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***Helicobacter pylori*'s cholesterol uptake impacts resistance to docosaheptaenoic acid**

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Abstract *Helicobacter pylori* colonizes half of the world population and is associated with gastric cancer. We have previously demonstrated that docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid known for its anti-inflammatory and antitumor effects, directly inhibits *H. pylori* growth in vitro and in mice. Nevertheless, the concentration of DHA shown to reduce *H. pylori* mice gastric colonization was ineffective in vitro. Related to the auxotrophy of *H. pylori* for cholesterol, we hypothesize that other mechanisms, in addition to DHA direct antibacterial effect, must be responsible for the reduction of the infection burden. In the present study we investigated if DHA affects also *H. pylori* growth, by reducing the availability of membrane cholesterol in the epithelial cell for *H. pylori* uptake. Levels of cholesterol in gastric epithelial cells and of cholesteryl glucosides in *H. pylori* were determined by thin layer chromatography and gas chromatography. The consequences of epithelial cells' cholesterol depletion on *H. pylori* growth were assessed in liquid cultures. We show that *H. pylori* uptakes cholesterol from epithelial cells. In addition, DHA lowers cholesterol levels in epithelial cells, decreases its de novo synthesis, leading to a lower synthesis of cholesteryl glucosides by *H. pylori*. A previous exposition of *H. pylori* to cholesterol influences the bacterium response to the direct inhibitory effect of DHA. Overall, our results suggest that a direct effect of DHA on *H. pylori* survival is modulated by its access to epithelial cell cholesterol, supporting the notion that cholesterol enhances the resistance of *H. pylori*. The cholesterol-dependent resistance of *H. pylori* to antimicrobial compounds raises new important aspects for the development of new anti-bacterial strategies.

Introduction

H. pylori colonizes half of the world population and is associated with dyspepsia, chronic gastritis, ulcer disease and gastric cancer. (Peek and Crabtree, 2006) Eradication of this infection leads to a reduction in the recurrence of peptic ulcer disease and prevents the development of gastric precancerous lesions (Talley et al., 2008), highlighting the need for new therapeutic strategies to treat *H. pylori* carriers. Usually, the first-line option for eradicating *H. pylori* infection is a triple treatment that consists of a proton pump inhibitor (PPI) and two antimicrobial agents. (Malfertheiner et al., 2007) However, the increasing incidence of bacteria strains resistant to these antibiotics is partially responsible for failure of treatment reported for triple therapy. (Gisbert et al., 2010) Thus, new alternative therapies that improve the eradication rate of *H. pylori* and that circumvent the antibiotics resistance are of great interest. According to a previous study (Thompson et al., 1994), we have demonstrated that polyunsaturated fatty acids (PUFAs) and in particular docosahexaenoic acid (DHA), which is a n-3 PUFA, hold an anti-*H. pylori* growth action. DHA inhibits *H. pylori* growth in a concentration dependent manner in vitro, and this was confirmed in a murine model.(Correia et al., 2012) Our previous data also suggest that this inhibitory effect involves the incorporation of DHA into the bacterium's cell wall, inducing changes in the components of the cell wall and facilitating bacteria lysis. (Correia et al., 2012) Nevertheless, the concentration of 50 μ M of DHA shown to reduce *H. pylori* mice gastric colonization was not effective in vitro. (Correia et al., 2012) These results are in line with the fact that other mechanisms in addition to a DHA direct antibacterial effect on *H. pylori*, must be responsible for the reduction of

