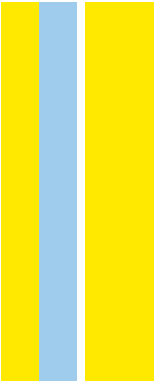


DOUTORAMENTO
PATOLOGIA E GENÉTICA MOLECULAR

Characterization of the adhesion and
pathogenicity mechanisms of gastric
non-*Helicobacter pylori* *Helicobacter*
species in humans

Rita Santos Matos

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2021



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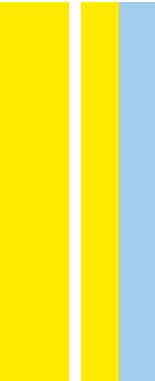


D.ICBAS 2021

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of gastric non-*Helicobacter pylori* *Helicobacter* species in humans

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**Characterization of the adhesion and pathogenicity
mechanisms of gastric non-*Helicobacter pylori*
Helicobacter species in humans**

Tese de Candidatura ao Grau de Doutor em Patologia e
Genética Molecular submetida ao Instituto de Ciências
Biomédicas Abel Salazar da Universidade do Porto.

Porto

2021

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Funding

PhD Fellowship (SFRH/BD/131159/2017) provided by the Portuguese Foundation for Science and Technology (FCT) of the Portuguese Ministry of Science, Technology and Higher Education.

Financiamento

Bolsa Individual de Doutoramento (SFRH/BD/131159/2017) da Fundação para a Ciência e Tecnologia (FCT) do Ministério da Ciência, Tecnologia e Ensino Superior.

Declaration

The author of this thesis declares that, in accordance with “n° 2, Alinea a, do Art.° 31° do Decreto-Lei n° 230/2009”, afforded a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted and/or submitted articles:

Scientific publications:

Matos R, De Witte C, Smet A, Berlamont H, De Bruyckere S, Amorim I, Gärtner F, Haesebrouck F (202). Antimicrobial Susceptibility Pattern of *Helicobacter heilmannii* and *Helicobacter ailurogastricus* isolates. *Microorganisms*. 8, 957; doi:10.3390/microorganisms8060957.

Matos R, Amorim I, Magalhães A, Haesebrouck F, Gärtner F, Reis CA (2021). Adhesion of *Helicobacter* species to the human gastric mucosa: A deep look into glycans role. *Frontiers in Molecular Biosciences*. 8:656439. doi: 10.3389/fmolb.2021.656439.

Matos R, Santos Sousa H, Nogueiro J, Magalhães A, Reis CA, Carneiro F, Amorim I, Haesebrouck F, Gärtner F. *Helicobacter* species binding to the human gastric mucosa. *Helicobacter*. e12867. doi: 10.1111/hel.12867.

Matos R, Taillieu E, De Bruyckere S, Rêma A, Santos Sousa H, Nogueiro J, Reis CA, Carneiro F, Haesebrouck F, Amorim I, Gärtner F. Presence of *Helicobacter* species in gastric mucosa of human patients and outcome of *Helicobacter* eradication treatment. *Journal of Personalized Medicine*. 12, 181. <https://doi.org/10.3390/jpm12020181>.

Matos R, Reis CA, Haesebrouck F, Amorim I, Gärtner F. The impact of *Helicobacter* species infection in human gastric cell lines (in preparation/ preliminary results)

This thesis also includes unpublished results.

Related scientific publications included in the Appendix section (Chapter 7):

Matos R, Fonseca KL, Mereiter S, Maceiras AR, Gomes J, Vilaplana C, Gärtner F, Rodrigues PNS, Reis CA, Saraiva M, Magalhães A (2021). *Mycobacterium tuberculosis* infection up-regulates sialyl-Lewis X expression in the lung epithelium. *Microorganisms* 9, 99 <https://doi.org/10.3390/microorganisms9010099>.

Fonseca KI, Maceiras AR, **Matos R**, Simões-Costa L, Sousa J, Cá B, Barros L, Fernandes AI, Mereiter S, Reis R, Gomes J, Tapia G, Rodríguez-Martínez P, Martín-Céspedes M, Vashakidze S, Gogishvilis S, Nikolaishvilis K, Appelberg R, Gärtner F, Rodrigues PNS, Vilaplana C, Reis CA, Magalhães A, Saraiva M (2020). Deficiency in the glycosyltransferase Gcnt1 increases susceptibility to tuberculosis through a mechanism involving neutrophils. *Mucosal Immunology* 13:836-848 <https://doi.org/10.1038/s41385-020-0277-7>.

Other scientific publications:

Freitas D, Campos D, Gomes J, Pinto F, Macedo JA, Matos R, Mereiter S, Pinto MT, Polónia A, Gartner F, Magalhães A, Reis CA (2019). O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. *EbioMedicine* 40:349-362 doi: 10.1016/j.ebiom.2019.01.017

Correia AS, **Matos R**, Gartner F, Amorim I, Vale N (2021). High Drug Resistance in Feline Mammary Carcinoma Cell Line (FMCm) and Comparison with Human Breast Cancer Cell Line (MCF-7). *Animals (Basel)* 6;11(8):2321 doi: 10.3390/ani11082321

ACKNOWLEDGMENTS

First, I would like to thank to my mentor and thesis supervisor Fátima Gärtner, for her support and kindness throughout all these years. She always had comfort and calm words to guide me in this journey, making me realize that everything is possible and helping me in this big process of becoming a scientist. Thank you!

To my co-supervisor Irina Amorim, I don't have enough words to thank you for all your support, openness, availability and friendship. I know that this was hard for everyone, but you gave me the opportunity to try and explore all my craziest ideas during these years. I made some mistakes for sure, but they were necessary for me to grow and became a better person and scientist.

I owe a deep thank to Freddy Haësebrouck, my co-supervisor. You received me in Ghent, and I felt so welcomed for everyone. You always pushed me to see a little bit forward and take an overall look to everything, showing me that there is always something more that we can do... we just need to keep trying! Thank you so much!

I hope I did not disappoint you!

I would also like to thank to Prof. Celso Reis, Ana Magalhães and Joana Gomes from Glycobiology in Cancer. You helped me in this path, sometimes even without notice it, and I am very thankful for your support and kind words during this journey.

To all my colleagues at Glycobiology in Cancer group, thank you for always being there for me. But more importantly, thank you for your kindness, advises and your support in the discussion of the work and all the difficulties and anxieties related with a PhD thesis. A special word for Francisca Diniz and Joana Rodrigues, thank you so much for your friendship, for being always there for me, for listening my problems and making me laugh even in the worst days!

Tânia, Lara, Cláudia, Bárbara... Thank you for your friendship and for being my confidants! You are such a joy in my life, and I don't have words to thank you for your daily reminder that life is much more than just work!

To my entire family, thank you for always being there and never doubt that I will do it and celebrated with me every conquer and achievement! To my mom and dad, that gifted me every day with love and support words, even being so away. To my brother and grandmother, I am so sorry that you had to listen my problems and confessions. Thank you for simple view of everything and helping me to relativise and see that there is always another way.

The *Helicobacter* genus is composed of at least 45 different species. These bacteria are Gram-negative, spiral-shaped and able to colonize the gastrointestinal tract of multiple hosts. *Helicobacter (H.) pylori* is the most studied species and known to infect the human gastric mucosa of approximately half of the worldwide population. Infection by *H. pylori* leads to a chronic inflammation, which can persist and evolve to more severe gastric disorders, such as peptic ulcers, dysplasia, intestinal metaplasia and gastric carcinoma. Furthermore, individuals infected by *H. pylori* can also present MALT lymphoma. The mechanisms underlying the adhesion and colonization of the human gastric mucosa by *H. pylori* have been widely studied in the last years. The major intervenients include the BabA and SabA bacterial adhesins, and the LeB and sialylated glycans expressed at the superficial epithelium. Currently, when infection by this pathogen is detected, patients are submitted to an eradication treatment, usually consisting of a proton pump inhibitor and 2 antibiotics (metronidazole and clarithromycin/amoxicillin) (standard triple therapy). Typically, these treatments eliminate the bacteria and avoid the progression to pre-neoplastic lesions. However, the efficacy of these therapies decreased in the last decades, mostly due to acquired resistance to the antibiotics used. Regarding this, new therapeutical approaches are urgently needed to treat *H. pylori* infection.

Besides *H. pylori*, several other *Helicobacter* species have been described colonizing the stomach of animals, such as pigs, dogs and cats, and humans, usually named non-*Helicobacter pylori Helicobacter* (NHPH) species. *H. suis* is one of the most common NHPH species, and it is known to infect the porcine gastric mucosa. Canine- and feline-associated NHPHs include *H. felis*, *H. salomonis*, *H. bizzozeronii* and *H. heilmannii*. These bacteria have also been found in the human gastric mucosa, and have been associated with gastric disorders, such as gastritis, peptic ulcers and MALT lymphoma. Actually, the occurrence of MALT lymphoma is higher after infection by NHPH than with *H. pylori*. The occurrence of human infection highlights the zoonotic potential of the NHPHs, which can cross the species barrier and be transmitted from animals to humans. Interestingly, acquired resistance to several antibiotics may also occur in NHPHs. These NHPHs lack the major adhesins involved in binding of *H. pylori* to the gastric mucosa, so the mechanisms used to colonize both humans and animals remain unknown.

Generally, this doctoral project has three aims: 1) determine the occurrence of *Helicobacter* spp. infection and the efficacy of the eradication treatment in the human Portuguese population; 2) determine the antimicrobial susceptibility pattern of some NHPH species to several antimicrobial compounds and possible acquired resistance mechanisms; and 3) evaluate the effects of NHPH species infection, at cellular level, namely the effect on cell viability, induction of apoptosis and inflammatory response, using gastric cell lines as *in vitro* models.

O género *Helicobacter* é composto por pelo menos 45 espécies diferentes, todas Gram-negativas e caracterizadas pela sua forma helicoidal. Estas bactérias são capazes de colonizar o trato gastrointestinal de vários hospedeiros. *Helicobacter* (*H.*) *pylori* é a espécie mais estudada e conhecida por infetar a mucosa gástrica humana de aproximadamente metade da população mundial. Infecção por *H. pylori* resulta numa inflamação crónica, que pode persistir e evoluir para condições clínicas mais severas, como úlceras, displasia, metaplasia intestinal, carcinoma gástrico e linfoma de MALT (tecido linfoide associado à mucosa). Os mecanismos usados pela *H. pylori* para aderir e colonizar a mucosa gástrica humana têm sido amplamente estudados nos últimos anos. Os principais intervenientes incluem as adesinas BabA e SabA, expressas na membrana bacteriana, e o LeB antigénio e estruturas sialiladas expressas à superfície das células epiteliais. Hoje em dia, quando um indivíduo é diagnosticado com infecção por *H. pylori*, este é submetido a um tratamento de erradicação, que consiste na administração de uma bomba de prótons e dois antibióticos diferentes (metronidazole e claritromicina/amoxicilina), num esquema chamado de terapia tripla. Normalmente, este tratamento resulta na eliminação do patógeno e evita a possível progressão para lesões pré-neoplásicas. No entanto, nos últimos anos, a eficácia deste tratamento tem descido abruptamente, principalmente devido à aquisição de resistências aos antibióticos usados. Tendo em conta tudo isto, novas estratégias terapêuticas para o tratamento deste tipo de infeções são urgentes.

Para além da *H. pylori*, outras espécies de *Helicobacter* têm sido também descritas no estômago de múltiplos animais, incluindo porcos, cães e gatos, e humanos. Estas são normalmente chamadas de não-*Helicobacter pylori* *Helicobacter* (NHPH), e inclui, por exemplo, a *H. suis*, conhecida por infetar principalmente suínos, *H. felis*, *H. salomonis*, *H. bizzozeronii* e *H. heilmannii*, que colonizam a mucosa gástrica canina e felina. Infecção por estes agentes está também associada a várias doenças gástricas, como gastrite, úlceras e linfoma de MALT. Na verdade, a ocorrência de linfoma de MALT é maior na infecção por NHPH do que na infecção por *H. pylori*. A infecção humana por NHPH evidencia o potencial zoonótico destas bactérias, que podem ultrapassar a barreira da espécie e ser transmitidas de animais para humanos. Mais interessante ainda, diversos estudos têm demonstrado a aquisição de resistência a vários antibióticos nestas espécies de NHPH gástricas. Ao contrário da *H. pylori*, estas NHPH não expressam os principais intervenientes conhecidos na adesão à mucosa gástrica humana. Infelizmente, os mecanismos usados por estes patógenos para infetar e

colonizar o epitélio gástrico permanecem desconhecidos, e mais estudos são necessários para compreender os mecanismos subjacentes à infecção por estas bactérias.

