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Secção Autónoma de Ciências da Saúde

Sandra Manuela Carvalho Alves

Exploring the role of peroxisomes in *Helicobacter pylori* infection

Estudo do papel dos peroxissomas na infecção por *Helicobacter pylori*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Daniela Maria Oliveira Gandra Ribeiro, Investigadora Principal no Laboratório *Organelle Dynamics in Infection and Disease*, Centro de Biologia Celular e Departamento de Biologia da Universidade de Aveiro; da Doutora Maria de Fátima Camões Sobral Bastos, Investigadora de Pós-doutoramento no Laboratório *Organelle Dynamics in Infection and Disease*, Centro de Biologia Celular e Departamento de Biologia da Universidade de Aveiro e da Doutora Carla Marina Gonçalves Leite, Investigadora de Pós-doutoramento no Laboratório *Epithelial Interactions in Cancer*, Instituto de Patologia e Imunologia Molecular da Universidade do Porto.

Dissertation presented to the University of Aveiro to fulfil the necessary requirements to obtain a Masters' Degree in Molecular Biomedicine, held under the scientific guidance of Dr. Daniela Maria Oliveira Gandra Ribeiro, Principal Investigator at Organelle Dynamics in Infection and Disease Laboratory, Centre for Cell Biology and Department of Biology of University of Aveiro; Dr. Maria de Fátima Camões Sobral Bastos, Post-doc Researcher at Organelle Dynamics in Infection and Disease Laboratory, Centre for Cell Biology and Department of Biology of University of Aveiro, and Dr. Carla Marina Gonçalves Leite, Post-doc Researcher at Epithelial Interactions in Cancer Laboratory, Institute of Molecular Pathology and Immunology of the University of Porto.

Dedico este trabalho aos meus amigos e a toda a minha família, especialmente ao meu pai, pelo apoio incondicional durante todo o meu percurso académico.

o júri

presidente

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"If you want to go fast, go alone.
If you want to go far, go together."
(African Proverb)

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palavras-chave Peroxissomas, mitocôndrias, infecção por *Helicobacter pylori*

resumo

Helicobacter pylori é uma bactéria gram-negativa microaerofílica. Os seres humanos são o seu principal reservatório e estima-se que cerca de 50% da população mundial encontra-se infetada, embora apenas cerca de 20% dos pacientes apresentem sintomas. *H. pylori* coloniza a mucosa gástrica humana e está associada a várias doenças gastrointestinais, como gastrite crónica, úlcera péptica e cancro gástrico.

Os peroxissomas são organelos de membrana simples, que se encontram virtualmente em todas as células eucarióticas. Nestes ocorrem várias reações metabólicas, com destaque para a oxidação dos ácidos gordos, a biossíntese de lípidos e a desintoxicação do peróxido de hidrogénio.

É cada vez mais aceite que os peroxissomas desempenham muito mais do que simples funções metabólicas. Em estudos anteriores foi demonstrado que os peroxissomas estão envolvidos na resposta imune em infeções virais. Para além disso, foi sugerido que tanto os patogénios bacterianos como os virais podem ser expostos ao reconhecimento peroxissomal.

Assim, torna-se importante estudar melhor o papel dos peroxissomas em resposta a infeções.

No presente trabalho foram analisadas possíveis alterações peroxisomais relativamente à sua morfologia, número e função enzimática, em células infetadas, com o principal objetivo de explorar o possível papel dos peroxissomas na infeção *por H. pylori.*

Os resultados obtidos sugerem que a infeção por *H. pylori* não afeta significativamente a morfologia, número ou localização dos peroxissomas. No entanto, os resultados sugerem que a infeção por *H. pylori* afeta a quantidade de catalase peroxissomal, provavelmente devido a um aumento das EROs no meio celular, resultante da infeção bacteriana.

keywords Peroxisomes, Mitochondria, *Helicobacter pylori* infection

abstract

The *Helicobacter pylori* is a gram-negative microaerophilic bacterium. Humans are the principal reservoir and it has been estimated that about 50% of the world population is infected by this bacterium, even though only about 20% of the patients present symptoms. The *H. pylori* colonizes the human gastric mucosa and is associated to several gastrointestinal diseases, such chronic gastritis, peptic ulceration and gastric cancer.

Peroxisomes are membrane-enclosed subcellular organelles, which can be virtually found in all eukaryotic cells. They are involved in several metabolic reactions, with emphasis for the fatty acid oxidation, lipid biosynthesis and hydrogen peroxide detoxification.

It is increasingly accepted that peroxisomes are more than simple metabolic organelles within cells. Previous studies of viral infections have demonstrated that peroxisomes are involved in the cellular innate immune response. It has also been suggested that all bacterial and viral pathogens may be exposed to peroxisomal recognition.

Thus, it becomes important to better study the role of the peroxisomes in response to infections.

In the present work we have analysed the possible peroxisomal alterations in morphology, number and enzymatic function in infected cells with the main aim of exploring a possible role for peroxisomes in *H. pylori* infection.

The obtained results suggest that *H. pylori* infection does not affect significantly the peroxisomal morphology, number or localization. However, the results obtained suggested that *H. pylori* infection affects the amount of peroxisomal catalase, probably due to an increase of ROS in the cellular environment, as a consequence of the bacterial infection.

ABBREVIATIONS

BabA – Blood group antigen adherence adhesin

CagA – Cytotoxin associated gene A

CagE – Cytotoxin associated gene E

Cag PAI – Cag Pathogenicity Island

DLP – Dynamin-like protein

Drp – Dynamin-related protein

ER – Endoplasmic reticulum

FA – Fatty acid

HpaA – *Helicobacter pylori* adhesion A

HP-NAP – Neutrophils-activating protein

IceA – Induced by contact with epithelium

LPS – Lipopolysaccharide

MAVS – Mitochondrial antiviral signaling protein

MOM – Mitochondrial outer membrane

MOMP – Mitochondrial outer membrane permeabilization

mtDNA – Mitochondrial DNA

OMPs – Outer membrane proteins

PBD – Peroxisomes biogenesis disorder

PEDs – Single peroxisomal enzyme/transporter deficiencies

Pex – Peroxin

PMP – Peroxisomal membrane protein

PTS – Peroxisomal targeting signal

ROS – Reactive oxygen species

SabA – Sialic acid binding adhesion

SES – Socioeconomic status

SOD – Superoxide dismutase

TLRs – Toll-like receptors

T4SS – Type Four Secretion System

VacA – Vacuolating cytotoxin A

VLCFA – Very-long-chain fatty acids

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1. INTRODUCTION

1.1. Peroxisomes

1.1.1. The multifaceted organelle

The interest in the study of peroxisomes and their physiological functions and alterations has considerably increased in recent years.

Peroxisomes are crucial ubiquitous subcellular organelles that can be found virtually in all eukaryotic cells. This organelle is present in all tissues but is more abundant in liver and kidney (F. Camões et al., 2009; Delille et al., 2006; M. Islinger & Schrader, 2011; Schrader & Fahimi, 2008).

Peroxisomes are constituted by a single lipid bilayer membrane with embedded peroxisomal membrane proteins (PMPs) surrounding a fine granular matrix, which may contain crystalline inclusions of matrix enzymes, such as is schematized in Figure 1.1 (F. Camões et al., 2009; M. Islinger & Schrader, 2011; Ribeiro et al., 2012). Their diameter ranges between 0.1 µm and 1 µm in typical human cells (F. Camões et al., 2009).

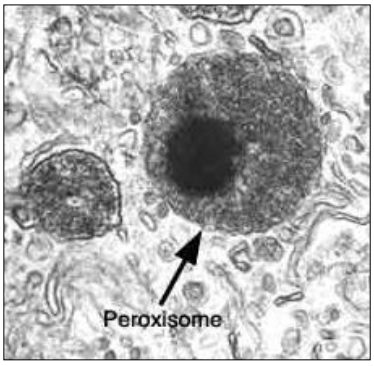

Figure 1.1 – Schematic representation of the peroxisome. *From facult.une.edu.*

Peroxisomes are highly dynamic, versatile, metabolically active and interconnected organelles. Their dynamics is associated to a great plasticity, hence, peroxisomes present an ample variety of different shapes and they have the vital capacity of changing their morphology to adapt to physiological changes in the cellular environment (M. Islinger et al., 2010). Peroxisomes can present different shapes ranging from small, spherical compartments to tubular reticular networks, and move along the cytoskeletal track (F. Camões et al., 2009; Schrader et al., 2003).

1.1.2. The life of the peroxisome: from the beginning to the end

Interestingly, the peroxisomes have distinctive features, which have often been in disagreement with existing dogmas in cell biology, particularly relating to their biogenesis and protein import, which differs substantially from the other organelles such as mitochondria (Delille et al., 2006). All proteins of peroxisomes are encoded by nuclear genes, because this organelle does not possess molecules of DNA or protein translation machinery (Schrader & Fahimi, 2008). The majority of the peroxisomal matrix and membrane proteins are consequently synthesized on free polyribosomes present in the cytosol and then post-translationally imported directly into pre-existing peroxisomes (Delille et al., 2006; Schrader & Fahimi, 2008).

The peroxisome biogenesis encompasses three main stages: the formation of the peroxisomal membrane, the import of proteins into the peroxisomal matrix, and the proliferation of the organelle. Distinct machineries are involved in this process. Peroxins are *PEX* genes-encoding proteins required for the biogenesis of the peroxisomes. Several peroxins identified are implicated in the import of matrix proteins and contribute to the formation of the coupling and translocation machinery at the peroxisomal membrane (Baker & Paudyal, 2014; Delille et al., 2006; Schrader, 2006). For example, Pex 5p and Pex 7p are cytosolic receptors that mediate the screening and internalization of peroxisomal matrix proteins which contain PTS1 (carboxy-terminal peroxisomal targeting signal 1) or PTS2 (peroxisomal targeting signal type 2) sequences (Baker & Paudyal, 2014; Delille et al., 2006; Schrader, 2006). However, the selection and insertion of peroxisomal membrane proteins (PMPs) are less understood and require other molecules than those involved in mechanism of peroxisomal matrix import (Delille et al., 2006). Pex 19p is the main protein that binds the PMPs in the cytoplasm and recruits them to the Pex 3p receptor at the organelle's membrane (Fujiki et al., 2006).

Peroxisomes can be formed according the *growth and division* model, which defends that the organelles can multiply from pre-existing peroxisomes (Figure 1.2) (Baker & Paudyal, 2014; Smith & Aitchison, 2013). This is a multistep process that includes peroxisome elongation/growth, constriction and final fission/division, forming spherical peroxisomes that can be asymmetrical or symmetrical (Smith & Aitchison, 2013). The conserved PMP Pex 11 is required for the elongation step and the GTPase DLP1 (dynamin-like protein 1) and the adaptors Fis1p and Mff play essential roles on the final fission (Itoyama et al., 2013; J. Koch & Brocard, 2012; Smith & Aitchison, 2013).