68 *H. pylori* load in the stomach of infected mice treated with DHA. In this context, we
69 cannot exclude that the direct action of DHA on epithelial cells impacts its anti-
70 *Helicobacter* effect besides affecting bacteria. In fact, it is well recognized that DHA
71 is incorporated into cell membrane of eukaryotic cells leading to alteration of
72 membrane physical properties such as permeability, lateral diffusion, lipid packing,
73 domain formation and cholesterol availability/distribution.(Shaikh et al., 2004)
74 Interestingly, cholesterol rich-platforms within cell membranes have been implicated
75 as cell gates for pathogens.(Xiong et al., 2009) Moreover, the genus *Helicobacter*
76 has the unique feature of assimilating free cholesterol (FC) extracted from epithelial
77 lipid rafts into its bacterial outer membrane, allowing its enzymatic
78 glycosylation.(Wunder et al., 2006) The *cgt H. pylori* mutant, which lacks the ability
79 to biosynthesize the glycosyl cholesterol and retains FC without glycosylation, is
80 easily phagocytized by macrophages and induces activation of antigen-specific T
81 cells. These features highlight the importance of cholesterol metabolism in the
82 bacteria survival and ability to colonize.(Shimomura et al., 2004; Wunder et al., 2006)
83 Moreover, the translocation of *H. pylori* virulence factors, such as vacuolating
84 cytotoxin A (VacA) and cytotoxin associated gene A (CagA), via the type IV secretion
85 system (TFSS), depends on the FC level of epithelial cell, as they are blocked by
86 cholesterol depletion (ex: cyclodextrins).(Lai et al., 2008; Patel et al., 2002) The
87 glycosyl cholesterol also modifies lateral-phase segregation in epithelial cell
88 membranes, reorganizing the architecture of the membrane and promoting the
89 formation of a functional TFSS.(Wang et al., 2012) Hence, glycosylation of
90 cholesterol plays an important role in *H. pylori* survival.

91 Given the known ability of n-3 PUFAs, and DHA in particular, to interfere with
92 cholesterol's distribution in the epithelial cell membrane and given the auxotrophy of
93 *H. pylori* for cholesterol, we hypothesized that besides its direct antibacterial effect,
94 DHA holds an indirect effect on *H. pylori* growth. This indirect effect might be
95 achieved through mechanisms that involve DHA ability to interfere with cholesterol
96 in epithelial cells. Additionally, statins, that as DHA, affect cholesterol metabolism,
97 increase the eradication of *H. pylori* infection when used as co-adjuvant to standard
98 triple therapy (Nseir et al., 2012). In this study we aim to further characterize the
99 mechanisms by which DHA holds an anti-*H. pylori* activity. We investigate if in
100 addition to its direct bactericidal action, DHA exerts an indirect effect on *H. pylori* by
101 modulating epithelial cells' cholesterol uptake.

MATERIAL AND METHODS

Fatty acids, *H. pylori* strains and cell culture conditions

DHA was obtained from *Cayman Chemical Company* (Michigan, USA) with a degree of purity of 99% in ethanol 0.06% (v/v). The *H. pylori* strains used were: SS1 (Lee et al., 1997) 26695 (ATCC 700392) obtained from the American Type Culture Collection (ATCC, Rockville, MD). *H. pylori* was grown on blood agar base 2 plates (Oxoid, Lyon, France) supplemented with 10% defibrinated horse blood (bioMérieux, Marcy l'Etoile, France). Plates were incubated at 37°C for 48h under microaerobic conditions (7% O₂, 10% CO₂; anoxomat system). Cell infections were performed with *H. pylori* grown in liquid cultures. Briefly, bacteria were inoculated to an initial optical density of 0.03 at 600 nm into liquid Brucella broth (BB) (Oxoid) supplemented with 10% fetal calf serum (FCS). *H. pylori* grown for 18-20h yields approximately 5x10⁸ colony forming units (CFU)/ml. Bacterial cholesterol analysis was performed with *H. pylori* strain 26695, due to the fact that this strain has a functional TFSS, described to be sensitive to host epithelial cholesterol. (Wang et al., 2012)

Human gastric epithelial cells (AGS: gastric adenocarcinoma, CRL-1739, ATCC-LGC®) were grown in RPMI medium 1640 supplemented with 10% FCS and 1% of penicillin-streptomycin under 37°C and under 5% CO₂ in humidified air. For cell viability assays, cells were trypsinized and counted using trypan blue (Sigma-Aldrich) staining. Liquid cultures of *H. pylori* were added to AGS cells in serum free medium for at least 18h, at a multiplicity of infection (MOI) of 100:1 and kept under agitation for 1h, 6h or 24h at 37° C. Treatment with cholesterol rich-microdomains disruptors in AGS cells was performed with methyl-β-cyclodextrin (MBCD; Sigma-

Aldrich). Briefly, AGS cells were washed with PBS three times and treated with 2.5 mM of MBCD for 1h at 37°C. To remove MBCD cells were washed with PBS three times.