De uma forma geral, este projeto de doutoramento tem como objetivo determinar a ocorrência da infecção gástrica por espécies do género *Helicobacter*, e avaliar a eficácia do tratamento de erradicação na população portuguesa. Para além disso, determinar os padrões de suscetibilidade antimicrobiana de algumas espécies de NHPH a vários compostos e possíveis mecanismos de resistência. Por último, caraterizar os mecanismos de adesão usados pelas NHPH para infetar a mucosa gástrica humana, avaliando os potenciais efeitos a nível molecular e celular, nomeadamente na indução de apoptose e estimulação de um processo inflamatório.

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Abbreviations

α – Alpha

AGS – Human gastric epithelial neoplastic cell line

AlpA – Adherence-associated protein, HopC

AlpB – Adherence-associated protein, HopB

BabA – Blood-antigen binding protein A

β – Beta

BHI – Brain Heart Infusion

BLAST(p) – Basic local alignment (protein) search tool

BSA – Bovine serum albumin

CagA – Cytotoxin-associated gene A

cag PAI – Cytotoxin-associated pathogenicity island

cDNA – Complement DNA

CLSI – Clinical and Laboratory Standards Institute

Ct – Threshold cycle value

DAB – Diaminobenzidine tetrachloride

DMSO – Dimethyl sulfoxide

DNA – Desoxyribonucleic acid

dNTP – Deoxyribonucleotide triphosphate

DU – Duodenal ulcers

dupA – Duodenal ulcer promoting gene cluster

E-cad – Epithelial cadherin

ECL – Enterochromaffin-like cells (or neuroendocrine cells)

ELISA – Enzyme-linked immunosorbent assay

ER – Endoplasmic reticulum

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FlaA – Flagellin A gene

FldA – *H. pylori* flavodoxin protein

FITC – Fluorescein isothiocyanate

FUT – Fucosyltransferase

Y - Gamma

GC – Gastric cancer

GES-1 – Human normal gastric epithelial cell line

GI – Gastrointestinal

gyrA – Gyrase A gene

gyrB – Gyrase B gene

h – Hour

H. – *Helicobacter*

H&E – Haematoxylin and eosin

HCl – Hydrochloric acid

Hof – *Helicobacter* outer membrane protein family

HofE – *Helicobacter* outer membrane protein E

HofF – *Helicobacter* outer membrane protein F

Hom – *Helicobacter* outer membrane

Hop – *Helicobacter* outer membrane porin

HopB – Adherence-associated protein, AlpB

HopC – Adherence-associated protein, AlpA

HopD – LacdiNAc-binding adhesin, LabA

HopH – Outer inflammatory protein A, OipA

HopP – Sialic acid-binding adhesin, SabA

HopQ – *Helicobacter pylori* outer membrane adhesin

HopS – Blood-antigen binding protein A, BabA

HopZ – *Helicobacter pylori* outer membrane protein

Hor – *Helicobacter* outer membrane porin-related

HS1 – *Helicobacter suis* type strain

HtrA – High temperature requirement

iceA – Epithelium gene A

Ig – Immunoglobulin

IL – Interleukin

IM – Intestinal Metaplasia

LabA – LacdiNAC-binding adhesin, HopD

Le – Lewis antigen

LeA – Lewis a antigen, fucosylated blood group antigen type 1

LeB – Lewis b antigen, di-fucosylated blood group antigen type 1

LeX – Lewis x antigen, sialyl-dimeric Lewis x glucosphingolipid antigen type 2

LeY – Lewis y antigen, di-fucosylated blood group antigen type 2

LPS – Lipopolysaccharide

MALT – Mucosa-associated lymphoid tissue

MAPK – Mitogen-activated protein kinase

MG – Modified Giemsa

MIC(s) – Minimal Inhibitory Concentration(s)

min – Minute

MKN – Human gastric epithelial neoplastic cell line

ml – Millilitre

MUC – Mucin

μl – Microliter

NAC – N-acetylcysteine

NHPH(s) – Non- *Helicobacter pylori* *Helicobacter* (helicobacters)

NF- κ B – Nuclear factor kappa-light-chain-enhancer

NSAID – Non-steroidal anti-inflammatory drugs

OipA – Outer inflammatory protein A, HopH

OMPs – Outer membrane proteins

PBS – Phosphate-buffered saline

PCR – Polymerase Chain Reaction

Pgb – Plasminogen binding proteins

pH – Potential of hydrogen, measure of acidity or basicity

p.i. – Post-infection

PPI(s) – Proton pump inhibitor(s)

PrtC – Bacterial collagenase

qPCR – Quantitative polymerase chain reaction

RER – Rough endoplasmic reticulum

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

RUT – Rapid urease test

SabA – Sialic acid binding adhesin

SAT – Stool antigen test

sLe^a – Sialyl-Lewis a antigen, sialylated blood group antigen type 1

sLe^x – Sialyl-Lewis x antigen, sialylated blood group antigen type 2

spp. – Species

T4SS – Type IV secretion system

Tip α – Tumor necrosis factor α -inducing protein

UBT – Urea breath test

UDE – Upper digestive endoscopy

Ulex – Ulex *Europaeus* Agglutinin I (UEA I)

UreA - Urease subunit A

UreB – Urease subunit B

VacA – Vacuolating cytotoxin A

VIP – Vasoactive intestinal polypeptide

WHO – World Health Organization

WT – Wild-type

1 CHAPTER

General Introduction

Scientific Aims

Paper I

CHAPTER 1

General Introduction

- Histology of the stomach
- Gastric *Helicobacter* species: morphology, prevalence, pathogenesis, diagnosis and treatment
- Glycans and their role in the host-pathogen interaction

Paper I

Adhesion of *Helicobacter* species to the human gastric mucosa: A deep look into glycans role

Scientific Aims

1. Histology of the stomach

The stomach is an organ of the digestive system, divided into four different anatomical regions: the cardia and the gastroesophageal junction, the fundus, the body and the antrum (Junqueira LCU 2005; Soybel 2005).

The gastric mucosa is one of the first layers of the stomach wall, from inside to outside. It consists of an epithelium, the **superficial gastric epithelium**, whose cells are characterized by the production of a viscous and alkaline mucus that adheres to the surface epithelium. This epithelium is supported by the *lamina propria* and a thin smooth muscle layer (*muscularis mucosae*). The second layer is the submucosa, composed of dense irregular connective tissue, and then a smooth muscle layer (circular and longitudinal) in line with the serosa (Dellman 1998; Junqueira LCU 2005; Wilcock 2013).

Histologically, the fundus and the body present an identical microscopic structure, usually called oxyntic mucosa. Therefore, the human stomach is composed of only three histological regions, characterized accordingly to the nature of the glands (Junqueira LCU 2005; Soybel 2005).

Functionally, the gastric mucosa can be divided into two different parts: the **acid-secreting** and the **non-acid secreting** region (Soybel 2005). The body and the fundus constitute the acid-secreting regions characterized by acid- and pepsinogen-secreting mucosa. In these regions, almost all the *lamina propria* is occupied by glands, which are composed of several types of cells: the chief cells, the neuroendocrine cells, the parietal cells and the mucous-neck cells.

At the base lie the **chief (zymogenic) cells**, characterized by their abundant rough endoplasmic reticulum (RER) and the secretion of pepsinogen and lipase. Upon contact with the acidic gastric juice, the pepsinogen is converted into pepsin, responsible for stomach's ability to initiate proteins digestion (Dellman 1998; Soybel 2005).

The middle region is composed mainly by the **parietal (or oxyntic) cells**. These cells present a typical morphology characterized by their large size, rounded or pyramidal, with an eosinophilic cytoplasm due to the high number of mitochondria and extensive intracellular canaliculi. The parietal cells are responsible for the production hydrochloric acid (HCl) and the intrinsic factor (a glycoprotein that binds to Vitamin B12, essential for maturation of red blood cells). These cells are usually interspersed among the mucous-neck cells (Dellman 1998; Junqueira LCU 2005; Soybel 2005).

The **mucous-neck cells** present an irregular shape, with the nuclei placed at the basal pole of the cell and the secretory granules close to the apical pole. They are

responsible for the secretion of a soluble mucous only under vagal stimulation. The mucins present on this mucous are different from those secreted by the surface mucous epithelial cells (Junqueira LCU 2005; Soybel 2005).

The **neuroendocrine (or enterochromaffin-like - ECL) cells** are specialized endocrine cells usually intercalated between parietal cells and smaller immature cells. These cells secrete several compounds including gastrin, which stimulates the production of HCl, histidine decarboxylase, essential for histamine production, glucagon, serotonin, substance P and vasoactive intestinal polypeptide (VIP) (Dellman 1998; Soybel 2005).

The antral mucosa is characterized by the presence of **gastrin-secreting G cells** and **somatostatin-secreting D cells** (alkaline-secreting surface epithelium). The antrum and pylorus region are also composed by a non-acid secreting mucosa, constituted by simple glands (mucous- and HCO₃-secreting surface epithelial cells) (Soybel 2005).

2. Gastric *Helicobacter* species

The *Helicobacter* genus is composed by a group of Gram-negative, helical-shape bacteria that can be found in a wide range of animals, including mammalian, avian, reptilian and amphibian hosts (Flahou B 2016; Schrenzel MD 2010). The genus comprises at least 46 different *Helicobacter* species identified in the gastrointestinal (GI) tract, however there is no information available for all of them, and some of species have not yet a formal name (Flahou B 2016; Gruntar I 2020; Fox 2002; Smet A 2018).

Helicobacter species can be divided into two different groups, according to the site of colonization: gastric and enterohepatic species. The gastric *Helicobacter* species colonize the stomach of several animals, being able to survive in this unique environment (Labigne A 1991; Solnick and Schauer 2001; Weeks et al. 2000). The enterohepatic *Helicobacter* species can be found on the mucosal surface of intestinal tract and/or in the liver (Solnick and Schauer 2001).

For several decades, the stomach was considered a sterile microenvironment due to its acidic conditions. The first isolation of a bacterial infection of the human stomach was performed by Marshall and Warren in 1984, who associated this bacterial infection with gastritis and gastric ulcers (Marshall BJ 1984). Initially, the bacterium was named *Campylobacter pylori*. However, phenotypic and genotypic analysis demonstrated that it did not belong to the *Campylobacter* genus, resulting in the

description of a new genus: the *Helicobacter* genus (Goodwin CS 1989). The bacterium was then renamed as *Helicobacter (H.) pylori*.

Besides *H. pylori*, other *Helicobacter* species have also been described as able to colonize the GI tract of several different animals. Interestingly, the first reports of the presence of gastric spiral shaped bacteria in the lower intestinal tract of animals were already done in 1881 by Rappin and in 1883 by Bizzozero. A few years later, Salomon also reported the observation of organisms in the stomach of dogs, cats and a brown Norway rats. However, due to the lack of proper identification tools at that time, the bacteria were not correctly identified and named. Fortunately, the development of several molecular techniques allowed the identification of these organisms, that are usually named as **non-*Helicobacter pylori* *Helicobacter* (NHPH)** species (Haesebrouck F 2009). So far, 17 NHPH species have been described, some of them with zoonotic potential. The more relevant gastric *Helicobacter* species, their morphologic features and natural host are listed in **Table 1**.

Table 1. Overview of some of the most relevant gastric *Helicobacter* species, including morphologic features and their natural host.

<i>Helicobacter</i> species	Morphology	Natural host	Reference
<i>H. pylori</i>	2.5-5.0µm long / 0.5-1.0µm wide 4-8 monopolar flagella No periplasmic fibrils	Human	(Goodwin CS 1989; Marshall BJ 1984)
<i>H. felis</i>	5-7.5µm long / 0.4µm wide 14-20 bipolar flagella Periplasmic fibrils Tight coil	Dog, cat, cheetah, New Guinea wild dog, rabbit	(Lee A 1988)
<i>H. suis</i>	2.3-6.7µm long / 0.9-1.2µm wide 4-10 bipolar flagella No periplasmic fibrils Tight coil	Pig, mandrill monkey, rhesus macaque, cynomolgus macaque	(Baele M 2008; De Groote D 1999)
<i>H. bizzozeronii</i>	5-10µm long / 0.3µm wide 10-20 bipolar flagella No periplasmic fibrils Tight coil	Dog, cat	(Hanninen ML 1996)
<i>H. salomonis</i>	5-7µm long / 0.8-1.2µm wide 10-23 bipolar flagella No periplasmic fibrils Little coil	Dog, cat, rabbit	(Jalava K 1997)
<i>H. heilmannii</i>	3-6.5µm long / 0.6-0.7µm wide 4-10 bipolar flagella No periplasmic fibrils Tight coil	Dog, cat, lynx, bobcat, cheetah, tiger, leopard, puma	(Haesebrouck 2011)
<i>H. ailurogastricus</i>	3-5.5µm long / 0.5-0.7µm wide	Cat	(Joosten M 2016)

6-8 bipolar flagella No periplasmic fibrils Tight coil

2.1 Prevalence and clinical outcome of infections with gastric *Helicobacter* species

2.1.1 Human infection by *Helicobacter pylori*

H. pylori is a spiral, flagellated, Gram-negative, microaerophilic bacteria, known to infect half of the worldwide population, being considered a major public health issue (Hooi JKY 2017; Huang Y 2016; Rodrigues MF 2019; van Amsterdam K 2006). The prevalence varies within the geographical region, being Oceania (24.4%), Western Europe (34.3%) and North America (37.1%) the regions with the lowest *H. pylori* prevalence (Hooi JKY 2017). On the other hand, Africa (79.1%), South America (69.4%), Latin America and Caribbean (63.4%) and Asia (54.7%) are the regions with the highest infection rate (Hooi JKY 2017). Interestingly, Portugal presents one of the highest *H. pylori* infection rates, with a prevalence described as 86.4% (Hooi JKY 2017). The reason for this is not clear. The major differences in the *H. pylori* prevalence are usually related to the level of urbanization, sanitation and access to clean water (Borges SS 2019; Hooi JKY 2017). The high prevalence of *H. pylori* is generally correlated to low socio-economic status of the population (Hooi JKY 2017).