It has also been suggested that the peroxisomes are capable of forming *de novo* from the endoplasmic reticulum (ER) (Figure 1.2) (Baker & Paudyal, 2014; Veenhuis & Klei, 2014). It is suggested that PMPs accumulate at the ER and are afterwards incorporated into pre-peroxisomal vesicles (vesicles that have budded from the ER) that fuse to form mature peroxisomes (Van der Zand et al., 2010; Van der Zand et al., 2012).

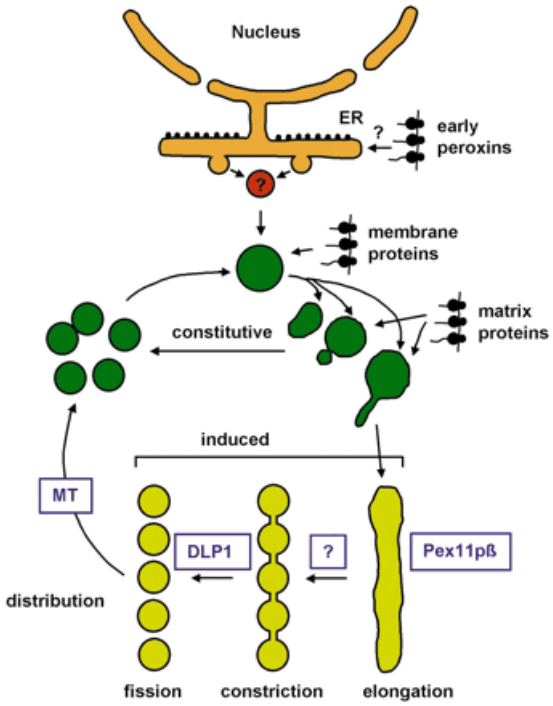

Figure 1.2 - Schematic view of dynamic peroxisome formation. *From A. Koch et al., 2004.*

Peroxisomes possess the ability to proliferate and multiply, or be degraded in response to nutritional and extracellular environmental stimuli (Delille et al., 2006; Schrader, 2006). Usually peroxisome proliferation comprises an increase in the number of peroxisomes, and an induction of peroxisomal enzymes, with emphasis to proteins involved in fatty acid β-oxidation (Delille et al., 2006).

When necessary, the peroxisome proliferation is reversed and the excess particles are removed by autophagy. Macroautophagy is a mechanism of sequestration by autophagy, where the organelles, such as peroxisomes, are first sequestered within autophagosomes that subsequently fuse with lysosomes/vacuoles where the digestion occurs (Baker & Paudyal, 2014; Schrader & Fahimi, 2008).

1.1.3. Peroxisomal functions

Peroxisomes are considered "multi-purpose" organelles, with specific metabolic functions that differ according to the organism and cell type, development stage of the organism and environment conditions (F. Camões et al., 2009; M. Islinger et al., 2010). They contribute to numerous biochemical pathways, such as a large variety of anabolic and catabolic reactions, being essential for human health and development (M. Islinger et al., 2012).

The most notables functions of the peroxisomes are related to the metabolism of the hydrogen peroxide and lipids, especially the β-oxidation of fatty acids (FA) (Delille et al., 2006; M. Islinger et al., 2011; Ribeiro et al., 2012). Peroxisomes have also an important role in other metabolic processes such lipid biosynthesis (bile acids, cholesterol and dolichol, fatty acid elongation and phospholipids necessary for nerve cell myelination), fatty acid α-oxidation, regulation of acyl-CoA/CoA ratio, protein/amino acid metabolism, glycerol biosynthesis, glyoxylate detoxification and catabolism of purines, prostaglandins and eicosanoids (Wanders & Waterham, 2006). Furthermore they contribute to signaling, development and ageing (M. Islinger & Schrader, 2011). New biological functions have been discovered in recent years, such pheromone production, polyamine metabolism, GPI-anchor biosynthesis, degradation of polyunsaturated dicarboxylic long-chain fatty acids, H_2O_2 signaling in hypothalamic neurons and viral innate immune defense (M. Islinger et al., 2012).

Fatty acids β-oxidation

β-oxidation is the principal mechanism of breakdown of fatty acid molecules. When fatty acids are more complex, like the case of the very long chain fatty acids (VLCFA), the degradation occurs initially in peroxisomes (Figure 1.3). This process is similar to the one that occurs in the mitochondria; both organelles contain their specific set of fatty acids β-oxidation enzymes, which catalyse similar reactions. The β-oxidation in mitochondria includes three steps: activation, transport and oxidation. The product of the last step suffers oxidation to become a molecule with two carbons, acetyl-CoA. However, in the peroxisome, due the fact that there is no respiratory chain, the electrons captured by FAD in the first oxidation react with O_2 to produce H_2O_2, making this mechanism a heat-generating process. In the second oxidation, the NADH has to be exported out of the peroxisome to be reoxidized in mitochondria. Finally, the chain-shortened acyl-CoA

esters have to be transported into the mitochondria where the process is completed (F. Camões et al., 2014; Delille et al., 2006).

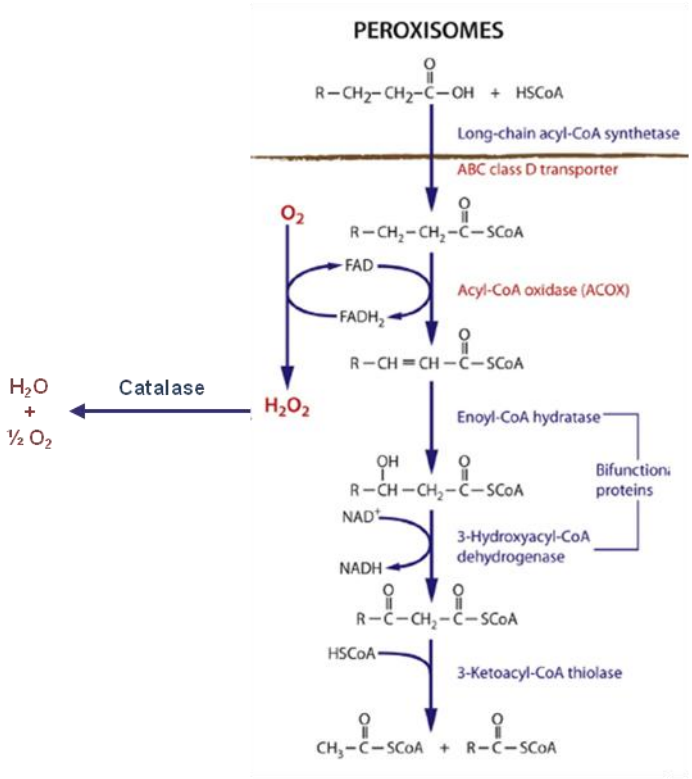

Figure 1.3 – Peroxisomal fatty acid β-oxidation pathway, including the reaction of detoxification of H_2O_2 by catalase. *Adapted from F. Camões et al., 2015.*

Peroxide Metabolism

Catalase is a peroxidase that exists in nearly all aerobically respiring organisms (Masters, 1995). This enzyme is an important regulator of oxidative stress and inflammation and is also noteworthy in protecting cells from toxic effects of hydrogen peroxide. In living tissue, the catalase enzyme decomposes hydrogen peroxide (H_2O_2) into water (H_20) and oxygen (0_2). H_2O_2 is a product of several normal metabolic pathways generated in the peroxisome, including the fatty acids *B*-oxidation (Figure 1.3). Catalase also utilizes the H_2O_2 to oxidize a variety of other substrates, that are especially important in liver and kidney cells, where peroxisomes detoxify various toxic molecules (Alberts et al., 2002; Schrader & Fahimi, 2006).

1.1.4. Peroxisomal disorders

There are many disorders associated with the peroxisomes, and often a simple change in a gene may lead to a very severe disease, sometimes even lethal. The peroxisomal disorders are usually classified in two groups: (1) the peroxisomal biogenesis disorders (PBDs) and (2) the single peroxisomal enzyme/transporter deficiencies (PEDs).

PBDs are a group of severe developmental brain disorders with a prevalence of 1:50.000. These disorders are caused by a mutation in peroxin *(PEX)* genes, which, such referred before, are related to peroxisome biogenesis (Waterham & Ebberink, 2012). PBDs usually lead to death during the childhood. This group of diseases includes the Zellweger syndrome (ZS), the neonatal adrenoleukodystrophy (NALD) and the infantile Refsum´s disease (IRD) and rhizomelic chondrodysplasia punctata (RCDP) type 1 (Delille et al., 2006; Waterham & Ebberink, 2012).

The single peroxisomal enzyme deficiencies are also based on a genetic mutation that affects a protein involved in one of the peroxisomal functions. The PEDs can be subdivided into distinct subgroups on the basis of the peroxisomal metabolic pathway affected: ether phospholipid (plasmalogen) biosynthesis, fatty acid β-oxidation, peroxisomal α-oxidation; glyoxylate detoxification, and H_2O_2 metabolism (Wanders, 2004). An example of PEDs is the metabolic disorder characterized by a total or close to total absence of catalase enzyme activity in erythrocytes, named acatalasemia, well-known as acatalasia or catalase deficiency (Wanders, 2004).

1.1.5. Peroxisomes – more than simple metabolic organelles

It is increasingly accepted that peroxisomes are more than simple metabolic organelles. Peroxisomes are active and interact functionally and morphologically with other organelles. For example, peroxisomes and mitochondria keep a very close interrelationship, which includes metabolic cooperation, such as in the degradation of fatty acids; contribute to cellular ROS homeostasis; share a redox-sensitive relationship and coordinated biogenesis by sharing key proteins of their division machinery, like Drp1 (dynamin-related protein 1), Fis1 and Mff (Hettema & Motley, 2009; Schrader, 2006). This cooperative interaction probably influences the functionality of both organelles in health and disease. In addition to the mitochondria, peroxisomes also interact with the ER and lipid droplets (Hettema & Motley, 2009; Odendall & Kagan, 2013; Schrader, 2006). Recently it has also been shown that peroxisomes play an

important role in the cellular antiviral immune defence mechanisms (Odendall & Kagan, 2013). The recognition of cellular infection by microorganisms is mediated by several receptor families, leading to production of different effectors that establish the immune response (Akira et al., 2006; Mogensen, 2009). These receptors recognize different pathogen associated molecular patterns, such as bacterial lipopolysaccharides, flagellin, lipoproteins and double-stranded RNA (Odendall & Kagan, 2013). In viral infections, the receptor RIG-I recognizes the viral genome and interacts with the proteins MAVS (mitochondrial antiviral signaling protein) at the mitochondrial and peroxisomal membranes which activates a signaling cascade that culminates with the production of compounds that will interfere with the viral life-cycle and propagation (Odendall & Kagan, 2013). The peroxisomal MAVS induces the rapid interferon-independent expression of defense factors while mitochondrial MAVS activates an interferon-dependent signaling pathway with delayed kinetics, amplifying and stabilizing of the antiviral response (Odendall & Kagan, 2013).