Incubation of AGS cells with 25-benz-oxa-diazol- (NBD)-labeled cholesterol and with DHA

Stock solutions of 25-benz-oxa-diazol-labeled cholesterol (NBD-cholesterol) (Avanti Polar Lipids) were solubilized in ethanol and stored at -20°C, at a final concentration of 5 µg/ml NBD-cholesterol. AGS cells were used as cholesterol suppliers for *H. pylori* uptake, because of previous demonstration that the bacteria extracts cholesterol from epithelial cells in a more efficient way than it extracts from liquid medium. (Wunder et al., 2006). In order to verify that epithelial cells had cholesterol available for *H. pylori* uptake, confluent AGS monolayers cells grown in a serum-free RPMI medium for 3h, were labeled with NBD-cholesterol (5 µg/ml NBD-cholesterol and ethanol concentration 0.25% v/v). Cells were then washed with PBS to remove unbound NBD-cholesterol and treated with DHA 50µM in RPMI medium for 12h. This concentration was shown to be within the range of non-toxic concentrations according to trypan blue exclusion test (data not shown). After DHA treatment, AGS cells were vigorously washed with PBS and put into a DHA-free fresh medium for 18h to exclude other sources of cholesterol than the ones present in epithelial cell membranes. Cells were then infected with *H. pylori* at MOI of 100:1 for 3h.

DHA treatment of *H. pylori* cultures

Stock solutions of DHA were diluted in BB enriched with 10% FCS and used at concentrations of 100µM and 250µM. To establish *H. pylori* growth curves, 18-20h bacteria cultures were diluted 100-fold in 10 ml of medium with or without DHA to an initial OD of 0.03. Each experiment consisting in *H. pylori* culture incubated with DHA at 100 µM and 250 µM and a control non-treated culture were performed in triplicate. Cultures were incubated under microaerophilic conditions, as described above. Every 12h, during a 48h period, a 200 µL sample of each bacterial culture was isolated, the OD measured and aliquots serially diluted and plated on blood agar petri dishes. After 48h of incubation, the number of viable bacteria was determined by colony forming units (CFU) counting.

Analysis of DHA and n-3 PUFAs composition in AGS cells

AGS cells used for fatty acid composition experiments grew in 75-cm² flasks as previously described. Lipids were trans-esterified directly from the membrane pellets by acid-catalysed transmethylation with methanolic-HCl (0.6M, 80°C, 2h), using tricosanoic acid as internal standard (Sigma). Fatty acid methyl esters (FAME) were extracted with hexane, taken to dryness under a nitrogen stream, then resuspended in dichloromethane and stored at -20°C. The chromatograph (Chrompack, CP-9001, the Netherlands) was equipped with a flame ionisation detector (250°C) and CP-Sil 88capillary column (Varian), with temperature gradient and splitless injection. EPA and DHA were quantified from calibration curves with internal standard, submitted to the entire analytical protocol, being expressed on a mass per cell count basis.(Ranjekar et al., 2003)

Free and total cholesterol quantification

AGS cells used for cholesterol profile analysis experiments grew in 75-cm² flasks as previously described. Cells were then washed with PBS and the gastric cell pellets were extracted with 2:1 (v/v) chloroform-methanol (50 mg/L BHT), sonicated for 5 minutes and gently shaken overnight. Aqueous NaCl solution (0.9%) was added in accordance with Folchet al.(Riff et al., 2005) The lower chloroform phase was collected, dehydrated with anhydrous sodium sulphate, taken to dryness under a gentle N₂ stream and reconstituted with hexane. Based on the initial cell counts, an accurate volume of lipid solution corresponding to 100,000 cells (5 to 10 µL) was spotted on a silica 60 HPTLC plate (Merck). A mixture of hexane: diethyl ether; acetic acid (70:30:1) was used for elution and 13mM α -naphthol in sulphuric acid/methanol (3/97; v/v; at 160 °C) for staining.(Riff et al., 2005) Free and esterified cholesterol from Sigma (USA) was used for band identification.