H. pylori infection is commonly acquired during childhood and it can persist for lifetime without clinical signs (Algood and Cover 2006; Huang Y 2016). The transmission route of *H. pylori* is not completely understood, but it can be direct (interpersonal) or indirect (environmental), occurring through oral-oral, gastric-oral or fecal-fecal route (Huang Y 2016; Brown 2000). The bacterium is usually clustered in families and transmitted from person to person (Ansari S 2017; Clyne M 2019).

H. pylori presents host tropism (humans) and tissue tropism (gastric epithelial cells or mucous layer) (Oleastro M 2013). The infection of the human gastric mucosa with this pathogen is closely related to **chronic gastritis**, **duodenal ulcers (DU)**, **gastric ulcers**, **gastric adenocarcinoma** and **gastric mucosa-associated lymphoid tissue (MALT) lymphoma** (Ahn HJ 2015). Almost all individuals infected by *H. pylori* develop chronic gastritis, but the infection can remain asymptomatic as well. *H. pylori* is also considered the principal causative agent of peptic ulcer disease (10-15% of the infected individuals) and gastric cancer (GC) (1-3% of the individuals) (Ahn HJ 2015; Bento-Miranda M 2014; De Falco M 2015; Hooi JKY 2017; van Amsterdam K 2006). Infection by *H. pylori* has also been associated with MALT lymphoma in 0.1% of the *H. pylori*-infected individuals (Ahn HJ 2015; De Falco M 2015; Parsonnet J 1991; Doulberis M 2020). Indeed, *H. pylori*

was classified as a Carcinogen type I in 1994 by the World Health Organization (WHO) due to its association with GC development (IARC 1994). *H. pylori* is considered a necessary but not sufficient agent for gastric carcinogenesis (Ahn HJ 2015), since the disease outcome results from a complex interaction between host, environmental and bacterial factors (Ahn HJ 2015; van Amsterdam K 2006).

The preferential colonization site of *H. pylori* is the non-acid secretory gastric mucosa in the antrum and cardia regions, but it can also be found in the oxyntic mucosa of the fundus and body regions (Rodrigues MF 2019). The bacteria adhere to the gastric epithelial cells inducing several histopathological changes, such as local inflammation. The type of disease that can evolve from this inflammation depends on the colonization site: a predominant chronic corpus localization leads to increased risk of gastric ulcers and GC, while a predominant chronic antrum gastritis might lead to a DU phenotype (Ahn HJ 2015; Correa and Piazzuelo 2012). When the colonization occurs mainly in the corpus region it is usually accompanied by atrophy of parietal and chief cells resulting in a decreased acid and pepsinogen secretion. On the other hand, the opposite phenotype, which is an increased acid- and gastrin-secretion, is observed when the bacteria colonize the antrum region of the stomach (Kusters JG 2006). Interestingly, the DU phenotype is related to a lower risk of GC occurrence (Ahn HJ 2015).

Typically, the *H. pylori*-associated inflammation results in a chronic inflammatory process, with recruitment of lymphocytes, macrophages, plasma cells and even neutrophils to the site of inflammation (Algood HM 2007; Blaser N 2019; De Falco M 2015; Rodrigues MF 2019). This non-atrophic chronic gastritis can evolve and lead to atrophic gastritis (loss of gastric glands and parietal cells, glandular hyperplasia and mucin depletion), intestinal metaplasia (IM) (replacement of gastric epithelial cells for intestinal-type epithelium, with presence of goblet cells with or without gland distortion), dysplasia, and, lately, to gastric adenocarcinoma (Huang Y 2016; Rodrigues MF 2019). The gastric adenocarcinoma phenotype appears more frequently when the bacteria colonize the proximal region of the stomach (Ahn HJ 2015). *H. pylori* has been closely associated with both diffuse- and intestinal-type gastric adenocarcinoma, characterized by death of glandular structures and poorly cohesive cells that can infiltrate the gastric wall (Ahn HJ 2015). A schematic representation of the gastric carcinogenesis is presented in **Figure 1**.

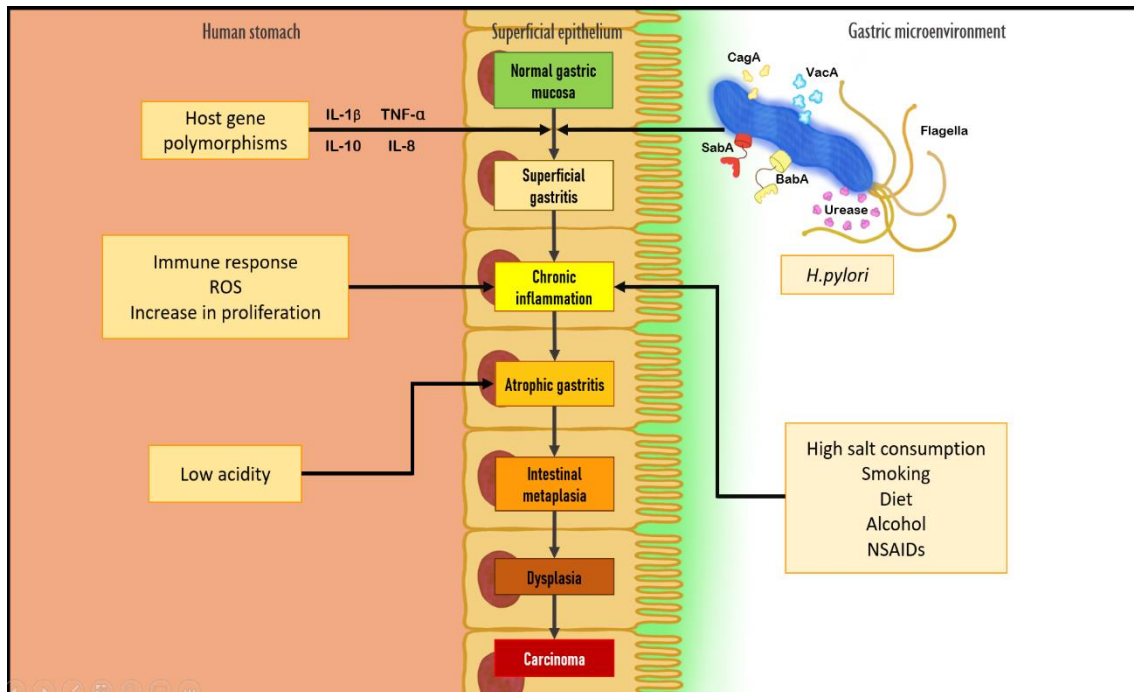


Figure 1. The human gastric carcinogenic pathway. The impact of *H. pylori* and host and environmental factors in the development of gastric lesions, and the evolution to gastric carcinoma. Illustration performed by Mário Jorge Ribeiro (ICBAS-UP), adapted from Wroblewski *et al.*, 2020.

2.1.2 Prevalence of gastric Non-*Helicobacter pylori* *Helicobacter* (NHPH) species in animals

Gastric NHPH represent a group of closely related but distinct *Helicobacter* species, colonizing preferentially the stomach of different animal species (Haesebrouck F 2009; Joosten M 2013). *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. ailurogastricus* and *H. heilmannii* are mainly found in dogs and cats, while pigs are the major host of *H. suis* (Bento-Miranda M 2014; Haesebrouck F 2009). Besides these principal hosts, some of the NHPH mentioned can be found in other animals, as presented in Table 1.

For several years, NHPH were referred to as “*H. heilmannii*”, but more recently they were divided into two different taxa: type 1 and type 2 (Haesebrouck 2011). *H. heilmannii* type 1 was shown to be *H. suis* (Baele M 2009; De Groote D 1999; Haesebrouck F 2009; O'Rourke JL 2004b). The *H. heilmannii* type 2 group includes the known canine- and feline-associated organisms: *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii*, *H. ailurogastricus*, *H. cynogastricus* and *H. baculiformis* (Baele M 2009; Haesebrouck F 2009; Joosten M 2016).

H. felis is a spiral-shaped *Helicobacter*, that was first isolated from a cat stomach (Lee A 1988), but later it was also found in the stomach of dogs (Kusters JG 2006;

Mohammadi M 1996). The presence of *H. felis* is very common in these animals, being found in approximately 25% of dogs (Hong S 2015), and in more than 50% of the dogs and cats (Neiger R 1998; Van den Bulck K 2005a). However, it is not clear if an infection of the canine and feline stomach by *H. felis* is associated with gastritis (Cattoli G 1999; Eaton KA 1996; Simpson KW 1999a; Simpson KW 1999b, 2000). For that reason, it has been hypothesized that *H. felis* can be part of the normal feline and canine gastric microbiota (Kusters JG 2006). However, studies in murine models showed that *H. felis* infection induces gastritis, epithelial cell proliferation and apoptosis (Chen M 1992; Court M 2002), indicating that this could be a good model to explore the zoonotic potential of *H. felis*. Gerbils infected with *H. felis* develop antral gastritis, accompanied by epithelial cells proliferation in the cardia and corpus at 4 weeks p.i, while in a mice model, it needs at least 8 weeks after infection to observe the same phenotype (Court M 2002).

Some studies described the presence of a six long spiral-shaped *Helicobacter* species in wild feline, human, canine and feline gastric biopsies (Hwang CY 2002; Neiger R 1998; O'Rourke JL 2004b). However the isolation and cultivation of the organism at that time was not possible, and it was provisionally named “Candidatus *H. heilmannii*” (Hwang CY 2002; Neiger R 1998; O'Rourke JL 2004b). Later, the bacterium was successfully isolated and characterized as ***Helicobacter heilmannii*** by Smet and co-workers in 2012. In dogs and cats, the prevalence varies between 20% to 100% (Hwang CY 2002; Neiger R 1998; Van den Bulck K 2005a; Wiinberg B 2005). *H. heilmannii* infection can be associated with chronic active gastritis, peptic ulcers and chronic vomiting but its pathogenic significance remains ambiguous, and it might be strain-dependent (Amorim I 2015; Hwang CY 2002; Joosten M 2013; Kubota-Aizawa S 2017; Smet A 2012). Interestingly, *H. heilmannii* infection in a mouse model results in the induction of gastric B-cell MALT lymphoma (O'Rourke JL 2004b).

H. suis is the main *Helicobacter* species found in the pig stomach, but it can also be found in non-human primates (De Bruyne E 2012; Grasso GM 1996; Haesebrouck F 2009), which probably represent the original hosts (Flahou B 2018). The prevalence of *H. suis* in pigs varies according to the age, being very low before weaning, and increasing gradually until slaughter age (De Bruyne E 2012; Hellemans A 2007). The infection rate ranges from 8% to 95% at slaughter age, with the majority of the studies reporting 60% or more *H. suis*-infected animals (Barbosa AJA 1995; Casagrande Proietti P 2010; Choi YK 2001; De Witte C 2017; Grasso GM 1996; Mendes EN 1990, 1991; Queiroz DMM 1996; Roosendaal R 2000). Infection of the porcine mucosa with this bacterium is closely associated with chronic gastritis (Ayles HL 1996; De Bruyne E 2012; Mendes EN 1991; Queiroz DMM 1996), and is involved in development of ulcerative lesions of the non-glandular part of the stomach (De Witte C 2016). Ulceration of the non-glandular part of

the stomach is found in 20-90% of pigs at slaughter age (Ayles HL 1996; Guise HJ 1997) and has been associated with sudden death, decreased feed intake and decreased daily weight gain (Ayles HL 1996; De Bruyne E 2012; De Witte C 2016; Haesebrouck F 2009).