A recent study have shown that the intracellular bacteria *Chlamydia* is able to hijack peroxisomes and make use of their enzymatic capacity to produce bacteria-specific phospholipids (Boncompain et al., 2014). However, not much more is known concerning the role of peroxisomes on the establishment or even the response against bacterial infections.

1.2. Helicobacter pylori

1.2.1. The Infectious agent

Helicobacter pylori is a small spiral–shaped gram-negative bacterium that belongs to the *Enterobacteriaceae* family (Figure 1.4) (Dunn & Cohen, 2000). It has a circular chromosome and is enclosed within two membranes that consist of an inner (cytoplasmic) membrane, periplasm with peptidoglycan, and an outer membrane of phospholipids and LPS (Lipopolysaccharide) (Figure 1.4). The bacterium measures between 2 and 4 µm in length and have a diameter between 0.5 and 1 µm. *H. pylori* usually has two to six unipolar sheathed flagella of around 3 µm in length that are important for its rapid motility through the viscous mucus that overlay the epithelial cells of the gastric mucosa (Figure 1.4) (Kusters et al., 2006; O'Toole et al., 2000).

H. pylori is a microaerophilic pathogen and its optimal growth occurs in the presence of 5 to 15% of oxygen (Kusters et al., 2006). The bacterium is generally viewed as an extracellular pathogen that is etiologically associated with various gastroduodenal diseases and, in 1994, it was considered a type I carcinogen according to IARC (International Agency for Research on Cancer), a subordinate organization of the WHO (World Health Organization).

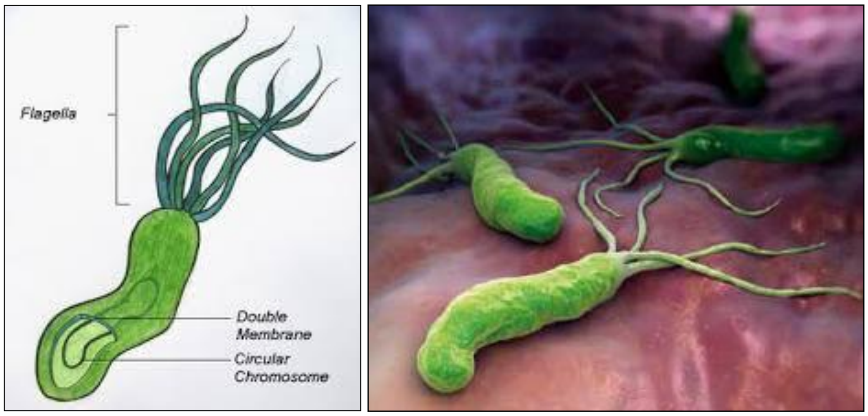

Figure 1.4 - **The spiral morphology and composition of *H. pylori.*** *From © 2008 Michelle Wiepjes and iStock.com/iLexx.*

1.2.2. Possible routes of transmission

The mode of transmission of *H. pylori* infection is still poorly understood. Possible routes of transmission comprise, for example, person-to-person contact, iatrogenic route and water route.

Person-to-person contact is considered the most likely transmission route, although exact transmission from one person to another is unknown (Duynhoven & Jonge, 2001). It is thought to occur by oral-oral or faecal-oral contamination (Brown, 2000; Duynhoven & Jonge, 2001; Mladenova et al., 2006). The oral-oral transmission is the most probable, especially in developed countries, and includes saliva, dental plaque and refluxed gastric contents or vomit (Duynhoven & Jonge, 2001).

The iatrogenic route includes transmission from one infected patient to another patient or to staff members following endoscopy (Azevedo et al., 2007; Brown, 2000). Nosocomial transmission of *H. pylori* is currently the unique proven mode of transmission; however in quantitative terms it is considered to be insignificant.

Another possible route of transmission of *H. pylori* is through the water due to faecal contamination (Brown, 2000). *H. pylori* can survive for several days in tap water in its infectious bacillary form and in river water for several months in a coccoid form (Brown, 2000; Vincent, 1995). Although there is still controversy regarding whether or not *H. pylori* coccoid forms are metabolically active or dead bacterium, or whether they can revert to original spiral shape, drinking water from stream, swimming in a stream and swimming in a swimming pool has been suggested as risk factors for the occurrence of infection, especially in developing countries (Brown, 2000; Goodman et al., 1996).

Although the principal reservoir of the bacterium *H. pylori* appears to be the human stomach, *H. pylori* was also been isolated from non-human primates and domestic cats (Azevedo et al., 2007; Brown, 2000; Mohamed & El-gohary, 2012). Nevertheless, results suggest that infection is most likely uncommon in cats and so it should not be considered a public health problem. *H. pylori* DNA was also detected in sheep (Brown, 2000; Mohamed & El-gohary, 2012; Momtaz et al., 2014) and the domestic housefly was suggested as a reservoir of *H. pylori,* however it seems improbable that this operates as a vector for *H. pylori* transmission (Brown, 2000; Mladenova et al., 2006).

Some studies indicate that is possible to decrease the risk of infection through increasing consumption of fruits, vegetables, vitamin C and high levels of beta-carotene, for example (Brown, 2000).

1.2.3. Prevalence of the H. pylori infection and risk factors

H. pylori infection has high prevalence in the entire world, being one public health problem for developing and developed countries. There is about 70% of prevalence in developing countries and about 30-40% of prevalence in developed countries. The prevalence of *H. pylori* infection depends broadly, for example, of the geographic area, age, race, ethnicity and socioeconomic status (SES) (Brown, 2000). Infection usually occurs during childhood, being a risk factor the age up to 10 years (Azevedo et al., 2007; Duynhoven & Jonge, 2001; Mladenova et al., 2006). The variation in age at acquisition of infection seems to be one of the factors to explain different effects on the gastric mucosa, which in turn result in different clinical outcomes.

H. pylori is a successful pathogenic bacterium considering its worldwide prevalence of in approximately 50% of the population. Although all *H. pylori*-infected individuals present histological gastritis, only 15-20% develops more severe clinical outcomes such as gastroduodenal ulcers, MALT lymphoma and gastric cancer. It is thought that the risk of development of infection and these diseases depends on differences in host genetic susceptibility to particular strains of *H. pylori*, genetic differences of the *H. pylori* strains and environmental factors (Brown, 2000; Yamaoka, 2010).

In the host genetic factors are included ABO blood group, Lewis blood-group antigen and polymorphism in inflammatory genes and cytokines. For example, it was established that blood group "O" is a risk factor in the pathogenesis of duodenal ulcer disease however, is not a risk factor for acquiring *H. pylori* infection (Pathol, 1991). Several host Lewis antigens on gastric epithelium, such as Lewis b (Le^b), Le^x and sialyl-Le^x, have been demonstrated act as receptors for *H. pylori* adhesions thus facilitating the bacterial colonization (Backert et al., 2011; Sheu et al., 2003). Another example is the host polymorphism in genes encoding interleukin-1 beta and the interleukin-1 receptor-antagonist (genes that regulate inflammatory responses) that were suggested to be associated with development of gastric cancer (J. C. Machado et al., 2001).

Several environmental factors are associated with *H. pylori* positivity, such as smoking, alcohol consumption, diet, and high salt consumption. A recent study demonstrates that a high salt intake promotes the carcinogenic effects of $CagA^+$ *H. pylori* strains, constituting a risk factor for gastric cancer (Gaddy et al., 2013). Some studies have evaluated the possible association between *H. pylori* infection and active smoking (Brown, 2000), though with contradictory results. Regarding

the alcohol consumption, especially at moderate to high levels, studies demonstrate a negative association with *H. pylori* infection (Brown, 2000).

1.2.4. Diseases associated with H. pylori infection

H. pylori infection causes gastric inflammation which can lead to several gastroduodenal diseases (Figure 1.5) (Brown, 2000; Dunn & Cohen, 2000; Kusters et al., 2006). *H. pylori* is the major etiologic agent of chronic gastritis (CG), which consists in a cellular infiltrate of immune cells in the human gastric mucosa (Kusters et al., 2006). In 5% of *H. pylori*-positive patients the gastritis can lead to development of gastric atrophy. *H. pylori* is also associated with the development of peptic ulcers, either gastric or duodenal ulcers, and *H. pylori*-positive patients have 10 to 20% lifetime risk of developing peptic ulceration (Kusters et al., 2006). Peptic ulcers are considered as mucosal wounds with a diameter of at least 0.5 cm penetrating the *muscularis* mucosa. Duodenal ulcers generally occur around 20 and 50 years old and gastric ulcers usually occur approximately at 40 years old (Kusters et al., 2006).

Infection by *H. pylori* has been associated with an increased risk of developing gastric cancer, which occurs in 1 to 2% of infected individuals, and with mucosa-associated lymphoid tissue (MALT) lymphoma that occurs in less than 1% of *H. pylori*-positive patients (Sepulveda, 2013; Suzuki et al., 2009).

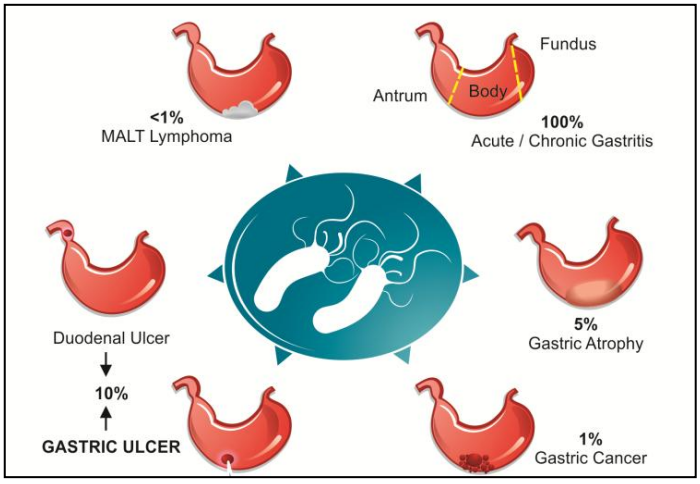

Figure 1.5 - **Schematic representation of diseases´ outcome of *H. pylori* infection**. *From Negrei & Boda, 2014.*

Currently, *H. pylori* infection diagnosis rely on several tests like biopsy-based tests, serological tests and breath urea test. Serologic test detects IgG antibodies produced against *H. pylori,* but IgA and IgM antibodies can be used as well (Brown, 2000). A “gold standard” method for detect

patients with *H. pylori* infection is an endoscopy to obtain a biopsy for testing by rapid urease test and/or for histological analysis The non-invasive breath urea test is also a "gold standard" method for *H. pylori* diagnosis and for confirmation of eradication after therapy (Brown, 2000).