From the remaining lipid solution, a 150 µL portion was transferred to a glass micro-vial and 5- α -cholestane (Sigma) was added as internal standard. An accurate portion was used for FC quantification, being taken to dryness (dessicated), derivatized with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (80 µl; 80°C, 1h) (Fluka, Spain) and directly evaluated by gas chromatography-mass spectrometry GC-MS (details below). The remaining volume was used for the analysis of total cholesterol, with a previous hydrolysis (0.36M KOH; 70°C; 30'+30') as described by Pelletier et al. and the lipid extract derivatized as for free cholesterol.(Pelletier et al., 1987)

GC–MS analyses were performed on an Agilent (USA) gas chromatograph 6890 interfaced (280 °C) to a MSD-5973N mass selective detector and equipped with a DB-5MS column (30 m × 0.25 mm I.D., 0.25 µm; J&W Scientific, USA). Electron impact mass spectra were measured at acceleration energy of 70 eV. Data acquisition was performed in the full-scan mode during the optimization of the method (mass range 50–600). Quantification was carried out in selective ion monitoring (SIM) mode with a dwell time of 30 ms and using the *m/z* 129, 329, 353, 268, 443, and 458 for cholesterol, and *m/z* 217, 357 and 372 for the internal standard. AGS cells protein concentration was determined by Lowry's method. (Lowry et al, 1951)

Cholesteryl glucosides analysis

Lipids were extracted as described above for the cholesterol analysis and reconstituted in chloroform:methanol (2:1). Each sample was applied sequentially in a 20x20 silica 60 glass plate (Merck), eluted with chloroform/methanol/ammonia (40/10/0.4, vol/vol) and stained with 13mM α -naphthol in sulphuric acid/methanol (3/97; v/v; at 160 °C) as described by Wunter *et al.* (Wunder et al., 2006) Glycolipids isolated from *H. pylori* lipids were used as reference, after fractionation by column chromatography on silicic acid and confirmation by HPTLC. (Hirai et al., 1995; Shimomura et al., 2004)

Cholesterol metabolism in AGS cells upon DHA and *H. pylori* infection

We were interested in assessing if DHA and *H. pylori* infection in AGS cells impaired cholesterol metabolism. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA-r) is the rate-limiting enzyme in cholesterol biosynthesis that catalyses mevalonate formation; Low Density Lipoprotein receptor (LDLr) is the preferential receptor for cholesterol uptake in epithelial cells. (Go, W. and Mani, A., 2012) (Kaur, G. et al, 2012) We have assessed both their expressions by immunoblotting. Anti-HMGCoA-r (Upstate, 1:200), anti-LDL-r (Abcam, 1:200) and anti- α -tubulin (Sigma, 1:10000) as loading control were used on protein extracts from AGS cells treated with DHA for 24 hours and infected with *H. pylori* strain 26695. Briefly, cells were lysed in cold Catenin lysis buffer 1% Triton X-100 (Sigma) in PBS enriched with a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma). The proteins were quantified using a modified Bradford assay (Bio-Rad). Thirty- μ g of proteins were eluted in sample buffer and loaded in 7.5% or 12% SDS-PAGE, depending on the molecular mass of molecules of interest. The proteins were then electroblotted onto a Hybond ECL membrane (Amersham Biosciences). Membranes were blocked with 5% non-fat milk and 0.5% Tween-20 in PBS and immunoblotted. Donkey anti-rabbit (Amersham Biosciences) or sheep anti-mouse (Amersham Biosciences) HRP-conjugated secondary antibodies were used as required, followed by ECL detection (Amersham Biosciences). Immunoblots were quantified with the Quantity One Software (Bio-Rad).

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Student's *t*-test was used

to assess differences in cell lipid composition profile. All statistical tests were two-sided. Differences were considered significant when $P < 0.05$.