In 1996, a helical-shaped bacterium, morphologically distinct from *H. felis*, was isolated from the canine mucosa (Hanninen ML 1996). Later, using DNA-DNA hybridization technique, it was considered a new specie and named as *H. bizzozeronii*. This bacterium can be found mostly in the canine stomach (Priestnall SL 2004; Van den Bulck K 2005a; Wiinberg B 2005), sometimes in association with chronic vomiting (Baele M 2009; Geyer C 1993; Hermanns W 1995; Kondadi PK 2012).

H. salomonis was isolated from dogs and characterized for the first time by Jalava in 1997. The major host of *H. salomonis* are dogs and cats (Jalava K 1997). Similar to *H. bizzozeronii*, infection of canine gastric mucosa by this pathogen has been associated with chronic vomiting by some authors although it may also be present in the stomach of animals without disease signs (Baele M 2009; Geyer C 1993; Hermanns W 1995).

H. ailurogastricus was identified by Joosten *et al.* in 2016. This *Helicobacter* species is closely related to *H. heilmannii*, with several morphological similarities. Indeed, these two *Helicobacter* species cannot be distinguished by means of 16S rRNA or *ureAB* gene sequencing, the two techniques frequently used for differentiation between gastric *Helicobacter* species (Joosten M 2016). Therefore, the only way to distinguish *H. ailurogastricus* from *H. heilmannii* is using a genome-sequencing-based approach to analyse the complete genome content (Joosten M 2016). The prevalence of this new *H. ailurogastricus* species in animals and its pathogenic role is not clear yet.

The presence of helical-shaped bacteria has been demonstrated in the stomach of dogs in the last decades. These *Helicobacter* organisms were detected in 67-86% of the healthy dogs and in 61-100% of dogs showing chronic vomiting (Baele M 2009; Geyer C 1993; Hermanns W 1995; Hwang CY 2002). *H. bizzozeronii* is described as the most frequent species in the dog gastric mucosa (Ekman E 2013; Priestnall SL 2004; Van den Bulck K 2005a; Wiinberg B 2005). Other *Helicobacter* species, namely *H. salomonis*, *H. heilmannii* and *H. felis* have also been found in the canine stomach (Amorim I 2014; Ekman E 2013; Hong S 2015), and co-infection with two or more species in dogs is very common (Amorim I 2014; Ekman E 2013).

Overall, in cats, gastric *Helicobacter* organisms have been detected in 41-100% of the animals, with animals with chronic vomiting presenting higher prevalence (Geyer C 1993; Hwang CY 2002; Lecoindre P 2000; Neiger R 1998; Otto G 1994; Papasouliotis K 1997; Tabrizi AS 2009; Takemura LS 2009; Weber AF 1958).

2.1.3 Prevalence of non-*Helicobacter pylori* *Helicobacter* (NHPH) species in humans

NHPH species have a preferential host species, but they may cross the species-barrier and occasionally colonize the gastric mucosa of other hosts (Amorim I 2014; Haesebrouck F 2009). Due to the possible transmission from animals to humans, NHPH infection is considered a zoonosis (Haesebrouck F 2009). Living in close proximity of infected animals is a potential risk factors for humans to contract NHPH infection (Lavelle JP 1994; Meining A 1998). The intensity of the contact is also important, since higher incidence of NHPHs have been reported in farmers, staff of slaughterhouses, veterinarians, pet owners and children having intense contact with pets (De Bock M 2006; Joosten M 2013). Furthermore, *H. suis* can also be transmitted to humans by manipulation or consumption of contaminated pork meat (De Cooman L 2013).

The prevalence of human NHPH infection has been described as very low, being found in approximately 0.2-6% of the gastric biopsies from human patients with gastric disease (Flahou B 2018; Haesebrouck F 2009). NHPH infection in humans can be associated with gastric disorders, such as chronic gastritis, peptic ulcers and MALT lymphoma (Amorim I 2014; Debongnie JC 1995, 1998; Haesebrouck F 2009; Joosten M 2013; Joosten M 2016; O'Rourke JL 2004a; Overby A 2017; Smet A 2018; Trebesius K 2001; Van den Bulck K 2005a). Interestingly, the risk of developing MALT lymphoma is higher with NHPH (1.47%) infection than with *H. pylori* (0.66%) infection (Morgner A 2000; Overby A 2017; Stolte M 1997; Stolte M 2002). Besides these main clinical outcomes, NHPH infection can also be associated with other symptoms, including nausea, vomiting, acute or chronic epigastric pain, dyspepsia, reflux esophagitis, heartburn, abdominal pain, decreased appetite, among others (Baele M 2009; Haesebrouck F 2009; Heilmann KL 1991; Sýkora J 2003; Wuppenhorst N 2013; Yang H 1998; Yang H 1995). The prevalence of NHPH infection in humans might be underdiagnosed due to several factors, such as occurrence of mild symptoms, or even the absence of symptoms, after NHPH infection and especially the difficult diagnosis and identification (Haesebrouck F 2009). Interestingly, *H. suis* DNA was detected in gastric biopsies from a high percentage (27.0%) of human patients with idiopathic parkinsonism (Blaecher C 2013). In a mouse model, *H. suis* was shown to affect the blood-cerebrospinal fluid barrier resulting in inflammation and neurodegeneration (Gorle N 2018), but its possible role in the pathogenesis of chronic central nervous disorders in humans remains to be determined (Gorle N 2020).

H. suis has been described as the most prevalent NHPH species in humans, representing 13.9% (De Groote D 2005) and 30.9% (Van den Bulck K 2005b, 2005a) of

NHPH infection in humans (Baele M 2009; Bento-Miranda M 2014; Haesebrouck F 2009; Padra M 2018). Amongst the NHPH, the prevalence varies according to the study, the techniques used and the geographical region. Overall, based on PCR techniques, *H. salomonis* has been found in 21% of the samples, *H. felis* in 15%, *H. heilmannii* in 8% and *H. bizzozeronii* in 4% of the gastric samples (Bento-Miranda M 2014; Van den Bulck K 2005a). Interestingly, co-infection with two or more NHPH species or with *H. pylori* and NHPH has been observed in 11-16% of the NHPH-infected individuals (Bento-Miranda M 2014; De Groote D 2005; Liu J 2015; Van den Bulck K 2005b).

Besides the main difference in prevalence, *H. pylori* and NHPHs also present different colonization patterns in the human stomach. For instance, studies with *H. suis* and *H. heilmannii* demonstrated that these organisms have a more focal distribution, mostly restricted to the antrum region (Stolte M 1997). These bacteria can be found mainly inside the canaliculi of parietal cells, in contrast to *H. pylori* which can be found preferentially in the mucous layer or close to the epithelial cells (Joo M 2007).

2.2 Role of virulence-associated factors in pathogenesis of gastric *Helicobacter* infections

In last years, several studies have used *in vitro* and *in vivo* models, including cell lines and animals, to evaluate the pathogenic potential of *H. pylori* and NHPH species. These experiments allowed identification of several mechanisms and molecules used by the pathogens for colonization and adhesion to the gastric mucosa.

Due to the high prevalence of *H. pylori* among the world's population this has been the target of several studies and some of the virulence factors and the host ligands underlying the colonization process were unlocked. However, little information is available regarding the virulence factors of NHPH, including their adhesion mechanisms. The virulence factors are key molecules for *Helicobacter* spp. pathogenesis, being involved in colonization, induction of gastric lesion and escape to the immune system, allowing a successful infection and colonization.

2.2.1 Colonization of the gastric mucosa

2.2.1.1 Survival in the acidic gastric microenvironment, motility and chemotaxis

The stomach is a unique microenvironment whose main characteristic is the low pH. All known gastric *Helicobacter* spp. possess a **urease** enzyme, responsible for the neutralization of the gastric acid, helping the survival of the bacteria (Haesebrouck F 2009; Kusters JG 2006). Additionally, these microorganisms also possess **flagella**,

essential for the bacteria to swim toward the more neutral pH of the gastric mucosa, favouring bacterial survival (Camilo V 2017; Haesebrouck F 2009; Kusters JG 2006). These two characteristics, together with urea- and bicarbonate-mediated chemotaxis, allow the bacteria to penetrate into the viscous mucus layer to reach the gastric epithelium, with neutral pH which favours the growth conditions needed (Gu 2017; Haesebrouck F 2009; Kusters JG 2006; Yoshiyama H 2000).

The urease enzyme is encoded by two subunit genes, *ureA* and *ureB*, and five accessory genes (*ureE*, *ureF*, *ureG*, *ureH* and *ureI*), essential for the molecule assembly (Mobley 1996). Urease hydrolyses urea, leading to the release of ammonia, which neutralizes the hydrochloric acid present in the stomach. This creates a neutral microenvironment around the bacterium, that can survive and move toward the gastric epithelium (Mobley 1996). Besides this role in colonization, urease may be involved in the development of gastric lesions. Urease induces damage to the mucosal barrier, due to the urease-mediated ammonia production. Additionally, urease also stimulates an inflammatory response, with recruitment of neutrophils and macrophages and stimulation of IL-8 and NF- κ B production (Beswick EJ 2006; Harris PR 1996; Mobley 1996; Wroblewski LE 2010).

Additionally, other chemoreceptors, including TlpA, TlpB, TlpC, TlpD, aCheA kinase and multiple coupling proteins, have also been described as important for the chemotaxis of *Helicobacter* (Camilo V 2017).

All the gastric *Helicobacter* spp. possess flagella, but the amount and distribution vary between the species. Each flagellum is formed by a body, hook and filament, encoded by several genes (Gu 2017). These genes and the assembly of the flagella were widely studied for *H. pylori* (Allan E 2000; Gu 2017; Josenhans C 2002; Lowenthal AC 2009; Radin JN 2013; Ryan KA 2005; Smith TG 2009), however, so far, the exact composition of the flagella of NHPHs remains to elucidate.

2.2.1.2 Adhesion of gastric *Helicobacter* species

The infection of *H. pylori* in the human gastric mucosa occurs in two different phases. First, the bacteria move through the mucus layer until the epithelial surface is reached (described in section 2.2.1.1), and then *H. pylori* firmly attaches to the host cells via outer membrane proteins OMPs (Huang Y 2016). The interaction between the adhesin molecules present on the bacterial surface and the receptor molecules on the host cells is essential for the attachment of the bacteria to the epithelial surface (Ansari S 2017). The bacterial attachment is important for survival of *H. pylori* in the gastric mucosa for several reasons: 1) protection from the washing out during mucous shedding

and peristaltic movements, 2) access to nutrients from the damaged host epithelial cells, 3) it allows the delivery of toxins and other virulence factors to the host cells, and 4) facilitates the development of persistent infection (Ansari S 2017; Aspholm M 2006; Kao CY 2016; Magalhaes A 2010b). The colonization triggers the expression of several virulence factors, that will induce host tissue damage (Huang Y 2016; Kao CY 2016).

The *H. pylori* OMPs can be divided into five families: family 1 includes the **Hop** (*Helicobacter* outer membrane porin; 21 members) and the **Hor** (Hop-related; 12 members); family 2 is composed by the **Hof** (*Helicobacter* OMP; 8 members), family 3 are the **Hom** (*Helicobacter* outer membrane; 4 members), family 4 are the **iron-regulated OMPs** (6 members), and finally family 5 includes the **efflux pumps OMPs** (3 members) (Ansari S 2017; Bauwens E 2018; Oleastro M 2013). The majority of these bacterial OMPs belong to the Hop proteins and can act as transport channels (porins) or adhesins, crucial molecules for the attachment to the human gastric mucosa (Oleastro M 2013). The major intervenients in the *H. pylori* binding to the gastric epithelial cells are the blood group-antigen binding protein A (**BabA** or HopS) and the sialic acid-binding adhesin (**SabA** or HopP). The BabA adhesin binds to fucosylated blood-group antigens H-type 1 and Lewis B (LeB) antigens (Benktander J 2012; Borén T 1993; Ilver D 1998). The SabA adhesin recognizes α 2,3-sialylated structures, such as sialyl-Lewis X (sLeX) and sialyl-Lewis A (sLeA), which expression is induced by the inflammation caused by *H. pylori* (Linden SK 2008; Magalhaes A 2015; Mahdavi J 2002).

However, not all *H. pylori* strains express the BabA or SabA adhesins, indicating that other OMPs might be involved in the binding to the gastric epithelium as well (Kao CY 2016; Oleastro M 2013). These OMPs include the lacdiNac-binding adhesin (LabA or HopD), adherence-associated proteins AlpA (HopC) and AlpB (HopB), OipA outer inflammatory protein A (HopH), HopQ, HopZ, neutrophil-activating protein (NAP) and heat shock protein 60 (HSP60) (Ansari S 2017; Bauwens E 2018; Benktander J 2018; Benktander J 2012; Odenbreit S 1999; Oleastro M 2013; Rossez Y 2014).