1.2.5. Colonization

H. pylori is able to colonize the surface of antrum and corpus of human stomach, with a tropism for the intercellular junctions of gastric epithelial cells. The infection persists for decades, unless treated with antibiotics (Kusters et al., 2006). Indeed, although it was isolated at the first time by Warren and Marshall, in 1982, the bacterium has evolved with humans for thousands of years (58.000 years) and is well adapted to survival in the gastric mucosa for almost the entire lifetime of the host; furthermore spontaneous eradication from the human stomach can be considered as a rare event (Pacifico et al., 2008).

Colonization mechanism of *H. pylori* is characterized by four steps: (1) penetration in the crypts of the stomach surface epithelium; (2) adaptation to the environment; (3) attachment to epithelial cells and finally (4) inflammation of gastric mucosa and the increase of reactive oxygen and nitrogen species, which results in chronic gastritis. After exploration of host defense mechanisms, *H. pylori* acquires nutrients and can have a successful replication. Colonization of the gastric mucosa requires a complex adaptive process (Testerman et al., 2001). In this context the main features that allow *H. pylori* development in the stomach are: its motility and penetration into the viscous mucus layer via flagella and spiral morphology, ability to change the pH of the stomach, production of urease enzyme, presence of bacterial adhesins that bind to the surface of epithelial cells, induction of inflammatory response and production of virulence factors like CagA and VacA cytotoxin (Figure 1.6).

Urease is produced in high levels, constituting about 6% of the total bacterial protein of *H. pylori*. Urease enzyme hydrolyzes urea into ammonia and carbon dioxide, increasing this way the pH in the stomach, which can protect the bacterium from the gastric acidity (Marais et al., 1999).

In the human stomach, the bacteria can be found in two different localizations: free in mucus layer or adhered to epithelial cells of the gastric tissue, mainly at intercellular junctions (Marais et al., 1999). Various *H. pylori* proteins had already been associated to a role in the attachment of the bacterium to epithelial cells (Backert et al., 2011), like *H. pylori adhesion A* (HpaA), *blood group antigen-binding adhesin* (BabA), *sialic acid binding adhesin* (SabA) and the *outer membrane*

proteins (OMPs). Around 4% of the *H. pylori* genome is predicted to encode OMPs, some of which can act as adhesions, like OipA (Yamaoka, 2010).

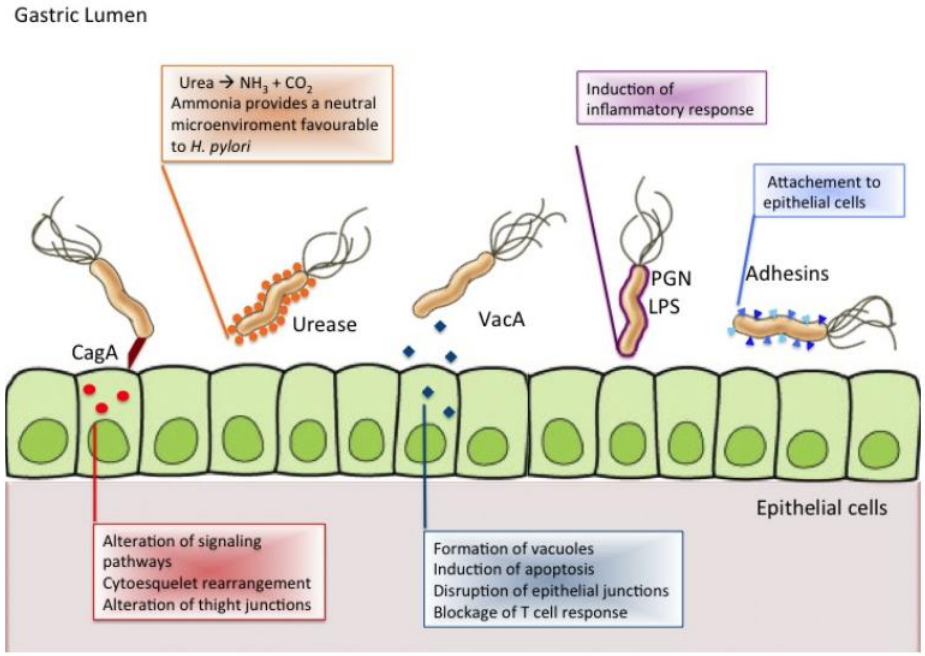

Figure 1.6 – *Helicobacter pylori* virulence factors activities. *From Morales-guerrero et al., 2001.*

1.2.6. Pathogenesis and virulence factors

Chronic active gastritis is the primary condition related *to H. pylori* colonization. However, a cascade of *H. pylori*-associated disorders can be activated in the infected mucosa until the development of gastric cancer, which is one of the most common malignancies in the world (Figure 1.7) (Kusters et al., 2006). *H. pylori* infection can lead to development of the gastric atrophy - with loss of stomach cells and impaired digestive system -, which can lead to metaplasia - characterized by transformation of the stomach lining - and, after, to dysplasia, corresponding to initial stages of stomach cancer. Dysplasia can, then, evolve to gastric cancer. Indeed, *H. pylori* is a carcinogenic and the most fascinating and best studied risk factor for gastric cancer (Wadhwa et al., 2013). It was already shown that its virulence factors induce chronic inflammation, mucosal damage and multiple alterations in the gene expression, including increase of oncoproteins (A. M. D. Machado et al., 2010; Wadhwa et al., 2013). Furthermore, *H. pylori* induces the expression of Toll-like receptors (TLRs), resulting in proliferation of gastric cells, and is also associated to genetic

and epigenetic alterations, which can promote transformation, invasion and metastasis of host cells (Polk & Peek, 2010; Sepulveda, 2013; Suzuki et al., 2009; Wadhwa et al., 2013).

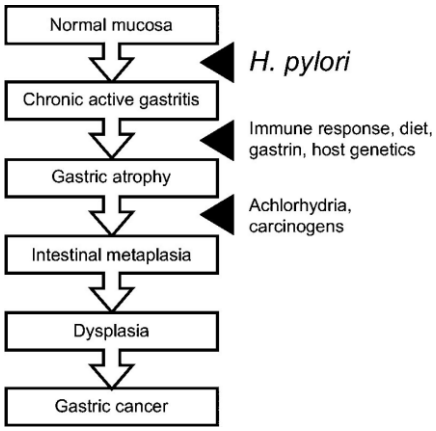

Figure 1.7 – Model representing the role of *H. pylori* and other factors in gastric carcinogenesis, based on the cascade proposed by Correa et al, 1975. *From Kusters et al., 2006.*

The pathogenicity of *H. pylori* is associated with the genome of the bacterium, which can be correlated with the severity of induced gastric lesions. *H. pylori* produces a number of virulence factors, such VacA (*vacuolating cytotoxin A*), CagA (c*ytotoxin associated gene A*), CagPAI (C*ag Pathogenicity Island*), CagE (c*ytotoxin associated gene B),* IceA (i*nduced by contact with epithelium),* HP-NAP (neutrophils-*activating protein),* and BabA that play an important role in disease development.

CagA

The *cagA* was the first strain-specific gene identified in *H. pylori.* The structure of the *cagA* gene reveals a 5´ region highly conserved however with a 3´region that possesses a variable number of repetitive sequences, leading to variation of the length of the CagA protein. Strains with more repetitive sequences seem to be associated to higher levels of CagA antibody, more severe degrees of atrophy and reduced survival in a low pH (Graham & Yamaoka, 1998; Kusters et al., 2006). The *cagA* gene is considered a marker for infection associated with a higher level of inflammation and to presence of CagPAI (Kusters et al., 2006). The exact function of CagA is unknown, but appeared to be a major virulence factor, which perturbs host cell signalling and in this manner promotes disturbance of epithelium and gastric carcinogenesis, and lead to production of IgG and IgA specific by the organism. The antigenic protein CagA is produced by about 60% of the strains and it is present only in *H. pylori* strains that are associated with severe forms of gastroduodenal disease (type I strains) (Graham & Yamaoka, 1998; Orodovsky et al., 1996).

CagPAI

CagPAI possesses about 31 genes and can be founded in about 60% of the western strains (Kusters et al., 2006). Furthermore CagPAI contains genes that encode components of the T4SS (Type Four Secretion System) which consists in a multiproteic complex that acts like a needle and injects bacterial effectors molecules into the host epithelial cell, such CagA protein and peptidoglycan components (Backert et al., 2011; Guillemin et al., 2002; Terradot & Waksman, 2011). This process can allow the bacterium to modulate host signalling pathways, including the expression of proto-oncogenes. The presence of the CagPAI corresponds to greater degree of virulence of strains (Kusters et al., 2006).

CagE

The *cagE* gene belongs to the CagPAI and is associated with an increased production of interleucine-8 (IL-8) by the epithelial cells of gastric mucosa, which increasing the presence of neutrophils in gastric mucosa of infected patients (Dunn & Cohen, 2000; Kusters et al., 2006).

VacA

The *vacA* gene is virtually present in all strains of *H. pylori* and presents two variable parts, *s* (encodes the signal peptide) and *m* (allele of middle region). The combination in mosaic of these alleles determines the production of cytotoxins responsible for degree of virulence of *H. pylori* (Basso et al., 2008). The virulence factor VacA is produced by approximately 50% of *H. pylori* strains (Cover, 1996). VacA is associated to severe inflammation, ulceration and increased risk of gastric cancer development (Kusters et al., 2006). This protein promotes formation of large intra-cellular vacuoles within cultured mammalian epithelial cells, increase cell permeability, mitochondrial network fragmentation and epithelial cell apoptosis (Figure 1.8) (Cover, 1996; Jain et al., 2011; Palframan et al., 2012). The VacA induces also the formation of selective channels of anions in epithelial cells, leading to exudation of urea into the gastric mucosa that is important as substrate for urease enzyme function (Kusters et al., 2006; Palframan et al., 2012).

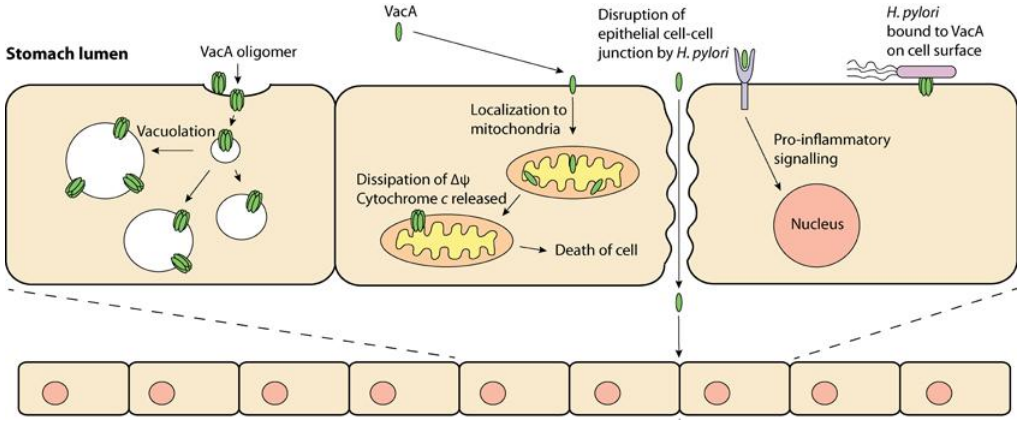

Figure 1.8 - VacA cytotoxin effects in epithelial cells. *From Palframan et al., 2012*.

