was able to induce the secretion of cytokines from these cells, the expressions of IFN-γ, IL-1b, IL-4, IL-6, IL-10, IL-17A were analyzed in the supernatants of AGS cells collected after 24h of co-colture with *Fn*. Interestingly, while IFN-g showed only a slightly increased at MOI 1000 as compared to control cells, *Fn* infection increased IL-4 and IL-6 from MOI 1000 to MOI 2000, as compared to uninfected cells (Figure 3).

DISCUSSION

The present study showed that *Fn* co-culture of AGS cells caused an increase in cell migration capacity and induced IL-4 and IL-6 secretion. It is known that IL-4 plays an important role in cancer cell proliferation and migration enhancing the malignant phenotype (Hallett et al., 2012). Moreover, increased plasma IL-4 levels were significantly higher in GC patients than in healthy controls (Cardenas et al., 2018). The preliminary results obtained in this study suggest that IL-4 could increase the migration ability of AGS cells. Further analysis will be needed to verify this hypothesis. Moreover, we demonstrated the adhesion of *Fn* to the plasma membrane of AGS cells and its presence in their cytoplasm. These data are in agreement with the evidence obtained in colorectal adenocarcinoma cells in which the bacterium is internalized by tumor cells (Casasanta et al., 2020). In conclusion, we hypothesize that future studies characterizing how *Fn* influences signaling pathways during cancer development will lead to targeted approaches that could potentially decrease the risk of progression to gastric cancer.

REFERENCES

Abed J, Maalouf N, Parhi L, Chaushu S, Mandelboim O, Bachrach G. Tumor Targeting by *Fusobacterium nucleatum*: A Pilot Study and Future Perspectives. Front Cell Infect Microbiol. 2017 Jun 30;7:295. doi: 10.3389/fcimb.2017.00295

Cárdenas DM, Sánchez AC, Rosas DA, Rivero E, Paparoni MD, Cruz MA, et al. Preliminary analysis of single-nucleotide polymorphisms in IL-10, IL-4, and IL-4Rα genes and profile of circulating cytokines in patients with gastric Cancer. BMC Gastroenterol. 2018 Dec 10;18(1):184. doi: 10.1186/s12876-018-0913-9

Casasanta MA, Yoo CC, Udayasuryan B, Sanders BE, Umaña A, Zhang Y, et al. Fusobacterium nucleatum host-cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. Sci Signal. 2020 Jul 21;13(641):eaba9157. doi: 10.1126/scisignal.aba9157

Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, Bray F. Cancer statistics for the year 2020: An overview. Int J Cancer. 2021 Apr 5. doi: 10.1002/ijc.33588

Flemer B, Warren RD, Barrett MP, Cisek K, Das A, Jeffery IB, et al. The oral microbiota in colorectal cancer is distinctive and predictive. Gut. 2018 Aug;67(8):1454-1463. doi: 10.1136/gutjnl-2017-314814

Gethings-Behncke C, Coleman HG, Jordao HWT, Longley DB, Crawford N, Murray LJ, Kunzmann AT. *Fusobacterium nucleatum* in the Colorectum and Its Association with Cancer Risk and Survival: A Systematic Review and Meta-analysis. Cancer Epidemiol Biomarkers Prev. 2020 Mar;29(3):539-548. doi: 10.1158/1055-9965

Hallett MA, Venmar KT, Fingleton B. Cytokine stimulation of epithelial cancer cells: the similar and divergent functions of IL-4 and IL-13. Cancer Res. 2012 Dec 15;72(24):6338-43. doi: 10.1158/0008-5472.CAN-12-3544.

Han YW. *Fusobacterium nucleatum*: a commensal-turned pathogen. Curr Opin Microbiol. 2015 Feb;23:141-7. doi: 10.1016/j.mib.2014.11.013

Hsieh YY, Tung SY, Pan HY, Yen CW, Xu HW, Lin YJ, et al. Increased Abundance of *Clostridium* and *Fusobacterium* in Gastric Microbiota of Patients with Gastric Cancer in Taiwan. Sci Rep. 2018 Jan 9;8(1):158. doi: 10.1038/s41598-017-18596-0

Kim M, Lee ST, Choi S, Lee H, Kwon SS, Byun JH, Kim YA, Rhee KJ, Choi JR, Kim TI, Lee K. *Fusobacterium nucleatum* in biopsied tissues from colorectal cancer patients and alcohol consumption in Korea. Sci Rep. 2020 Nov 16;10(1):19915. doi: 10.1038/s41598-020-76467-7

Maccelli A, Carradori S, Puca V, Sisto F, Lanuti P, Crestoni ME, Lasalvia A, Muraro R, Bysell H, Di Sotto A, Roos S, Grande R. Correlation between the Antimicrobial Activity and Metabolic Profiles of Cell Free Supernatants and Membrane Vesicles Produced by *Lactobacillus reuteri* DSM 17938. Microorganisms. 2020 Oct 24;8(11):1653. doi: 10.3390/microorganisms8111653

Piscione M, Mazzone M, Di Marcantonio MC, Muraro R, Mincione G. Eradication of *Helicobacter pylori* and Gastric Cancer: A Controversial Relationship. Front Microbiol. 2021 Feb 4;12:630852. doi: 10.3389/fmicb.2021.630852

Sahan AZ, Hazra TK, Das S. The Pivotal Role of DNA Repair in Infection Mediated-Inflammation and Cancer. Front Microbiol. 2018 Apr 11;9:663. doi: 10.3389/fmicb.2018.00663

Savino L, Di Marcantonio MC, Moscatello C, Cotellese R, Centurione L, Muraro R, et al. Effects of H_2O_2 Treatment Combined with PI3K Inhibitor and MEK Inhibitor in AGS Cells: Oxidative Stress Outcomes in a Model of Gastric Cancer. Front Oncol. 2022 Mar 16;12:860760. doi: 10.3389/fonc.2022.860760.

Servetas SL, Bridge DR, Merrell DS. Molecular mechanisms of gastric cancer initiation and progression by Helicobacter pylori. Curr Opin Infect Dis. 2016 Jun;29(3):304-10. doi: 10.1097/QCO.0000000000000248

Toma SC, Ungureanu BS, Patrascu S, Surlin V, Georgescu I. Colorectal Cancer Biomarkers - A New Trend in Early Diagnosis. Curr Health Sci J. 2018 Apr-Jun;44(2):140-146. doi: 10.12865/CHSJ.44.02.08.

Yamamura K, Baba Y, Miyake K, Nakamura K, Shigaki H, Mima K, et al. *Fusobacterium nucleatum* in gastroenterological cancer: Evaluation of measurement methods using quantitative polymerase chain reaction and a literature review. Oncol Lett. 2017 Dec;14(6):6373-6378. doi: 10.3892/ol.2017.7001

Zhou Z, Chen J, Yao H, Hu H. Fusobacterium and Colorectal Cancer. Front Oncol. 2018 Oct 15;8:371. doi: 10.3389/fonc.2018.00371