NHPH species also possess several OMPs, however genomic comparison with *H. pylori* demonstrated that the animal-associated NHPHs lack the major known *H. pylori* adhesins (Bauwens E 2018). Nevertheless, NHPHs can bind to both the human and animal gastric mucosa, suggesting that other OMPs might be involved in the adhesion. Indeed, recent studies showed that the canine, feline and porcine NHPHs express some Hof, Hor, Hom and Hop proteins (Arnold IC 2011; Bauwens E 2018; Liu C 2016), but further studies are needed to better understand the role of these molecules in the NHPH adhesion.

Genome sequence analysis of *H. felis* demonstrated that several OMPs and virulence factors are expressed by this bacterium. Hop, Hor, Hof, Hom, urease, GGT (gamma-glutamyl transpeptidase), NapA (neutrophil-activating protein A), HtrA (high temperature requirement A), collagenase and iron-uptake genes were found (Arnold IC 2011). The exact role of many of these molecules still needs to be investigated, but they are important for colonization, motility, chemotaxis, natural competence and virulence of *H. felis* in animals and humans (Arnold IC 2011).

H. heilmannii has been the target of some recent studies, and the presence of genes encoding the HofE and HofF OMPs was observed in the genome of this microorganism (Liu C 2016). These two molecules were classified as adhesins and described as important for the binding to the gastric mucosa, presenting higher *in vitro* affinity to gastric epithelial cells than to mucins. Additionally, these adhesins can activate IL-1 β expression, that in turns induces the production of MUC13 mucin (De Witte C 2016; Liu C 2016). Indeed, experiments with mutant strains lacking HofE or HofF exhibited a decreased *in vitro* binding to gastric mucins and epithelial cells, and also a lower gastric colonization level in the stomach of mice infected with these strains compared to the non-infected animals (Liu C 2016), highlighting that binding of these adhesins seems to occur preferentially to the gastric epithelial cells than to mucins. *H. heilmannii* genome sequence analysis also showed the presence of genes encoding some virulence factors such as iceA, HgrA and jhp0562-like glycosyltransferase (Joosten M 2016). Both species *H. heilmannii* and *H. ailurogastricus* contain a VacA-like autotransporter, but the mechanism and the effective role of this toxin remains unknown (Joosten M 2016).

The detailed host-pathogen interaction and the role of glycans in this process will be discussed later, in section 3.

The known molecules involved in the adhesion to the gastric mucosa are represented in Figure 2.

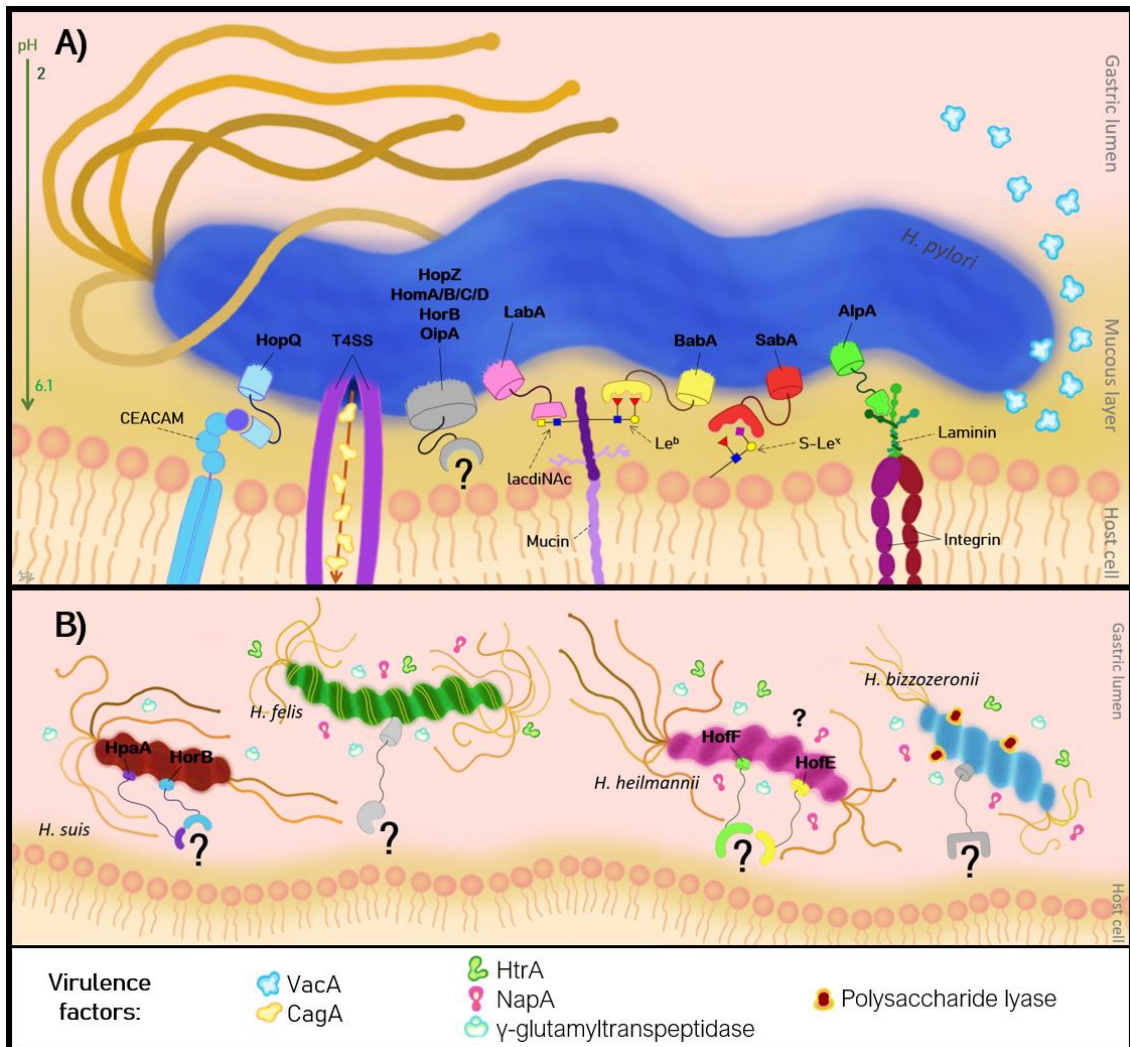


Figure 2. Pathogenesis of *Helicobacter* infection of the human gastric mucosa. (A) Interaction of *H. pylori* adhesins with the host cell receptors, and the virulence factors. (B) NHPH adhesins and virulence factors involved in the adhesion mechanism. Illustration adapted from Matos *et al.*, 2021.

2.2.2 Induction of gastric lesions

After binding to the gastric epithelial cells, the last step is initiated: the induction of host tissue damage, in which several virulence factors expressed by the bacteria play a role. The principal virulence factors used by *H. pylori* are the cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), but other components such as LPS can also be helpful in this process (Camilo V 2017; Kao CY 2016; Oleastro M 2013). These toxins are important for the deregulation of the host's intracellular signalling pathways (Ahn HJ 2015).

The *cag* pathogenicity island (**cagPAI**) contains 27-31 genes and encodes **CagA** and a type IV secretion system (**T4SS**) (Ahn HJ 2015; Akopyants NS 1998; Camilo V

2017; Crabtree JE 1991; Dewayani A 2021; Fischer W 2001; Kusters JG 2006; Odenbreit S 1999; Parsonnet J 1991). The *cagA* gene encodes the CagA toxin, and it is highly immunogenic being commonly used as an indicator for the presence of the *cagPAI* locus (Ahn HJ 2015). The T4SS binds to the host cell membrane, forming a syringe-like structure, that allow the entrance of several *H. pylori* components into the host cell, including the CagA toxin (Ahn HJ 2015; Fischer W 2001; Kusters JG 2006; Odenbreit S 1999; Tegtmeyer N 2011; Wroblewski LE 2010). After delivery by T4SS, CagA binds to the cell membrane inner surface and undergoes tyrosine phosphorylation by members of the Src and Abl family kinases, resulting in morphological changes, proliferation and alterations in the intracellular signalling pathway (Ahn HJ 2015; Akopyants NS 1998; Camilo V 2017; Kusters JG 2006; Odenbreit S 1999; Tegtmeyer N 2011; Wroblewski LE 2010). CagA triggers many downstream pathways in the host cells, including Ras, MAPK, β -catenin and extracellular signal-regulated kinase pathway (Ahn HJ 2015; Tegtmeyer N 2011; Wroblewski LE 2010). Furthermore, CagA can induce T cell activation and the production of proinflammatory cytokines, such as IL-1 β , IL-8 and NF- κ β (Akopyants NS 1998; Camilo V 2017; Crabtree JE 1991; Dewayani A 2021; Fischer W 2001). The prevalence of CagA-positive *H. pylori* strains differs according to the region, varying from 50-70% in the Western countries to 100% in East Asian countries (Akopyants NS 1998; Ansari S 2017; Blaser MJ 1995; Kao CY 2016). Individuals infected with CagA+ strains have harsh inflammation, severe gastritis, higher epithelial damage, higher steps of atrophy, higher risk of peptic ulcers and GC (Ahn HJ 2015; Akopyants NS 1998; Blaser MJ 1995; Camilo V 2017; Crabtree JE 1991; Fischer W 2001; Odenbreit S 2002; Parsonnet J 1991). Furthermore, CagA is associated with *H. pylori*-induced MALT lymphoma (Ahn HJ 2015; Camilo V 2017). The same clinical outcomes are observed in persons infected with CagA-negative strains, suggesting that this is not completely dependent on the *cagPAI* and other bacterial factors may also contribute for the development of a more severe phenotype (Ahn HJ 2015; Yamaoka Y 2014). NHPHs do not present *cagPAI* locus (Haesebrouck F 2009).

Another virulence factor used by *H. pylori* is the **VacA** (Camilo V 2017; Kusters JG 2006). The majority of *H. pylori* strains express VacA toxin, but differences in vacuolating activity can be observed between strains, with only 50% of the strains producing active and functional VacA (Kusters JG 2006; Wroblewski LE 2010). The *vacA* gene is composed of three regions: signal (s) region, middle (m) region and intermediate (i) region (Gebert B 2003; Wroblewski LE 2010; Yahiro K 2003). Each region is divided into 2 subtypes and different combinations can be observed, constituting several chimeric VacA molecules (Wroblewski LE 2010; Yahiro K 2003). Some chimeras have

been more often associated with gastric disorders, such as the VacA s1/m1 chimeric strain, which is associated with duodenal ulcers and GC in Western countries (Wroblewski LE 2010). Also, the subtype i1 has been correlated with higher incidence of GC (Wroblewski LE 2010; Yahiro K 2003). The VacA cytotoxin exerts multiple effects on the epithelial cells using different pathways, and some of the main results include: disruption of gastric epithelial barrier and loss of function; modulation of the inflammatory response; disruption of late endosomal compartments; decrease in mitochondrial transmembrane potential leading to the release of cytochrome c, activation of caspase-8 and induction of apoptosis *in vitro* (Cover TL 2003; Galmiche A 2000; Leunk RD 1988; Manente L 2008; Papini E 1994; Peek RM 1999; Willhite DC 2003; Wroblewski LE 2010). Most gastric NHPH species do not produce functional VacA, except for *H. cecorum* (Haesebrouck F 2009).

Triple positive *H. pylori* strains (BabA, VacA and CagA positive) have been associated with more severe gastric disorders, such as peptic ulcers and gastric adenocarcinoma (Benktander J 2018).

The gamma-glutamyl transpeptidase (**GGT**) is a virulence factor present in almost all the gastric *Helicobacter* spp., including *H. pylori* and NHPH species (Chevalier C 1999; Haesebrouck F 2009). GGT converts glutamine into glutamate and ammonia, and glutathione into glutamate and cysteinyl glycine (Flahou B 2011). In 2011, Flahou *et al.*, demonstrated that *H. suis* GGT induces apoptosis and necrosis in human gastric neoplastic cell line (AGS cell line) (Flahou B 2011).