RESULTS

DHA is incorporated into AGS cells

Our first goal was to test if DHA was incorporated into AGS cells upon medium supplementation. Cell cultures were treated with 50 μM of DHA for 12h, a concentration shown to be non-toxic according to trypan blue exclusion test (data not shown). Gas chromatography was performed to assess DHA content and n-3 PUFAs (DHA+EPA) content in cells. As shown in Figure 1, DHA supplementation in AGS cells medium significantly increased total n-3 PUFA content (4-fold compared to non-DHA treated cells) and DHA content (9-fold compared to non-DHA treated cells) in AGS cells, $P < 0.05$. These results confirm that DHA present in the medium was incorporated into AGS cells.

DHA decreases cholesterol availability to *H. pylori* in AGS cells

We postulated that DHA incorporation into AGS cells induces changes in cholesterol levels. Cholesterol can be present either in a free form or esterified, depending on epithelial cell dynamic and cholesterol metabolism.(Shimomura et al., 2004) Fractions of total and FC were assessed by gas chromatography analysis. Results presented in figure 2 correspond to the 24h time-point, but all the other time-points assessed reflect the same relation in cholesterol levels. DHA treatment decreased total cholesterol levels (4.4 ± 0.8 vs 7.1 ± 0.9 μg per mg of cell protein), $P < 0.05$ (Figure

2A). Similarly, the cholesterol free fraction, which is the preferential form of cholesterol extracted by *H. pylori* (Shimomura et al., 2004), is significantly decreased from AGS cells upon DHA treatment (5.4 ± 0.4 vs 2.8 ± 0.5 μ g per mg of cell protein, $P < 0.05$, (Figure 2A).

To confirm that *H. pylori* is indeed able to extract cholesterol from epithelial cells, we incubated AGS epithelial cells with *H. pylori* and then analysed FC amounts in cells after 24h of infection. Both total and FC cellular levels were significantly decreased by *H. pylori* infection comparatively to non-infected cells (3.3 ± 0.7 vs 7.1 ± 0.9 μ g per mg of cell protein, $P < 0.01$, and 2.1 ± 0.6 vs 5.4 ± 0.4 μ g per mg of cell protein, respectively, $P < 0.05$ (Figure 2A). These results were confirmed by TLC analysis showing that FC was significantly lower in AGS cells treated with 50 μ M of DHA or incubated with *H. pylori* 26695 when compared with control cells, $P < 0.05$ (Figures 2B). In conclusion, our results indicate that DHA incorporation into AGS cells leads to a decrease in the levels of total and free forms of cholesterol.

DHA treatment of cells results in a decreased cholesterol glycosylation by *H. pylori*

It was demonstrated that *H. pylori* glycosylates cholesterol by an enzymatic reaction following its extraction from epithelial cells (Wunder et al., 2006). In order to determine if the reduction of FC observed in DHA treated and infected cells results in a decrease of cholesterol uptake due to *H. pylori*, we performed a TLC analysis to measure cholesterol α -glycosylation by *H. pylori* on AGS cells either pre-treated or not with DHA. TLC results show that cholesterol α -glycosylation occurs in a time

dependant manner (Figure 3). Pre-treatment of AGS cells with 50 μ M of DHA for 12h before the co-culture with *H. pylori* significantly decreased the synthesis of α -glucoside cholesterol by bacteria. These results support the hypothesis that DHA leads to a reduction in the availability of cell cholesterol for *H. pylori* uptake.

Previous exposition of *H. pylori* to cholesterol decreases DHA anti-bacterial effect

It was previously shown that *H. pylori* resistance to antibiotics is enhanced in the presence of cholesterol. (McGee et al., 2011) Following this line of thought, we decided to investigate if the sensitivity of *H. pylori* to DHA could also be dependent on its access to epithelial cells' cholesterol. Briefly, AGS cells were incubated with NBD-cholesterol (NBD was used for fluorescent staining of cholesterol) and treated in three alternative ways: 1) incubation with DHA; 2) incubation with MBCD (cholesterol removal agent); 3) no incubation, as summarized in Figure 4A. Liquid cultures of *H. pylori* 26695 and SS1 were added to AGS cells at MOI of 100:1 for 3h. Bacteria were then retrieved and let to grow for 48h in liquid cultures either in DHA free medium or in the presence of 100 μ M or 250 μ M DHA; these concentrations were chosen because they are known to inhibit *H. pylori* growth (Correia et al., 2012). Aliquots were collected every 12h to analyse bacteria viability. As illustrated in Figure 4B, NBD-cholesterol was efficiently incorporated into AGS cells. *H. pylori* growth rate and viability were neither affected by NBD-cholesterol or by MBCD treatment (data not shown).