HP-NAP

The intensity of inflammation and neutrophil infiltration is connected with the severity of the damage induced in mucosa and DNA. The chemotactic factor HP-NAP promotes neutrophil adhesion to endothelial cells and stimulates the release of reactive oxygen, nitrogen and proteases by neutrophils (Dunn & Cohen, 2000; Kusters et al., 2006).

BabA

The factor adherence BabA linked to difucosylated Lewis^b (Le^b) blood group Ags found on epithelial cells. This allows the contact between the bacterium and the epithelium and facilitates the release of virulence factors like as CagA and VacA. The *babA* gene has two different alleles: *babA1* and *babA2*. The *babA2* appears to improve the colonization properties of *H. pylori* by the increase of colonization densities, which are important for the degree of mucosal inflammation and damage (Backert et al., 2011; Kusters et al., 2006; Rad et al., 2002).

A summary of some examples of adhesins and virulence factors associated with *H. pylori* is presented in Table 1.1.

Table 1.1 – Examples of adhesins and virulence-associated proteins of *H. pylori.*

Protein	Predicted role	Association with *H. pylori*-related disease
VacA	Disrupts endosomal maturation leading to cytoplasmic vacuolation; selectively increase the permeability of polarized epithelial cell monolayers resulting in barrier dysfunction at tight junction and induces mitochondrial damage, cytochrome c release and gastric epithelial cell apoptosis.	Has been directly implicated in the development of gastric and duodenal ulcers and increased risk of gastric cancer development.
CagA	CagA is associated with a prominent inflammatory response. Once in the host leads to cytoesquelet rearrangement. *cagA* gene is considered a marker for the of presence of CagPAI.	CagA is involved in gastric cancer development.
CagPAI	CagPAI is associated with increased interleukin-8 production and mucosal inflammation. And encodes a type IV secretation system.	Possibly associated to gastric cancer and duodenal ulcer disease.
IceA	The *iceA1* allele encodes CATG-recognizing restriction endonucleases.	*iceA1* has been associated with peptic ulcer disease, but this association is not universal.
HP-NAP	HP-NAP is reported to activate neutrophils and is a possible adhesin to mucin; possible function in protection of *H. pylori* DNA or iron storage.	Unknown
BabA	Binds to fucosylated Le^b blood group antigen on cells.	*babA2* allele has been implicated in peptic ulcer disease and gastric cancer.
SabA	Binds to sialyl-Le^x and sialyl-Le^a antigens and is involved in activation of neutrophils.	None
OipA	OipA has been reported to assist in IL-8 induction, but this association is not universal.	Expression of OipA is linked to *cag* status and development of duodenal ulcers and gastric cancer.

1.3. Aims of the Dissertation

The main aim of this master thesis was to evaluate a possible role for peroxisomes during *Helicobacter pylori* infection. For that were established two specific objectives:

- Evaluate if *H. pylori* infection in different time points induces alterations in the morphology and number of peroxisomes (in parallel to mitochondria) in gastric cells by fluorescence microscopy analysis.
- Evaluate the possible alterations in metabolic functions of peroxisomes upon *H. pylori* infection in different time points by western blotting analysis.

2. MATERIALS & METHODS

2.1. Cell culture

Human MKN28 gastric epithelial cell line derived gastric adenocarcinoma, and the monkey COS-7 fibroblast-like cell line, derived from the African green monkey kidney fibroblasts, were grown in RPMI (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone serum, GE Healthcare HyClone™, Utah, USA) and 1% (v/v) penicillin-streptomycin solution (Invitrogen) at 37 ºC in a humidified atmosphere of 5% CO_2 in air. Cells were grown in T75 or T25 plastic flask (TPP, Trasadingen, Switzerland) for protein extraction and in 6-well plates (TPP) with coverslips for immunofluorescence studies.

2.2. *H. pylori* strains and growth conditions

H. pylori strains 26695 (ATCC 700392; cag PAI^+; $cagA^+$; $vacA^+$: s1/m1), 60190 (ATCC 49503; cag PAI^+; $cagA^+$; $vacA^+$: s1/m1), and 84-183 (ATCC 53726; cag PAI^+; $cagA^+$; $vacA^+$: s1/m1) were obtained from the American Type Culture Collection (ATCC). *H. pylori* strains were cultured for 48 h in tryptic soy agar (TSA) plates supplemented with 5% sheep blood (Becton, Dickinson and Company, New Jersey, USA) and incubated at 37 ºC under microaerobic conditions. For the infection, bacteria grown in TSA plates were harvested in phosphate-buffered saline (PBS), pH 7.4, and added to the host cells at a multiplicity of infection (MOI) of 100. Bacterial density was estimated by the absorbance measurement at 600 nm.

2.3. Infection of mammalian cell lines

Cell lines were seeded in culture flask (T75 or T25) or in 6-well plates to reach forty to fifty confluence on the day of the infection, in complete medium without antibiotics. *H. pylori*, collected in PBS from TSA plates, was added to host cells at a MOI of 100 for different times points, and the cultures were maintained at 37 ºC under a 5% carbon dioxide atmosphere. Control cell cultures (non-infected cells) were performed in the same conditions as described above in the absence of bacteria. After the infection period, cells were washed 3 times in PBS plus $Ca2^+$/$Mg2^+$ to remove all bacteria (non-adherent and adherent), and lysed for western blotting analysis or fixed for immunofluorescence studies. The cell culture and the *Helicobacter pylori* infection were performed at IPATIMUP by our collaborators, as well as the preparation of lysates for western blotting analysis and the samples on coverslips for immunofluorescence analysis.

2.4. Western blotting analysis

2.4.1. Lysates preparation

Infected and non-infected cells were lysed in PBS, pH 7.4 with 1% Triton X-100 and 1% NP-40 lysis buffer with proteases (Protease Inhibitors Set, Roche Diagnostics GmbH, Penzberg, Germany) and phosphatases (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich Corporation, MO, USA) inhibitors. Cells were scraped with cold complete lysis buffer on ice, centrifuged at 14.000 rpm, 30 min, at 4 ºC. Soluble proteins were collected after centrifugation and stored at -80 ºC.

2.4.2. Total protein quantification (Bradford´s method)

The total protein concentration (µg) from the lysates was determined using the Bradford Protein Assay. Bradford Protein Assay is a simple method used to determine the total protein content of a sample; this method is based on the proportional binding of the dye Coomassie to proteins. Coomassie absorbs at 595 nm.

The Bradford reagent was diluted 1:5 with H_2O, to the necessary final volume. Tubes with known BSA concentrations (1-15 µg), were prepared using BSA stock solution with 1 µg/µl, and used as standards. Sample tubes were also prepared using the same volume of lysates. Duplicates were done for all the conditions. After, all tubes were filled up to 100 µl with 0.1 M NaOH, 1 ml of diluted Bradford was added to each tube and all were incubated for 15 min at room temperature (RT). The optical density at 595 nm (OD_{595}) of the samples and standards was measured using a spectrophotometer. A standard curve was created and the samples total protein concentration was calculated using Excel software.

2.4.3. SDS-PAGE

Sodium Dodecyl Suftate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique commonly used, which uses a discontinuous polyacrylamide gel as a support medium and SDS, an anionic detergent that denatures proteins and confers a net negative charge to the polypeptide in proportion to its length. In SDS-PAGE, the proteins are separated by an electric current according to their molecular weight, this means that heavy molecules have a slow migration and light molecules have a rapid migration.

Polyacrylamide gels were prepared with a 10% resolving gel on the bottom and a 4% stacking gel on top (composition at the Appendix). Samples with 60 µg of protein were prepared with loading buffer (composition at the Appendix), boiled for 5 min and loaded on the gels. A molecular weight marker was also loaded. The apparatus with gels were placed in a tank filled with running buffer (composition at the Appendix). The electrophoresis ran at 80 V and 300 mA for 2h to 2h 30 min.

2.4.4. Electrotransference

Separated proteins were transferred from the electrophoresis gel to a support membrane of nitrocellulose. A sandwich made with blotting paper, gel, membrane and blotting paper was assembled in the appropriate apparatus filled with transfer buffer (composition at the Appendix). The electrotransference occurred at 0.4 A and 100 V for 2h. After the transference, the membranes were stained with Ponceau S solution (composition at the Appendix) to verify the transference efficiency. Membranes where then washed with PBS and let dry for further analysis.

2.4.5. Immunoblotting

The membranes were hydrated with PBS, incubated with a 5% dry milk solution in PBS for 1h, for blocking and washed with TBS-T (Tris-buffered saline and 0.15% Tween 20). After, the membranes were incubated with the primary antibody, for 1-3h. The primary antibodies were diluted in TBS-T containing 3% dry milk, according to the table 2.1. After the incubation the membranes were washed with TBS-T, 3 times for 10 min. The membranes were then incubated for 1h with the secondary antibodies diluted 1:5000 in PBS. The secondary antibodies are conjugated with horseradish peroxidase (HRP) and were raised in goat against rabbit or mouse IgGs. Thus, it was used goat anti-rabbit or goat anti-mouse, depending on the primary antibody used. Finally, the membranes were washed with TBS-T, 3 times for 10 min. All the incubations and washes were done with agitation and at RT.

Table 2.1 – Primary antibodies used in western blotting analysis.

Target Protein	Antibody	Dilution	Expected MW	Product
Catalase	α-catalase rabbit polyclonal	1:500	64 KDa	sc-50508, Santa cruz biotechonology®
CagA (Cytotoxin associated gene A)	α-CagA rabbit polyclonal	1:750	120KDa	sc-25766, Santa cruz biotechonology®
GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase)	α-GAPDH mouse monoclonal	1:7500	37 KDa	sc-47724, Santa cruz biotechonology®

2.4.6. Chemioluminescence detection (ECL)

ECL™ is a light emitting non-radioactive method for the detection of immobilized antigens, conjugated with HRP antibodies. The membranes previously incubated with primary and secondary antibodies were incubated with the ECL detection solution for 1 min, in the dark at RT. The membranes were then exposed to x-ray films in a proper cassette for the appropriate time. After, the films were developed and fixed with the respective solutions.

2.4.7. Quantification and data analysis

The intensity of the obtained bands (protein expression) on film was quantified by densitometry using GS-880 calibrated imaging densitometer and Quantity One software (BioRad). The expression of proteins was expressed as percentage of control meaning that to the control was attributed 100%.