Besides these main virulence factors, *Helicobacter* species present other virulence factors that have been associated with development of gastric lesions in *H. pylori*, but for NHPHs the role of these molecules or homologs remains unclear. The duodenal ulcer promoting gene cluster (**dupA**) present in some *H. pylori* strains has been associated with higher risk of duodenal ulcers (Abadi T 2016). The increased risk of peptic and duodenal ulcers has also been associated with the expression of epithelium gene A (**iceA**) and *H. pylori* OMP **OipA** (Huang Y 2016). A homolog of iceA gene was found in the *H. heilmannii* genome (Joosten M 2016). Disruption of gastric epithelium, by cleavage of E-cadherin, can be caused by the **HtrA** of *H. pylori* (Harrer A 2017; Hoy B 2010). HtrA gene is present in the genome of *H. suis*, *H. felis* and *H. heilmannii* (Arnold IC 2011; Smet A 2013; Vermoote M 2012). Plasminogen binding proteins (**PgbA** and **PgbB**) allow the binding of *H. pylori* to plasminogen, interfering with the healing of gastric ulcers (Jönsson K 2004). Homologs of these proteins were found in the *H. suis* and *H. heilmannii* genome sequence (Smet A 2013; Vermoote M 2012). Other proteins,

including the production of bacterial collagenase (**PrtC**), the tumor necrosis factor α -inducing protein (**Tipa**) and the *H. pylori* flavodoxin protein (**FldA**), have also been described in the genome of *H. pylori* and NHPH species *H. suis*, *H. felis* and *H. heilmannii* (Chang C 1999; Kavermann H 2003; Smet A 2013; Suganuma M 2005; Vermoote M 2012).

The lipopolysaccharide (**LPS**) is a structural component of the cell wall of Gram-negative bacteria, including *Helicobacter* spp., with toxicity properties (Gottesmann M 2020; Monteiro MA 1998). For *Helicobacter*, one of the major components of LPS is the O antigen, a homologous of the Lewis blood group antigens H-type 1, LeA, LeX and LeY (Altman E 2005; Chmiela M 2019; Monteiro MA 1998; Van Amersfoort ES 2003). Expression of *H. pylori* O antigen at the bacterial surface can induce the expression of LeX antigens, promoting adhesion to the gastric epithelial cells (Altman E 2005; Chmiela M 2019; Edwards NJ 2000; Mahdavi J 2003). Additionally, the LPS expressed at the surface of *H. pylori* has also been described as playing a role in the suppression of the bacteria elimination (Chmiela M 2019). However, the exact role of LPS in the colonization is not completely understood (Gottesmann M 2020).

2.3 Diagnosis and treatment of infections with gastric *Helicobacter* species

2.3.1 Invasive and non-invasive diagnostic tests

Multiple diagnostic tests for detection of gastric *Helicobacter* infection are currently available. Tests can be divided into two groups: invasive or non-invasive (Abadi 2018; Wang YK 2015). These tests are used mostly for the detection of *H. pylori* in humans. However, some of these techniques can also be applied for the detection of NHPH species if required.

The **non-invasive tests** are usually the first choice for *Helicobacter* detection, because they are simpler, easier and faster. However, the percentage of false-negatives using these tests is high (Abadi 2018; Wang YK 2015). The stool antigen test (SAT), the urea breath test (UBT) and serologic evaluation are the most common non-invasive techniques used in the clinic, and the major advantage is to avoid endoscopy (Abadi 2018; Batts KP 2013; Best LM 2018; Sabbagh P 2019; Wang YK 2015).

The **UBT** test is based on the intrinsic ability of *Helicobacter* spp. to breakdown absorbed ^{13}C or ^{14}C -labeled urea into CO_2 , under acidic conditions (Abadi 2018; Atherton 1994; Ferwana M 2015). Urea is metabolized to ammonia and labeled-bicarbonate, which is transferred to the lungs and produces labeled- CO_2 , which is detected by the test (Abadi 2018; Atherton 1994; Ferwana M 2015; Wang YK 2015). However, false

positive results are possible due to the presence of other urease-producing organisms, such as *Staphylococcus* and *Streptococcus* species (Abadi 2018; Atherton 1994; Ferwana M 2015). The detection of NHPH infections by UBT is difficult. Although NHPH produce urease, the very focal colonization pattern, the relative lower bacterial density and variable distribution often leads to false negative results (Haesebrouck F 2009; Matsui H 2014). Also, UBT test does not differentiate NHPH from *H. pylori* (Haesebrouck F 2009).

The **SAT** is usually recommended whenever UBT test is not available or possible to perform. It is used for detection of initial *H. pylori* infection and to confirm eradication of the bacterium after the treatment (Abadi 2018; Gisbert JP 2006; Korkmaz H 2013; Shimoyama 2013; Wang YK 2015). This technique is very easy to perform and does not require any chemicals or expensive materials. The diagnosis is based on the detection of *H. pylori* antigens in stool samples, using monoclonal or polyclonal antibodies (Abadi 2018; Best LM 2018; Gisbert JP 2006; Korkmaz H 2013; Shimoyama 2013; Wang YK 2015). The SAT test cannot be used for NHPH identification (Goji S 2015; Haesebrouck F 2009).

Serologic tests are based on the detection of antibodies against *H. pylori* in blood samples, using enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassays (EIA) (Abadi 2018; Sabbagh P 2019). Unfortunately, this technique does not distinguish active from inactive infection (Abadi 2018; Sabbagh P 2019). Besides on blood samples (serum or plasma), this test can also be performed on saliva, dental plaques or urine, with less sensitivity (Abadi 2018). Serologic tests for diagnosis of NHPH infections in humans are not available.

The **invasive tests** require endoscopic biopsy collection and are generally performed only when there is no response to antibiotic therapy and when there are signs of problematic and symptomatic gastric disease (Abadi 2018; Wang YK 2015). Histology, culture, rapid urease test (RUT) and PCR are commonly used for detection of gastric *Helicobacter* infection (Abadi 2018; Wang YK 2015).

RUT test is based on the *Helicobacter* urease activity. When urea is converted into ammonia, an increase in pH is observed, which results in a colour change of the phenol red indicator (Abadi 2018; Best LM 2018; Wang YK 2015). Since all gastric *Helicobacter* spp. have a urease enzyme, the RUT test can be used for NHPH diagnostic, but species identification is not possible.

Histology is based on the detection of *H. pylori* in gastric biopsies, due to its characteristic helical-shape and specific localization (Abadi 2018; Wang YK 2015). Bacteria can be visualized using different stains, such as haematoxylin and eosin (H&E),

Giemsa, Warthin-Starry, or immunohistochemistry using an anti-*H. pylori* antibody (Batts KP 2013; Best LM 2018; Wang YK 2015). This technique allows the identification of gastric lesions associated with the infection. NHPH visualization is also possible during histological evaluation. Even though the morphology of NHPH is different from *H. pylori*, the differentiation may be difficult and further specific analysis is required for NHPH species identification (Bento-Miranda M 2014; Flahou B 2016; Haesebrouck F 2009; Happonens I 1996; Stolte M 1997). Besides this, the focal colonization of NHPH in the human gastric mucosa could lead to false negative results (Holck S 1997; Stolte M 1997).

Culture is the best technique to identify *H. pylori*, however the bacterium is very fastidious and requires specific growth conditions (microaerophilic conditions and blood-containing solid agar) (Abadi 2018; Best LM 2018; Wang YK 2015). This technique is time-consuming and laborious, but antimicrobial susceptibility tests can be performed to obtain more information. For NHPH, isolation and cultivation are much more difficult than for *H. pylori*, due to their extremely fastidious nature (Haesebrouck F 2009). Although *H. bizzozeronii*, *H. felis* and *H. suis* have been isolated from human patients (Kivisto R 2010; Rimbara E 2021; Schott T 2012; Wuppenhorst N 2013), culture is not a suitable technique for routine diagnosis of NHPH infections.

Detection of *H. pylori*-associated DNA using a specific-**PCR** can be performed on a variety of samples: gastric biopsies (invasive), saliva and faeces (non-invasive) (Abadi 2018; Best LM 2018; Choi YK 2001; Farshad S 2004, 2006; Wang YK 2015). Quantitative PCR (qPCR) can also be performed to obtain information about the number of bacteria present in the sample. The detection and differentiation of NHPH species is also possible using PCR or qPCR techniques. For some years, the genes most often used for *Helicobacter* species identification were (part of) 16S rRNA, *gyrB*, *ureAB*, *hsp60*, *iceA* and *lpsA* (Baele M 2004; Blaecher C 2013; Choi YK 2001; De Groote D 2001; Hannula M 2007; O'Rourke JL 2004b; Solnick JV 1993). However, tests based on these genes do not always allow differentiation between closely related gastric *Helicobacter* species (Joosten M 2016), and more specific approaches, such as analysis of the complete genome, are sometimes needed for identification at species level.

An overview of the invasive and non-invasive tests used for diagnosis of gastric *Helicobacter* infections is presented in Table 2.

Table 2. Overview of the tests available for the detection of *Helicobacter* species. In bold are signed the diagnostic tests that allow the identification of NHPH species.

Test	Type	Characteristics	Advantages	Disadvantages
Urea breath test (UBT)	Non-invasive	Sensitivity: >95% Specificity: >95%	-Simple and safe; -High specificity and sensitivity - Detection of eradication of infection	False-negatives
Stool antigen test (SAT)	Non-invasive	Sensitivity: >95% Specificity: >95%	-Fast, simple and not expensive -High specificity and sensitivity - Popular among the patients	False-negatives
Serology	Non-invasive	Sensitivity: >96% Specificity: 60-90%	-Cost-effectiveness cheapest -Widely available -Applicable to patients treated with antibiotics and PPIs -No false negative -Detection of early infection	- Failure to distinguish acute infection and previous contact, and to confirm cure after treatment
Endoscopy	Invasive	Low specificity Low sensitivity	-Visual observation of gastric lesions	-Low sensitivity and specificity -Aggressive for the patient
Rapid urease test (RUT)	Invasive	Sensitivity: 50-95% Specificity: >95%	-Fast, simple, not expensive	-Sensitivity and specificity are lower in gastric ulcers and IM
Histology	Invasive	Sensitivity: 95% Specificity: 80-90%	-Direct diagnosis of <i>H. pylori</i> -Investigation of eradication	-Dependent of personal validation -Time-consuming -Skills needed -High cost -False-negatives
Culture	Invasive	Sensitivity: 50-95% Specificity: >95%	-Determination of antimicrobial resistance	-Expensive and complicated -Time-consuming -Specific cultivation conditions
PCR	Invasive	Sensitivity: 80% Specificity: 100%	-High sensitivity and specificity -Tracking of antimicrobial resistance and mutations	-Expensive -Skills needed -Risk of contamination -False-positives (detection of foreign DNA)

2.3.2 Treatment of gastric *Helicobacter* infections: eradication and antimicrobial resistance

2.3.2.1 Eradication of *Helicobacter pylori*

Individuals with confirmed *H. pylori* presence are usually submitted to an eradication treatment, in order to avoid evolution to more severe gastric diseases, and to reduce the risk of transmission (Ansari S 2017; Batts KP 2013; Hung IF 2009). The best approach would be a personalized therapeutical scheme based on culture of the bacteria followed by antimicrobial susceptibility tests. However, as previously mentioned, the bacterial isolation and *in vitro* cultivation are extremely difficult, and for that reason a standard treatment is usually the first choice to treat *H. pylori* infection (Batts KP 2013; Papastergiou V 2014). This eradication treatment is recommended for active peptic ulcer disease, untreated confirmed history of peptic ulcer disease, low-grade stage I MALT-lymphoma or in case of locally excised early GC (Batts KP 2013). Eradication of *H. pylori* lead to a complete remission of MALT lymphoma in 62% of the patients (Hung IF 2009). When effective, the elimination of the bacteria prevents the development of pre-neoplastic lesions, including atrophy and IM, if not established yet (Hung IF 2009).

The **standard triple therapy** for *H. pylori* eradication consists of a proton pump inhibitor (PPI) combined with two different antibiotics, clarithromycin and metronidazole or amoxicillin, for 7 to 14 days (Bedoya-Gomez IJ 2020; Feng L 2016; Li B 2020; Papastergiou V 2014). The PPI is used to increase the antibiotic absorption in the stomach (Bedoya-Gomez IJ 2020). This treatment is effective in the elimination of the bacteria in 80% of the cases, however the efficacy has been decreasing in the last years due to occurrence of acquired resistance in *H. pylori* against clarithromycin and metronidazole (Bedoya-Gomez IJ 2020; De Francesco V 2010; Ghotaslou R 2015). The percentage of acquired resistance varies depending on the geographical region, and is correlated with consumption of antibiotics, so the eradication should be adapted according to these values (Gisbert JP 2015; Goderska K 2018). The major percentage of acquired resistance worldwide has been observed for metronidazole, with a mean value of 47.2% (30.5% North America - 75.0% Africa). Globally, *H. pylori* acquired resistance to clarithromycin is 19.7%, resistance to amoxicillin 14.7% and levofloxacin resistance 19%. A lower percentage of acquired resistance is observed for tetracycline with a mean value of 11.7% (Basu PP 2011; Ghotaslou R 2015; Goderska K 2018). The high incidence of acquired resistance led to the development of alternative therapeutical approaches for the regions with high incidence of acquired resistance. These alternative schemes can be used also as second line eradication regimen when the first treatment fails, and some of them are mentioned below (Gisbert JP 2015).