The effects of DHA on the growth of *H. pylori* following exposure to cells' cholesterol were analysed on strain 26695 (Figure 4C) and SS1 (Figure 4D). When *H. pylori* uptakes NBD-cholesterol from non-treated AGS cells, the presence of 100 μ M of DHA in the bacterial culture did not affect the bacteria growth. However, if cholesterol is previously removed from AGS cells by treatment with DHA or MBCD, 100 μ M of DHA significantly decreases *H. pylori* growth in both strains, $P < 0.05$. Similar effects were obtained with 250 μ M of DHA, $P < 0.05$. We thus observed that the sensitivity of *H. pylori* to DHA treatment is higher when bacteria are previously in contact with cholesterol depleted cells, compared to cells with a normal level of cholesterol. Hence, our results show that the direct inhibitory effect of DHA on *H. pylori* growth can be modulated by the accessibility of *H. pylori* to cholesterol from the host epithelial cells.

Cholesterol metabolism in AGS cells upon DHA and *H. pylori* infection

We have seen that DHA treatment in AGS cells regardless *H. pylori* infection significantly decreased the expression of both HMG-CoA reductase and LDL-receptor. MBCD did not hold a significant effect in the expression of these proteins. These results show that DHA decreases cholesterol levels through an interference in cholesterol metabolism *de novo* synthesis.

Discussion

N-3 PUFAs were identified as molecules holding an anti-*H. pylori* effect through mechanisms that interfere with the bacteria's cell wall integrity.(Thompson et al., 1994) We have demonstrated that high concentrations of DHA, an n-3 PUFA, directly inhibit *H. pylori* growth in vitro and its adherence to AGS cells. (Correia et al., 2012; Correia et al., 2013) However, the amount of DHA shown to reduce *H. pylori* mice gastric colonization was lower, suggesting that other mechanisms, in addition to the DHA direct antibacterial effect on *H. pylori*, should be accountable for the reduction of *H. pylori* load in the stomach of infected mice treated with DHA. Hence we hypothesized that DHA might modify key properties of epithelial cells that would challenge *H. pylori* infection. According to the auxotrophy of *H. pylori* for cholesterol, we aimed to determine if DHA, besides its direct bactericidal effect, holds an indirect action on *H. pylori* growth through modulation of cholesterol levels in the cellular membrane of epithelial cells. As reported in the literature, DHA can influence lipid properties of epithelial cells.(McMurray et al., 2011) It is advocated that the unique hairpin structure of DHA is incompatible with cholesterol presence. Therefore, the treatment of epithelial cells with DHA leads to its incorporation into membrane phospholipids which will cluster unevenly into microdomains DHA-rich and DHA-poor. As the DHA amount increases in cell membrane, cholesterol will be effluxed, since DHA and cholesterol are chemically incompatible. (Dusserre et al., 1995; McMurray et al., 2011) DHA is selectively incorporated into closely packed phospholipids, concentrated in the inner leaflet of the cell membrane, which will exclude cholesterol from the inner towards the outer leaflet of the membrane by

negative association.(Applegate and Glomset, 1986; Knapp et al., 1994) Cholesterol from the outer layer can be adsorbed by lipoproteins or other molecules and the efflux increases in epithelial cells.(Dusserre et al., 1995) The ability of DHA to affect membrane composition and structure raised the hypothesis that DHA could have an indirect effect on the ability of bacteria to survive and to colonize efficiently the gastric mucosa, by modulating the availability of cholesterol to *H. pylori* uptake in gastric epithelial cells, although not as efficient as MBCD that is described to remove more than 80% of cell membrane cholesterol. (Francis et al., 1999) These data led us to further analyse if the direct inhibitory effect of DHA on *H. pylori* growth could be influenced by accessibility of bacteria to the cells' cholesterol.