2.5. Immunofluorescence analysis

2.5.1. Samples preparation

After the infection period (as described at section 2.1), mammalian cells grown on coverslips were washed 3 times in PBS plus $Ca2^{+}/Mg2^{+}$ to remove all bacteria (non-adherent and adherent) and fixed with 4% para-Formaldehyde (pFA) for 20 min at RT. Then were washed and stored in PBS at 4 ºC. This procedure was also done in IPATIMUP by our collaborators.

2.5.2. Immunofluorescence procedure

To continue the immunofluorescence procedure, the coverslips to be used were transferred to a new multiwells, washed 3 times with PBS, permeabilized with 0.2% Triton X-100 for 10 min at RT and washed again 3 times with PBS. The samples were then blocked with 1% BSA in PBS for 10 min at RT, followed by the incubation with the primary antibodies at appropriate dilutions in PBS (Table 2.2) for 1h at RT. Each coverslip was incubated simultaneously with two different primary antibodies, which dilutions were prepared together. To prevent from drying, the multiwells were wrapped in wet paper. After the incubation the coverslips were washed 3 times with PBS. Then, the appropriate secondary antibodies were incubated for 1h as previously. The coverslips were once again washed 3 times with PBS. An incubation with the Hoechst solution, for 2 min, was also performed as previously. Each coverslip was dipped 2 times in dH_2O and mounted with a drop of Mowiol solution on slide.

Table 2.2 – Primary and secondary antibodies (and dye) used in IMF analysis.

Primary antibody	**Dilution**	**Product**
α-PMP70 mouse monoclonal	1:100	SAB4200181, Sigma-aldrich®
α-TOM20 rabbit monoclonal	1:100	612278, BD Biosciences
Secondary antibody and dye	**Dilution**	**Product**
Donkey α-Mouse IgG, Alexa 488	1:400	A21202, Invitrogen™
Donkey α-Rabbit IgG, TRICT	1:100	711-025-152, Jackson ImmunoResearch
Hoechst 33258 dye	1:2000	09460 Polysciences

2.5.3. Microscopy analysis

Microscopy analysis was done using an Olympus IX-81 inverted microscope (Olympus Optical Co.) equipped with a Plan Apo 100x/1.40 oil objective (Olympus Optical) and with the appropriate filter combinations.

Images were acquired with an F-view II CCD camera (Soft Imaging System) driven by Soft Imaging software. Obtained images were processed using the Soft Imaging Viewer.

3. RESULTS

3.1. Morphological analysis

Peroxisomes' remarkable dynamics is associated to a great plasticity, presenting an ample variety of different shapes and the capacity of changing their morphology to adapt to physiological changes in the cellular environment (M. Islinger et al., 2010). Alterations in peroxisome morphology have been observed under certain disease conditions such as carcinogenesis and even viral infections (Ribeiro et al., 2012). The study of peroxisome morphology and metabolism during bacterial infections is yet an unexplored field of research that we aimed at tackling with the experimental procedure followed in this master thesis. We have selected the bacterium *H. pylori*, due its high prevalence worldwide, causing several gastroduodenal diseases (Kusters et al., 2006). Furthermore, there are evidences that this bacterium directly affects the mitochondria, including mtDNA mutations and activating the cellular mitochondrial fission machinery in the infected cells (Jain et al., 2011; A. M. D. Machado et al., 2010).

In order to unravel a possible role for peroxisomes during *H. Pylori* infection, we started by performing a detailed analysis of the peroxisome morphology (in parallel to mitochondria morphology) in different time points after infection of gastric cells. MKN28 cells were infected with the highly pathogenic *H. pylori* 26695 and 60190 strains (*cagPAI*$^+$ and *vacA*$^+$ strains of *H. pylori*) to assure that the results obtained were not strain-specific. Cells were fixed in 4% paraformaldehyde immediately after infection, subjected to immunofluorescence with antibodies against peroxisomal and/or mitochondrial proteins and observed under a fluorescence microscope.

Two hundred cells were analysed per condition taking into account the size/shape and number of their peroxisomes in comparison to the non-infected controls in the following time points: 3h, 6h, 10h and 24h after infection. We considered cells containing "fragmented" peroxisomes/mitochondria as those whose organelles were smaller and in higher number when compared to the control cells. On the other hand, cells containing "elongated" peroxisomes/mitochondria presented longer and less organelles than in control cells.

As shown in Figure 3.1, Figure 3.2 and Figure 3.3 upon *H. pylori* infection, the mitochondrial network presents a progressive fragmentation over the time. At 24h post-infection, the mitochondria were practically all fragmented, especially in the samples of cells infected with the *H. pylori* 60190 strain, which appears to be more virulent that the *H. pylori* 26695. The results

show that the *H. pylori* infection (independently of the used strain) induced the transition of mitochondrial networks into significantly shorter punctiform organelles, which is similar to the results previously reported in other related studies (Ashktorab, 2004; Jain et al., 2011). And demonstrating that *H. pylori*-dependent fragmentation of mitochondria is not idiosyncratic to a single strain.

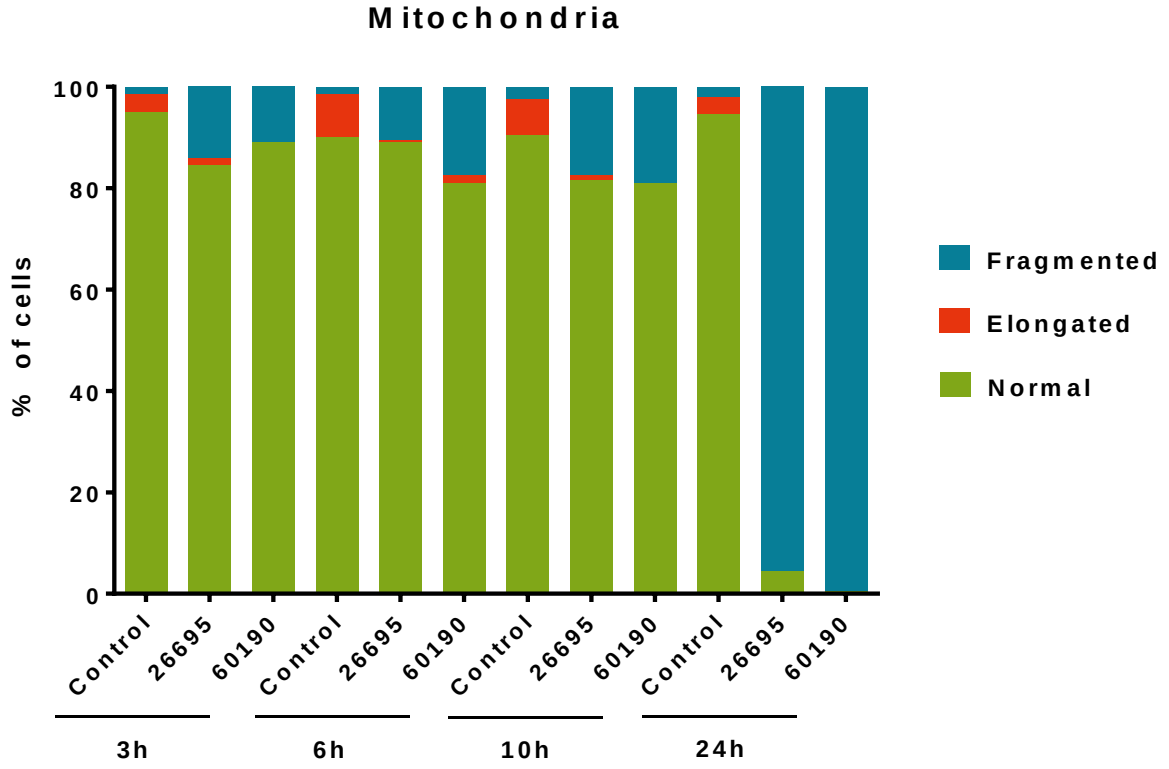

Figure 3.1 - **Morphology analysis of Mitochondria**. Percentage of MKN28 cells with *normal, fragmented* or *elongated* morphology of mitochondria in uninfected (control) and *H. pylori* infected (26695 and 60190 strains) MKN28 cells, in different time points.

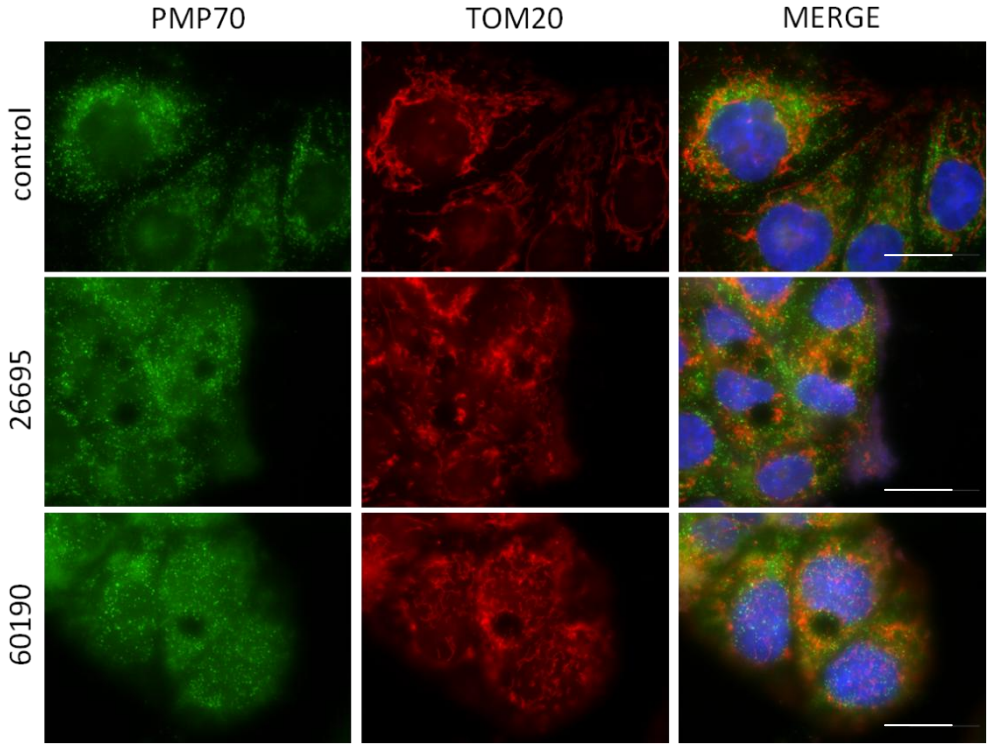

Figure 3.2 – Fluorescence microscopy analysis of peroxisomes and mitochondria in MKN28 cell line infected with *H. pylori* strains 26695 and 60190, 10h post-infection. Peroxisomes were marked with α-PMP70 and mitochondria were marked with α-TOM20. Nuclei are shown in blue. Scale Bars: 20 µm.