One of the most common schemes is the **bismuth-containing quadruple therapy** (Basu PP 2011; Gisbert JP 2015; Papastergiou V 2014). This therapy consists of treatment with a PPI, bismuth, metronidazole, and tetracycline, for 14 days, and is recommended in regions with high incidence of clarithromycin resistance ($\geq 20\%$) (Basu PP 2011; Ghotaslou R 2015; Papastergiou V 2014). **Non-bismuth quadruple therapy** (PPI + 3 antibiotics) is also recommended in these regions (Papastergiou V 2014). Another alternative for patients in these regions can be a **levofloxacin-based therapy**, that uses levofloxacin instead of clarithromycin in the standard triple therapy (Papastergiou V 2014). The use of this therapeutical scheme has to be surveyed due to the recent increase in levofloxacin resistance, associated with the increased use of this antibiotic (Papastergiou V 2014).

Sequential therapy is a simple dual therapy including a PPI and amoxicillin for the first 5 days, followed by PPI + clarithromycin + metronidazole for the second 5 days (Feng L 2016; Ghotaslou R 2015; Papastergiou V 2014).

Hybrid therapy consists of a PPI combined with amoxicillin for 7 days, and then quadruple therapy for another 7 days (Goderska K 2018; Papastergiou V 2014).

The efficacy of the eradication strategies described for *H. pylori*, for treatment of gastric infections with NHPH species in humans is not clear, due to the low percentage of confirmed infection. In a limited number of cases, individuals were treated with same strategies used for *H. pylori*, often with success (Haesebrouck F 2009). Recently, antimicrobial susceptibility tests have been performed on NHPH isolates in order to better understand if these species also present acquired resistance to the standard antibiotics used.

2.3.2.2 Antimicrobial resistance in gastric NHPH species

H. bizzozeronii and *H. suis* were successfully isolated from the human stomach and acquired resistance against tetracycline and metronidazole was observed for *H. bizzozeronii* (Schott T 2012), while a *H. suis* isolate showed acquired resistance to amoxicillin and higher MIC values for metronidazole (Rimbara E 2021). Besides these, antimicrobial susceptibility tests have also been performed on NHPH isolates obtained from canine, feline and porcine stomach. Canine- and feline- *H. felis*, canine *H. bizzozeronii* and canine *H. salomonis* isolates were tested against several antimicrobial compounds. Acquired resistance was not detected against ampicillin, clarithromycin, tetracycline, tylosin, enrofloxacin, gentamicin and neomycin. Some isolates presented decreased susceptibility against lincomycin and spectinomycin (Van den Bulck K 2005b). Two *H. felis* isolates presented acquired resistance to metronidazole (Van den Bulck K

2005b). The antimicrobial susceptibility pattern of porcine and non-human primates *H. suis* isolates was also evaluated. Some porcine *H. suis* isolates showed acquired resistance to fluoroquinolones, tetracycline, lincomycin or spectinomycin, mostly due to the presence of single nucleotide polymorphisms (SNPs) (Berlamont H 2019; Vermoote M 2011). Interestingly, the porcine *H. suis* isolates showed higher ampicillin, tetracycline, and doxycycline MIC values, in comparison to the primate *H. suis* isolates (Berlamont H 2019). The detection of SNPs on these isolates suggests a possible intrinsic decreased susceptibility of porcine *H. suis* isolates for ampicillin, an antibiotic commonly used to treat gastric *Helicobacter* infection in humans (Berlamont H 2019).

3. Host-pathogen interaction: role of the gastric glycophenotype

3.1 Glycosylation and glycan structures

Glycans are one of the major and fundamental macromolecules components of the cells (Hooper LV 2001; Pinho SS 2015). Glycosylation is a post-translational modification, defined as the covalent linkage of saccharides (single sugars) to other saccharides, proteins or lipids, forming different families of glycoconjugates such as glycosphingolipids, glycosaminoglycans and glycoproteins (Eichler 2019; Endo 2019; Mereiter S 2019; Pinho SS 2015). Glycans and glycosylation are essential for the normal function of the cells (homeostasis), being involved in multiple biological processes, such as cell-cell and cell-matrix interaction, protein folding, protection from proteases and subcellular targeting (Eichler 2019; Endo 2019).

Glycosylation can be divided in two families depending on the nature of the linkage. The biosynthesis and further modifications are tissue and cell specific and are controlled by glycosyltransferases (Pinho SS 2015). The *N*-glycosylation initiates on the endoplasmic reticulum (ER), and finishes in the Golgi apparatus, with major effects on function, stability and subcellular localization of the new synthesized molecules (Eichler 2019; Moremen KW 2012; Pinho SS 2015; Schjoldager KT 2020). The *N*-glycans results from the addition of a *N*-Acetylglucosamine (GlcNAc) to the amine group on an asparagine residue (Eichler 2019; Endo 2019; Magalhaes A 2010a; Mereiter S 2019; Pinho SS 2015). On the other hand, the attachment of a *N*-Acetylgalactosamine (GalNAc) to the hydroxyl group of a serine or threonine residue initiates the *O*-glycosylation pathway (Schjoldager KT 2020).

Alterations in the glycophenotype have been associated with more susceptibility to infection by several human pathogens (De Oliveira DM 2017; Etzold S 2014; Formosa-Dague C 2018; Thompson AJ 2019). Some examples of the role of glycans as important mediators in the host-pathogen interaction include *H. pylori* (Aspholm-Hurting M 2004; Benktander J 2012; Borén T 1994), *Mycobacterium tuberculosis* (Barnes DD 2017;

Fonseca KL 2020; Matos R 2021; Shukla S 2018), *Escherichia coli* (Bottomley AL 2020; Krishnan et al. 2014), Influenza virus (Air 2014; Bateman AC 2010; Byrd-Leotis L 2017; Cohen M 2016), and recently SARS-Cov-2 (Casalino L 2020; Clausen TM 2020; Watanabe Y 2020). The majority of the pathogen's express glycan binding proteins on their surface that recognize and bind to specific receptors on the surface of host cells (Ringot-Destrez B 2017).

3.2 The ABO(H) and Lewis antigens: role in adhesion of *Helicobacter pylori*

The ABO(H) histo-blood group antigens are formed by sequential addition of saccharide residues to glycoproteins and glycolipids (Yamamoto F 1990). The linkage of a fucose residue to the terminal galactose of the core chains, by an α 1,2-fucosyltransferase, results in the synthesis of the H antigens (H-type 1 and H-type 2) (Clausen H 1989; Koda Y 1996; Le Pendu J 1982; Oriol R 1986; Torrado J 2000). Blood-group A and B phenotypes are formed by the addition of a terminal galactose (GalNAc α 1,3 or Gal α 1,3, respectively) to the H antigen, by the action of a GalNAc or a D-galactosyltransferase, respectively (Clausen H 1989; Koda Y 1996; Le Pendu J 1982; Oriol R 1986). The Lewis antigens are terminal structures that decorate a huge number of glycan chains, and they can be classified as type 1 or type 2, based on their biosynthesis (Amorim I 2014; Magalhaes A 2015). Type 1 Lewis antigens (LeA, LeB and sLeA) are characterized by the Gal β 1,3GlcNAc linkage, while type 2 antigens (LeX, LeY and sLeX) present a Gal β 1,4GlcNAc linkage (Le Pendu J 1982; Oriol R 1986; Pinho SS 2015; Symmes BA 2018). These processes are highly regulated by the action of several glycosyltransferases, including fucosyltransferases and sialyltransferases (De Oliveira DM 2017; Harduin-Lepers A 2012; Pinho SS 2015). These blood-group glycan structures are also expressed in the surface mucous (Borén T 1993; Borén T 1994), in extracellular fluids, such as saliva, tears and breast milk, and on oral epithelial cells (De Oliveira DM 2017).

The biosynthetic pathway of the ABO(H) antigens is represented in Figure 3.

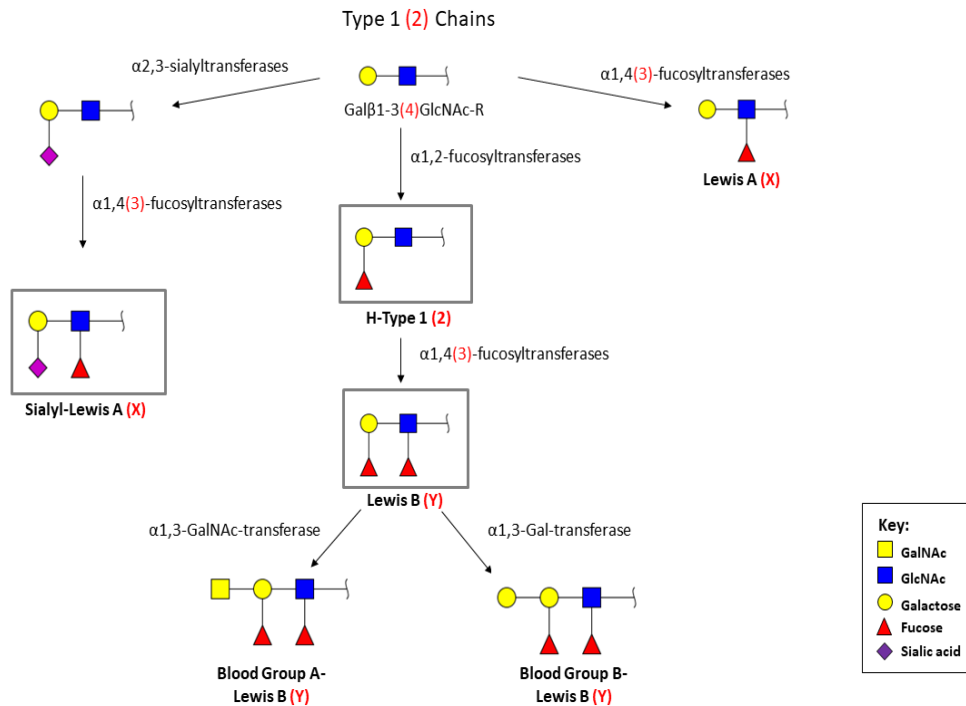


Figure 3. Biosynthetic pathway of the terminal histo-blood group antigens [ABO(H)]. Type 1 chains are characterized by the Galβ1,3GlcNAc linkage (represented in black), while type 2 chains display a Galβ1,4GlcNAc linkage (represented between parentheses and in red). Monosaccharide symbols follow the Symbol Nomenclature for Glycans (Neelamegham S 2019). The antigens signed in the grey box (LeB, H-type 1, sLeA, and sLeX) are the structures recognized by *H. pylori* BabA and SabA adhesins. Illustration adapted from Matos *et al.*, 2021.

The adhesion of *H. pylori* to the human gastric mucosa is mediated by surface receptors present on the host cells, that are recognized by bacterial adhesins. Indeed, only a small percentage of the bacteria attach to the epithelium. The majority are localized in the gastric pits and in the mucous layer (Clyne M 2019).

A successful infection and colonization occur in two steps: 1) *H. pylori* crosses the mucous layer and reaches the surface of the gastric mucosa, and 2) *H. pylori* adheres to the epithelial cells via OMPs (Borén T 1993; Borén T 1994; Huang Y 2016). The first interaction is mediated by the bacterial **BabA** adhesin, which binds to fucosylated blood-group antigens H-type 1 and LeB (Benktander J 2012; Borén T 1993; Ilver D 1998; McGuckin MA 2011). LeB antigen can be expressed on MUC5AC, MUC1 and MUC2 mucins (Ansari S 2017; Chmiela M 2019; Clyne M 2019; Mahdavi MR 2013). The inflammation resulting from *H. pylori* colonization of the gastric mucosa is accompanied by the remodelling of the glycosylation profile, with *de novo* expression of α2,3-sialylated glycans, such as sLeX and sLeA (Mahdavi J 2002). These structures are recognized by

the **SabA** adhesin, forming a second point of attachment to the host cells (Benktander J 2018; Linden SK 2008; Magalhaes A 2015; Mahdavi J 2002).

The lacdiNAc-binding adhesin LabA is another adhesin that has been suggested to be involved in bacterial adhesion (Rossez Y 2014). LabA binds specifically to the LacdiNAc residue located on MUC5AC mucins (Rossez Y 2014). This motif is widespread in the human body, e.g., stomach, colon, small intestine, salivary glands and bronchioles, but always co-localized with MUC5AC (Rossez Y 2014). However, the role of this adhesin and even the specificity to lacdiNAc motif is not clear yet, and further studies are needed (Mthembu YH 2020; Paraskevopoulou V 2021).