Most interestingly, molecules that are known to lower cholesterol levels, such as statins, have been shown to inhibit *H. pylori* eradication when used as co-adjuvants to standard triple therapy. This occurs through a mechanism not yet elucidated. Both n-3 PUFAs and statins have been shown to reduce bacterial growth in vivo, at lower concentration as compared to their in vitro effect. (Bergman et al., 2011) Recently, simvastatin was reported to increase the efficacy of triple therapy to eradicate *H. pylori*. (Nseir et al., 2012) Altogether, these observations support that their anti-*H. pylori* properties might be associated to their ability to interfere with cholesterol, rather than their bactericidal action *per se*. If confirmed, this could be a major breakthrough in terms of *H. pylori* infection treatment, since the involvement of cholesterol metabolism as a major target for *H. pylori* therapy opens new possibilities and strategies to manage the infection.

371 In this study we show that *H. pylori* uptakes cholesterol from epithelial cells and that
372 DHA effectively lowers total cholesterol and FC in epithelial cells.(Dusserre et al.,
373 1995; Wunder et al., 2006) We have also demonstrated that according to this lower
374 level of cholesterol in epithelial cells, *H. pylori* uptakes less FC and consequently
375 produces less cholesteryl glucosides. Low levels of cholesterol in the epithelial cell
376 membrane do indeed result in less cholesterol availability to *H. pylori* uptake. This
377 feature is of utmost importance, as *H. pylori* is auxothrophic for cholesterol. It
378 extracts FC (non-esterified) from host epithelial cells, modifies it by α -glycosylation
379 and incorporates the modified cholesteryl glucosides into its membrane.(Lee et al.,
380 2008; Patel et al., 2002; Shimomura et al., 2004; Wunder et al., 2006) This
381 dependence on cholesterol is very well illustrated by the fact that *cgt* knockout *H.*
382 *pylori* mutants that are unable to α -glycosylate cholesterol, do not colonize efficiently
383 the gerbil model.(McGee et al., 2011)

384 We have also observed that DHA interferes with cholesterol metabolism *de novo*
385 synthesis. Indeed, DHA is able to lower LDL-r and HMGCoA reductase (which
386 catalyses a critical step of converting HMG-CoA to mevalonic acid in the cholesterol
387 synthesis pathway) which might explain its effect in lowering cholesterol levels. This
388 reduction was already identified by others in epithelial cells, where incubation of
389 epithelial cells with DHA down-regulated the expression levels of important players
390 in cholesterol metabolism. (Goldstein, J, Brown, M, 1990)

391 Our observations propose that a previous exposition of *H. pylori* to cholesterol
392 renders the bacterium more effective in resisting the direct inhibitory effect of DHA.
393 However, if the cholesterol is previously removed by DHA or MBCD, *H. pylori*

becomes more sensitive to DHA exposure. These results indicate that *H. pylori* accessibility to cholesterol improves the resistance of the bacteria to the inhibitory growth effect of DHA. This increased resistance was also shown with antibiotics. In the same sense MBCD, which depletes epithelial cell cholesterol, is known to abrogate *H. pylori* infection both in vitro and in vivo, (Hildebrandt and McGee, 2009; Lai et al., 2008) Although the physiologic significance of incorporation of cholesterol and its derivatives into the *H. pylori* membrane is still not well established, our results support the hypothesis that cholesterol assimilation is important for *H. pylori*'s survival and growth.(Shimomura et al., 2004; Trainor et al., 2011)

In conclusion, in the present study, we have shown that DHA is efficiently incorporated into epithelial cells and is able to decrease cell membrane cholesterol levels, which confirms its recognized role in modulating the lipid profile of the cell membrane.(Rockett et al., 2011) DHA also decreases cholesterol de novo synthesis which overall can explain the decrease in cholesterol levels. Our findings also demonstrate that DHA decreases cholesterol uptake by *H. pylori* from epithelial cells, and that *H. pylori* sensitivity to DHA can be influenced by a previous exposure to cholesterol. These results are in line with reported observations showing that *H. pylori* that grows with cholesterol is up to thousand-fold more resistant to amoxicillin treatment than *H. pylori* grown in cholesterol-depleted medium.(McGee et al., 2011)