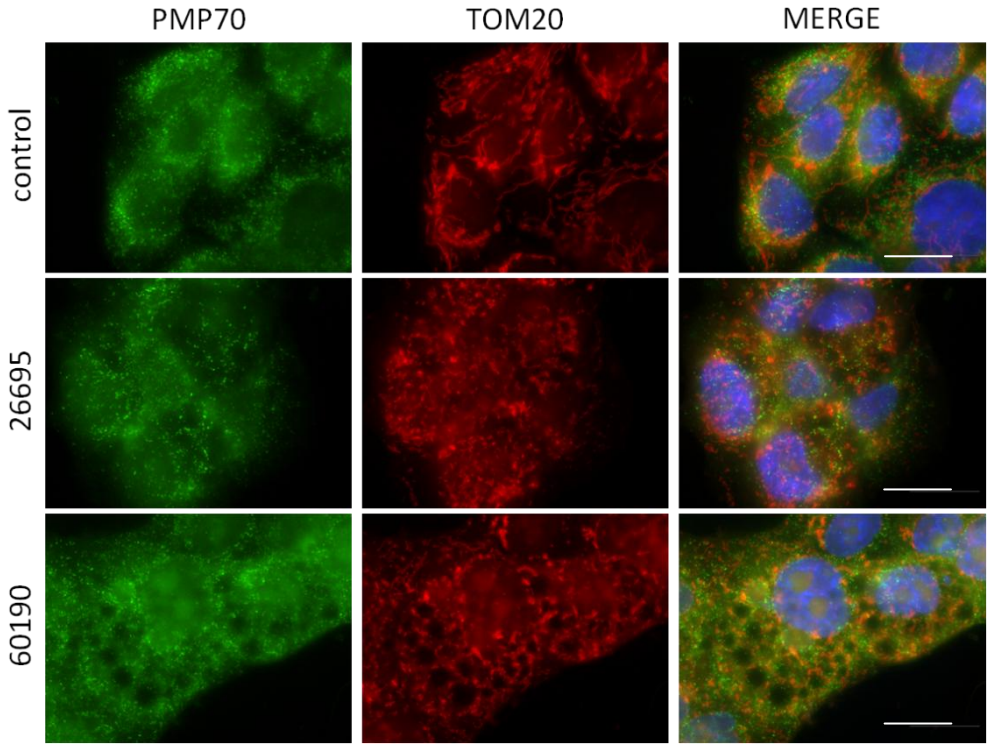

Figure 3.3 - Fluorescence microscopy analysis of peroxisomes and mitochondria in MKN28 cell line infected with *H. pylori* strains 26695 and 60190, 24h post-infection. Peroxisomes were marked with α-PMP70 and mitochondria were marked with α-TOM20. Nuclei are shown in blue. Scale Bars: 20 µm.

As shown in Figure 3.2, Figure 3.3 and Figure 3.4, no significant alterations were observed on peroxisome morphology after the *H. pylori* infection, at any of the observed time points. Our results, hence, suggest that mitochondrial morphology changes are relevant for the establishment of *H. pylori* infection but demonstrate no specific influence of peroxisomal morphology on this mechanism.

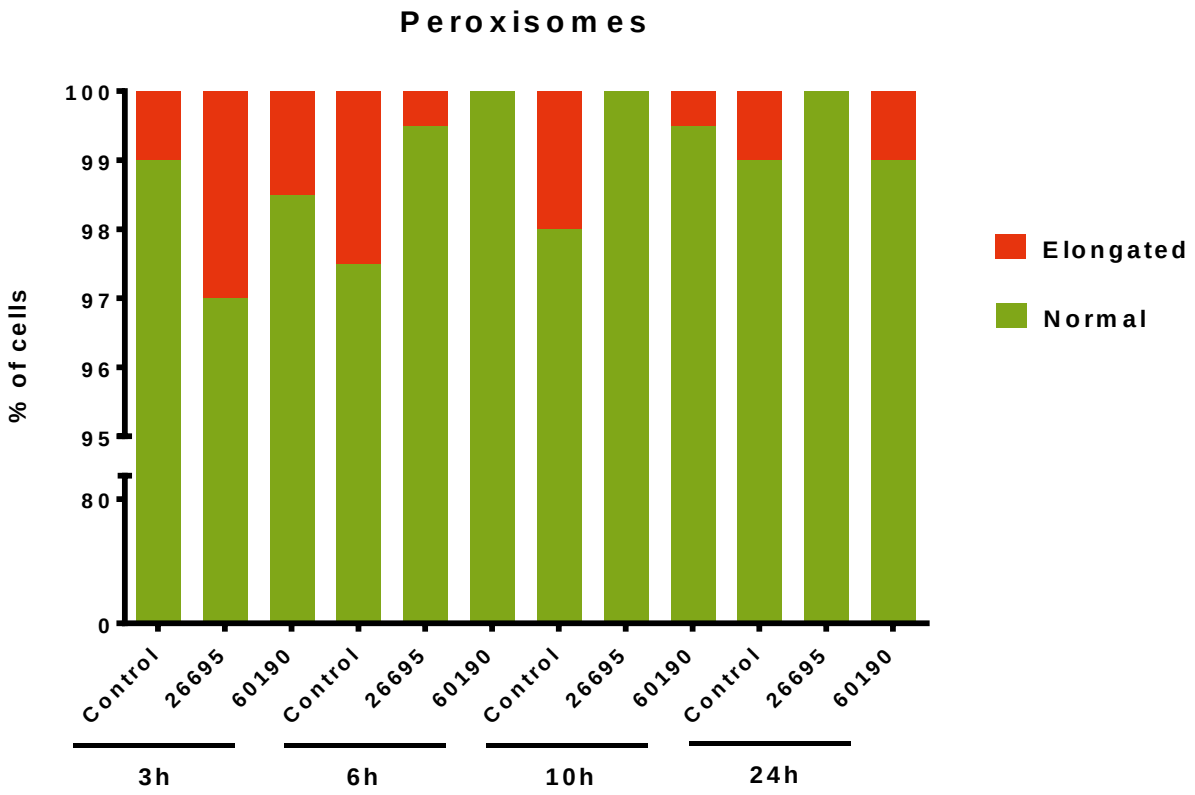

Figure 3.4 - **Morphology analysis of Peroxisomes**. Percentage of MKN28 cells with *normal* or *elongated* morphology of peroxisomes in uninfected (control) and *H. pylori* infected (26695 and 60190 strains) MKN28 cells, in different time points.

We have also analysed (in parallel to the previous analyses) the presence of vacuoles, which are strictly associated with the pathogenicity of VacA cytotoxin. Significant differences between the strains were not observed, as both are *vacA*$^{+}$ with the highly vacuolating genotype (s1/m1), and the expected slight rise in the number of vacuoles is observed in the last time points (Figure 3.5), in accordance with the increase of toxicity caused by the infection over the time.

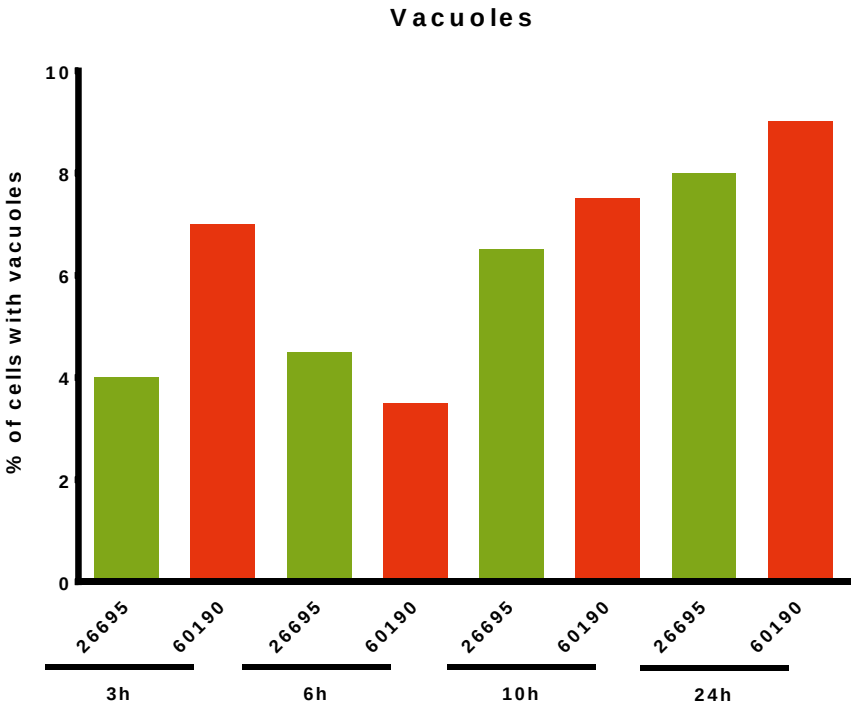

Figure 3.5 – Percentage of cells with vacuoles in MKN28 cell line, over the time in control and *H. pylori*-infected cells.

3.2. Metabolic analysis

Two of the main functions associated to peroxisomes are the β-oxidation of long- to very long-chain fatty acids and the metabolism of hydrogen peroxide, a reactive oxygen specie (ROS), by the catalase enzyme. Previous studies have demonstrated that *H. pylori* infection can induce ROS production and apoptosis in human gastric epithelial cells (Cytotoxin et al., 2003; Kuck et al., 2001; Tsugawa et al., 2012). High levels of ROS have been involved in cellular physiological and pathological mechanisms such as cell proliferation, apoptosis, differentiation, carcinogenesis, among others.

Besides the peroxisomal morphological analysis presented above, another aim of this study was the evaluation of the possible alterations in metabolic functions of this organelle upon *H. pylori* infection. With that in mind, we have used lysates of COS-7 cells infected with the *H. pylori* 26695 strain and analysed the presence and amount of the peroxisomal protein catalase, in comparison to the control uninfected cells. The cell lysates were subjected to western blotting analysis as previously described. The samples were analysed at different time points: 6h, 8h, 12h, 16h and 24h post-infection with *H. pylori.* GAPDH was used as loading control of the protein input in all membranes.

By analysing the expression of catalase in the different samples we could observe a rapid and significant increase in the protein levels in infected cells when compared to uninfected control cells, following by a clear decrease with the time post-infection as shown in Figure 3.6.