H. pylori cholesterol-dependent resistance to some anti-*Helicobacter* molecules such as bile salts, is also documented.(Trainor et al., 2011) Previous studies have implicated cholesterol as a crucial component for antibiotics resistance, which would enable it to modulate therapies efficiency. This concept may be very important for

417 the management of *H. pylori* infection since it supports the important notion that the
418 resistance of *H. pylori* to some antimicrobial compounds, which are or could be used
419 clinically to eradicate the infection, can be modulated by cholesterol uptake.
420 (Testerman et al., 2006; Trainor et al., 2011)
421

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Figures legend

Figure 1 Quantification of n-3 PUFA and DHA in gastric epithelial cells. AGS cells were incubated with DHA 50 μ M for 12h. Incorporation of n-3 PUFA and DHA was quantified by Gas Chromatography as described in material and methods. Student's t-test was performed to assess differences between AGS control cells and DHA treated cells. DHA content and n-3 PUFAs content in AGS cells were significantly different in DHA treated cells compared to non-treated cells, $p < 0.05$. Results are expressed as fold-increase compared with normalized control levels. Data are expressed as the mean $\pm$ standard deviation and are representative of three independent experiments.

Figure 2: Cholesterol levels in DHA-treated and *H. pylori*-infected AGS cells for 24 hours. A) Both DHA treatment and *H. pylori* infection decreased the levels of total cholesterol and free cholesterol in AGS cells. Results are expressed as μ g of cholesterol per mg of cell protein. B) Free and esterified cholesterol levels in AGS cells treated with DHA and incubated with *H. pylori* 26695 were assessed by TLC. C) Cholesterol metabolism in AGS cells in terms of expression of LDL-receptor and HMG-CoA reductase by Western Blot analysis.

Figure 3: *H. pylori* enzymatic glycosylation of cholesterol assessed by Thin Layer Chromatography (TLC).

Liquid cultures of *H. pylori* 26695 were added to AGS cells previously treated or not with 50 μ M of DHA. Cholesterol α -glycosylation significantly increased with *H. pylori*

incubation time ($p < 0.05$) in the absence of DHA treatment. Cholesterol α -glycosylation was significantly decreased in AGS cells treated with 50 μM of DHA in comparison with AGS cells not treated with DHA in all time points (one, six and 24h of *H. pylori* incubation), $p < 0.01$.

Figure 4: DHA efficiency in inhibiting *H. pylori* growth depends on the previous uptake of cholesterol by *H. pylori*. A) Description of the experiment: AGS cells were grown as described until reaching monolayers of cells, incubated with NBD-cholesterol for 3h and treated in three alternative ways: 1) incubation with DHA for 12 hours; 2) incubation with MBCD for 1 hour; 3) no incubation. Then, liquid cultures of *H. pylori* 26695 and SS1 were added to the AGS cells for 3h; the pelleted *H. pylori* was then grown in liquid cultures without DHA, with 100 μM of DHA, or with 250 μM of DHA, for 48h. B) AGS cells were grown on glass coverslips. AGS cells were preloaded with NBD-cholesterol. Samples were analyzed by laser scanning microscopy (magnification 40x). The experiment shows that NBD-cholesterol was effectively incorporated into AGS cells following a 3h incubation period (green staining represents NBD-cholesterol in AGS cells). C) Impact of cholesterol previous uptake on the inhibitory growth effect of DHA on *H. pylori*. Data are expressed as the mean $\pm$ standard deviation and are representative of three independent experiments. *(asterisk) Refers to significant differences in *H. pylori* growth between cells exposed to cholesterol and DHA-treated, MBCD-treated and AGS cells non-treated.

592

593 **Figure 1**

594
595 **Figure 2**

Figure 3

Figure 4