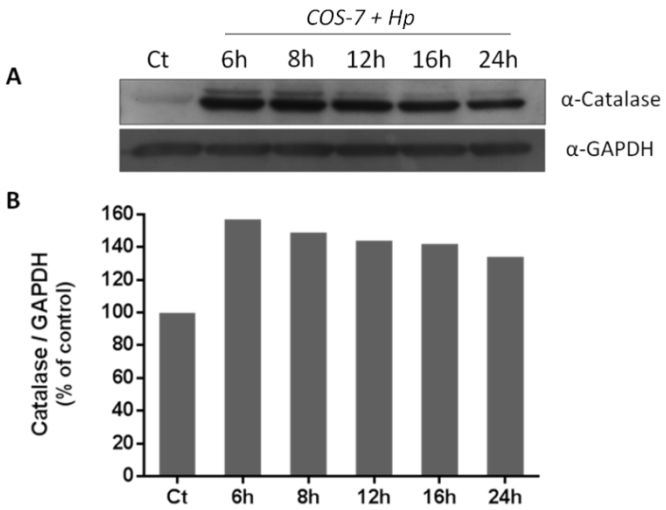

Figure 3.6 – **Effect of *H. pylori* infection on catalase expression in COS-7 cells.** Cells were infected with *H. pylori* and collected at the specified time points (6h, 8h, 12h, 16h and 24h). **A**. The expression levels of catalase was analysed by immunoblotting with specific antibody. GAPDH levels were also assessed as a loading control. **B**. Results obtained were quantified, catalase expression levels were normalised with GAPDH levels and values represented graphically as percentage of control (cells not infected).

As the *H. pylori* also contains catalase, we tested whether the α-catalase antibody also recognized the bacterial catalase from pure samples of *H*. pylori 84183 strain. Surprisingly in Figure 3.7 we observed that, indeed, the used antibody clearly also recognized the catalase from the *H. pylori.* However, it is not expected that the amount of bacteria that remains in the samples decreases throughout the time. In order to check this, we used an antibody against CagA, a specific virulence factor of *H. pylori*, and, as shown in Figure 3.7, there was no significant differences on the amount of bacteria present in the sample. With this we can conclude that the decrease observed in the catalase expression is due to a specific decrease on the peroxisomal catalase from the infected mammalian cells.

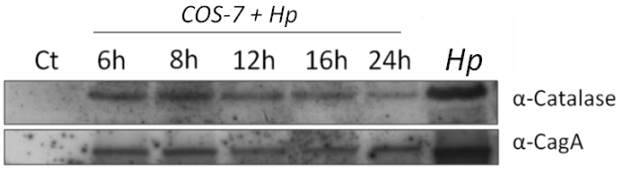

Figure 3.7 - Analysis of catalase and CagA expression in COS-7 cells and in pure bacterial lysate. The expression of catalase and CagA was analysed by western blotting in lysates of uninfected cells and cells infected with *H. pylori,* for 6h, 8h, 12h, 16h and 24h. A pure bacterial lysate was included to evaluate if the anti-human catalase antibody cross-reacted with the bacterial catalase. The CagA expression was used as a reference for the contribution of bacterial protein to the total protein of the samples.

4. DISCUSSION

Peroxisomes are crucial ubiquitous membrane-enclosed subcellular organelles, which can virtually be found in all eukaryotic cells. They are involved in several metabolic pathways, with emphasis for the β-oxidation of fatty acids, lipid biosynthesis and production and scavenging of ROS, in particular of hydrogen peroxide (M. Islinger et al., 2012; Schrader & Fahimi, 2006). However, peroxisomes are more than simple metabolic organelles: they are active and dynamic organelles that interact functionally and morphologically with other organelles, such mitochondria, ER and lipid droplets (Odendall & Kagan, 2013; Schrader, 2006). Indeed, peroxisomes and mitochondria exhibit a close interrelationship that includes metabolic cooperations, cross-talk and share of key components of their fission machinery (F. Camões et al., 2009; Schrader, 2006) . Alterations in metabolism, biogenesis, dynamic and proliferation in one organelle can potentially influence the other. Previous studies have demonstrated that peroxisomes are involved in innate immunity and protective responses of the cell, such as during viral infections, where they work in partnership with mitochondria (Odendall & Kagan, 2013). However, not much is yet known about the role of peroxisomes in bacterial infections.

The bacterium *H. pylori* was selected for this study, due the fact that is a human gastric pathogen of high prevalence all over the world (Brown, 2000; Kusters et al., 2006), and there are evidences that *H. pylori* infection directly affects the mitochondria and leads to cellular apoptosis (Fannjiang et al., 2004; Jain et al., 2011; Willhite & Blanke, 2004).

Our results have shown that, 10h after *H. pylori* infection, mitochondrial suffered a significant change from a reticulotubular to a punctiform phenotype, culminating at 24h post-infection with an almost complete mitochondrial fragmentation. These results are in agreement with previous studies that have also reported a change on mitochondrial morphology upon *H. pylori* infection (Ashktorab, 2004; Jain et al.,2011).

It has been shown that the chronic infection by *H. pylori* is associated with increased apoptosis within the gastric mucosa. This increase may alter the gastric environment (loss of specialized cells, cellular proliferation and gastric atrophy) to promote *H. pylori* persistence and contribute to gastric disease. VacA, in addition to be important for *H. pylori* colonization and disease pathogenesis is also essential and sufficient to promote mitochondrial network fragmentation (Jain et al., 2011). It has been reported that VacA disrupts the cellular dynamics of mitochondria during infection as a mechanism to modulate the host's apoptotic machinery and to induce gastric epithelial cell death (Cytotoxin et al., 2003; Fannjiang et al., 2004; Karbowski & Youle, 2003).

VacA is a toxin that traffics to the mitochondria and is then translocated across the mitochondrial outer membrane (MOM); in the interior induces mitochondrial dysfunction and mitochondrial outer membrane permeabilization (MOMP). Increased mitochondrial fission promotes MOMP within VacA-intoxicated cells; VacA - dependent MOMP requires activation of the proapoptotic effector Bcl-2-associated X protein (Bax) and effector Bcl-2 homologous antagonist/killer (Bak), leading to membrane depolarisation, cytochrome c release and subsequent cell death (Ashktorab, 2004; Parone et al., 2006; Yamasaki et al., 2006). It has been shown that VacA recruits and induces the activation of cellular mitochondrial fission machinery, including the dynamin-related protein 1 (Drp1) that localizes to focal sites of division on the MOM (Jain et al., 2011).

Peroxisomal morphological changes are often in concert with mitochondrial alterations. However, our morphological analyses have revealed no apparent changes in peroxisome morphology upon *H. pylori* infection. The morphology control of these two organelles seems to be orchestrated independently during infection. The fact that they share most proteins of their fission machinery may not be of relevance in this case and the recruitment of Drp1 to the mitochondrial membrane (inducing its fragmentation) may indeed be solely induced by the localization of VacA at this organelle.

A certain level of peroxisomal elongation could be expected in response to an increasing of ROS, caused by the infection and VacA (Schrader & Fahimi, 2006). However, we have not observed any relevant elongation when compared to control cells. The absence of a significant elongation can be due to the fact that the ROS levels present were not sufficient to cause this change in peroxisomal morphology. Moreover, it is highly likely that an increase of antioxidant proteins acted as the first line of defence against the increase of ROS in the environment. In fact, our results clearly show a dramatic increase in the amount of catalase between uninfected cells and cells infected with *H. pylori.* Oxidative stress represents a serious problem for *H. pylori* infection, as one of the host's primary defences against bacterial infection is the production of ROS, particularly hydrogen peroxide. To react to this cellular response *H. pylori* possesses sophisticated antioxidant defence mechanisms, including catalase, superoxide dismutase (SOD), thioredoxins, peroxiredoxins, alkylhydroperoxide reductase and NADPH quinone reductase. The structure of the bacterial catalase is closely related to other catalases, which explains why the α-catalase antibody used in our experiments also recognized the bacterial catalase. However, as shown by the levels of CagA, there is no significant change on the amount of bacteria present in the analysed samples. Hence, the increase of catalase levels in comparison to the control as well as its

decrease with the time post-infection is clearly due to the amount of the mammalian (peroxisomal) catalase.

The hyperlipidemia is a feature of bacterial infections and it has been suggested to be related to the elevation of the cytokine TNFα, which was related to the significant suppression of the expression of catalase and other peroxisomal proteins (Schrader & Fahimi, 2006). In this context, an elevation of cytokine TNFα promoted by *H. pylori,* could be a possible explanation for the slight decrease of catalase during infection.

All together our results suggest that, although peroxisomal morphology does not change upon *H. pylori* infection, this organelle seems to have an important role on the infection mechanism by contributing for the degradation of the ROS that are produced by the cell. Further experiments should be performed in order to confirm our results in lysates from gastric cells. The analysis of other peroxisomal proteins, such as the ones involved in the β-oxidation of very-long-chain fatty acids would also be pertinent. It would be also interesting to repeat these analyses in cells infected with mutant *H. pylori* lacking some virulence factors such as CagA or VacA. These and other analyses must be performed in order to better unravel the role of peroxisomes in *H. pylori* infection and pinpoint the mechanism involved.

5. CONCLUSION & FUTURE PERSPECTIVES

The obtained results suggest that *Helicobacter pylori* infection does not affect significantly the peroxisomal morphology, number or localization. However, in the context of metabolic analysis, the results suggested that *H. pylori* infection affects the amount of peroxisomal catalase, probably due to an increase of ROS in the cellular environment (as consequence of the bacterial infection) and the subsequent necessity of to increase their degradation.

However, more experiments are needed to confirm the observed results and it would also be relevant to analyse possible changes in other peroxisomal enzymes, such as the ones involved in the lipids β-oxidation. In addition, the enzymatic activity of catalase should also be evaluated. The analyses of all these proteins in samples of cells infected with mutant *H. pylori,* without some virulence factors such as CagA or VacA toxin, would also like lead to interesting results.

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7. APPENDIX

1x PBS: 1.39 M NaCl, 80 mM $NaHPO_4$, 0.0268 M KCl, 0.0147 M KH_2PO_4, pH 7.36, prepared from 10xPBS diluted in ddH_2O.

1x TBS-T: 100 mM Tris pH8, 1,5 M NaCl, 0,05% Tween20, prepared from 10x TBS -T diluted in ddH_2O.

SDS-PAGE

Polyacrylamide Gels

Table 7.2 - Composition of the running and stacking gels for SDS-PAGE. APS – ammonium persulfate, SDS – sodium dodecyl sulphate, PAA – polyacrylamide.

Components	10% Resolving Gel	Stacking Gel 4%
40% PAA	4.00ml	1.00ml
2 M Tris pH 8.8 /1M Tris pH 6.8	2.98ml	1.25ml
20% SDS	80µl	50µl
H_20	8.88ml	7.61ml
TEMED	8µl	10µl
10% APS	48µl	80µl

Loading buffer: 1M Tris pH 6.80, 10% Glycerol, 1M DTT, 20% SDS, β-Mercaptoethanol, 0.1% Bromophenol Blue.

Running Buffer 1x: 250 mM Tris, 1.9 M Glycin, 1% SDS.

Electrotransference

Transfer buffer: 0.05 M Tris, 0.4 M Glycin, 0.05% SDS, 20% Metanol.

Ponceau S solution: 0.2% Ponceau S in 3% Acetic acid

Protocol Immunofluorescence (IMF)

PFA 4%: 20 g PFA in 450 mL ddH_2O, 4 drops 1 M NaOH, 50 mL 10x PBS

Mowiol Solution (3:1 Mowiol/n-propyl-Galat)