The main *H. pylori* adhesins and the respective ligands are listed in Table 3.

Table 3. *Helicobacter pylori* adhesins and their respective ligands.

Adhesin	Receptors identified	Localization	Reference
BabA	LeB and terminal fucose, H1-antigen, A-antigen and B-antigen	Gastric epithelium	(Benktander J 2012; Borén T 1993; Ilver D 1998; McGuckin MA 2011)
	MUC5AC mucin with N-Acetylgalactosamine- β 1,4-N-Acetylglucosamine	Gastric mucous	(Kenny DT 2012; Linden S 2004)
	MUC1 mucin	Gastric mucous	(Linden SK 2009)
	MUC2 mucin	Gastric mucous	(Cohen M 2003)
	MUC5B mucin	Saliva	(Prakobphol A 2005; Walz A 2009)
	Secretory Ig A containing fucose-oligosaccharide motifs	Saliva	(Borén T 1993; Royle L 2003)
	Salivary Agglutinin	Saliva	(Issa S 2010)
SabA	sLeA antigen	Gastric epithelium	(Mahdavi J 2002)
	sLeX antigen	Gastric epithelium	(Mahdavi J 2002)
LabA	lacdiNAc motif	Gastric epithelium	(Rossez Y 2014)

The H phenotype is the more frequent in the population (70%), particularly in Western Europe and North America (De Oliveira DM 2017), and the expression of ABO(H) antigens is dependent on the expression of fucosyltransferase 2 (*FUT2*) (De Oliveira DM 2017; Heneghan M 1998). Individuals expressing *FUT2* gene, which is associated with the synthesis of LeB, the main ligand of *H. pylori* BabA adhesin, and

other blood-group antigens, expressed by epithelial cells, are called secretors (De Oliveira DM 2017; Kelly R 1995; Lee HS 2006; Magalhaes A 2009; Magalhaes A 2016; Murata K 1992; Orntoft TF 1991; Serpa J 2004; Silva LM 2009; Teixeira A 2002; Tsukazaki K 1991). Secretor individuals are usually more prone to infection by *H. pylori* and development of associated lesions (De Oliveira DM 2017; Heneghan M 1998).

The absence of ABO(H) antigens on the gastric epithelial cells is the main characteristic of the non-secretor phenotype (Heggelund JE 2017). In these non-secretor individuals, the interaction between SabA and the sLeX has been described as determinant of the *H. pylori* colonization (Benktander J 2018; Sheu BS 2006).

3.3 Mucins

Mucins are high molecular weight glycoproteins, expressed in several epithelial tissues, in a tissue-specific manner (Duarte HO 2016; Hollingsworth MA 2004). They can be divided into three subgroups: secreted-gel forming mucins, cell-surface mucins and secreted non-gel-forming mucins (Linden SK 2008). These molecules are essential for the binding of several pathogens, regulation of antimicrobial response, induction of tolerance mechanisms, induction of cell proliferation against pathogens and chemicals and enzymatic and mechanical damage (Heggelund JE 2017; Linden SK 2008; Magalhaes A 2010b).

Tissues characterized by dense expression of mucins are considered preferential sites of infection (Linden SK 2008). Pathogens are attracted to these binding sites and they can be trapped and then removed and eliminated (Linden SK 2008). However, pathogens seem to have developed a strategy to use these molecules for their benefit and to use them to establish a closer contact with the epithelial cells, favouring the infection and a successful colonization (Linden SK 2008).

A healthy human gastric mucosa is characterized by the presence of the membrane-bound MUC1 in the foveolar epithelial cells, and the secreted MUC5AC and MUC6, the main constituents of the gastric mucous layer (Ferreira B 2006; Linden SK 2008; Magalhaes A 2010b; Reis CA 1997, 2000; Symmes BA 2018). MUC5AC and MUC6 mucins can also be expressed by the foveolar epithelium or glands, respectively (Linden SK 2008; Magalhaes A 2010b; Reis CA 1997, 1999, 2000). Under pathological conditions, usually associated with *H. pylori* infection, such as intestinal metaplasia, *de novo* expression of MUC2 mucin can be observed in the gastric mucosa (Reis CA 1999; Teixeira A 2002).

Mucins and their crucial involvement in *H. pylori* binding to the human gastric mucosa have been widely studied and described in detail. The development of *H. pylori*-associated gastritis and GC could be associated with allelic variation on the MUC1-

encoding gene, and mice deficient in this mucin present a more severe chronic inflammation (Carvalho F 1997; McGuckin MA 2007; Sheng YH 2020; Vinall LE 2002).

In homeostasis, low levels of MUC13 mucin are detected in the epithelial cells of GI tract (Maher DM 2011). However, alterations in the expression of this mucin have been associated with increased risk of inflammatory diseases and inflammation-induced carcinoma (gastric and colon) (Gupta BK 2014; Maher DM 2011; Sheng YH 2012). Interestingly, MUC13 seems to have an important role in the infection by NHPH. Some studies suggested that the MUC13 expression could be induced by IL-1 β cytokine in mice infected with *H. suis* or *H. heilmannii*, being possible involved in the attachment to the epithelial cells (De Witte C 2016; Gorle N 2018; Liu J 2015).

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Scientific Aims

General aim:

This research project approached the following topics: determination of the antimicrobial susceptibility pattern of NHPH and possible acquisition of resistance mechanisms; evaluation of the efficacy of the currently available *H. pylori* eradication treatment; and exploring the effects of the NHPH infection *in vitro*, at cellular level.

Specific aims:

Paper I

The aim of this paper was to give an overview of the adhesion of gastric *Helicobacter* species to the human gastric mucosa, focusing on the role of glycans and glycosylation in the adhesion and colonization process.

Paper II

In the last years, the efficacy of the *Helicobacter pylori* eradication treatment decreased drastically, mostly due to acquired resistance to some of the main antibiotics used. The best approach to evaluate the possible acquisition of resistance mechanisms is using antimicrobial susceptibility tests. Analysis of the susceptibility pattern to the most common antibiotics is very useful not only in the human clinic but also in veterinary clinic. In the last years, the antimicrobial susceptibility pattern of *H. suis*, *H. felis*, *H. bizzozeronii* and *H. salomonis* isolates was evaluated, but for *H. heilmannii* and *H. ailurogastricus* isolates this was never performed. In this study, feline isolates of these two *Helicobacter* species were used to determine the susceptibility pattern to several antimicrobial compounds. Some isolates demonstrated possible acquired resistance/decreased susceptibility to some antibiotics tested.

Paper III

Portugal presents one of the highest percentages of acquired resistance to clarithromycin in *H. pylori*, one of the antibiotics used in the standard eradication therapy.

The prevalence of NHPH species in humans has been described as very low, but the values might be underdiagnosed due to the lack of proper diagnostic tools. In this study, we aimed to determine the occurrence of NHPH infection in humans and evaluate the efficacy of the eradication treatment in the elimination of the bacteria in a cohort of patients.

Paper IV

Previous studies reported that *H. pylori* and gastric NHPH species have different abilities to adhere to canine gastric mucosa and these differences may play a role in host adaptation (Amorim 2014). Therefore, the capacity to adhere and colonize the human stomach may also vary between different gastric *Helicobacter* species. In order to determine which NHPH species are more prone to bind to the human gastric tissue, *in vitro* binding ability assays were performed. Additionally, we also determined the gastric glycophenotype and the secretor status of these individuals, and its possible relation to binding of some NHPH species.

Paper V

Human infection by *H. pylori* leads to an inflammatory response with recruitment of immune cells and activation of several cytokines and other molecules. Furthermore, the binding of the bacteria results in the *de novo* synthesis of sialylated glycans, changing the gastric glycophenotype and working as a second point of attachment. However, the effects of NHPH infection on human cells are unknown. In this study, we intend to evaluate possible effects associated with infection by these agents, for instance on the cell viability, cytokine production, and on the activation of sialylated structures biosynthesis.

Paper I

Adhesion of *Helicobacter* species to the human
gastric mucosa:

A deep look into glycans role

Rita Matos, Irina Amorim, Ana Magalhães, Freddy Haësebrouck, Fátima
Gärtner, Celso A. Reis

Frontiers in Molecular Biosciences

2021

doi: 10.3389/fmolb.2021.656439



Adhesion of *Helicobacter* Species to the Human Gastric Mucosa: A Deep Look Into Glycans Role

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OPEN ACCESS

Edited by:

Yan Liu,
Imperial College London,
United Kingdom

Reviewed by:

Virgínia Tajadura-Ortega,
Imperial College London,
United Kingdom
Anton V. Zaslavov,
University of Turku, Finland

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Specialty section:

This article was submitted to
Structural Biology,
a section of the journal
Frontiers in Molecular Biosciences

Received: 20 January 2021

Accepted: 22 April 2021

Published: 07 May 2021

Citation:

Matos R, Amorim I, Magalhães A,
Haesebrouck F, Gärtner F and Reis CA
(2021) Adhesion of *Helicobacter*
Species to the Human Gastric
Mucosa: A Deep Look Into
Glycans Role.
Front. Mol. Biosci. 8:656439.
doi: 10.3389/fmolb.2021.656439

Helicobacter species infections may be associated with the development of gastric disorders, such as gastritis, peptic ulcers, intestinal metaplasia, dysplasia and gastric carcinoma. Binding of these bacteria to the gastric mucosa occurs through the recognition of specific glycan receptors expressed by the host epithelial cells. This review addresses the state of the art knowledge on these host glycan structures and the bacterial adhesins involved in *Helicobacter* spp. adhesion to gastric mucosa colonization. Glycans are expressed on every cell surface and they are crucial for several biological processes, including protein folding, cell signaling and recognition, and host-pathogen interactions. *Helicobacter pylori* is the most predominant gastric *Helicobacter* species in humans. The adhesion of this bacterium to glycan epitopes present on the gastric epithelial surface is a crucial step for a successful colonization. Major adhesins essential for colonization and infection are the blood-group antigen-binding adhesin (BabA) which mediates the interaction with fucosylated H-type 1 and Lewis B glycans, and the sialic acid-binding adhesin (SabA) which recognizes the sialyl-Lewis A and X glycan antigens. Since not every *H. pylori* strain expresses functional BabA or SabA adhesins, other bacterial proteins are most probably also involved in this adhesion process, including LabA (LacdiNAc-binding adhesin), which binds to the LacdiNAc motif on MUC5AC mucin. Besides *H. pylori*, several other gastric non-*Helicobacter pylori* *Helicobacters* (NHPH), mainly associated with pigs (*H. suis*) and pets (*H. felis*, *H. bizzozeronii*, *H. salomonis*, and *H. heilmannii*), may also colonize the human stomach and cause gastric disease, including gastritis, peptic ulcers and mucosa-associated lymphoid tissue (MALT) lymphoma. These NHPH lack homologous to the major known adhesins involved in colonization of the human stomach. In humans, NHPH infection rate is much lower than in the natural hosts.

Differences in the glycosylation profile between gastric human and animal mucins acting as glycan receptors for NHPH-associated adhesins, may be involved. The identification and characterization of the key molecules involved in the adhesion of gastric *Helicobacter* species to the gastric mucosa is important to understand the colonization and infection strategies displayed by different members of this genus.

Keywords: adhesion, gastric mucosa, glycans, glycosylation, *helicobacter*

GLYCOSYLATION AND GLYCAN STRUCTURES

Glycosylation is a post-translational modification and is defined as the covalent attachment of single sugars (saccharides) to other saccharides, proteins or lipids (Pinho and Reis, 2015; Eichler, 2019; Endo, 2019; Mereiter et al., 2019). This enzyme-mediated glycosidic linkage to specific residues on the target molecules follows the secretory pathway, beginning in the endoplasmic reticulum, in the case of *N*-glycosylation, and finishing in the Golgi apparatus, with major effects on function, stability and subcellular localization of the newly biosynthesized glycoconjugate (Moremen et al., 2012; Pinho and Reis, 2015; Eichler, 2019; Schjoldager et al., 2020). Glycosylation is an essential mechanism for the regulation of several biological processes, such as cell-cell and cell-matrix interaction, protein folding, protection from proteases and subcellular targeting (Eichler, 2019; Endo, 2019). Additionally, glycans have been described as crucial for the interaction and adhesion of several microorganisms to human cells (Etzold and Juge, 2014; Formosa-Dague et al., 2018; Thompson et al., 2019).

Protein glycosylation can be classified in families according to the nature of the linkage, being the biosynthesis and further modifications tissue and cell specific, controlled by glycosyltransferases (Pinho and Reis, 2015). The two main protein glycosylation pathways are the *O*- and *N*-glycosylation, that differ on the amino acid used for the attachment of the glycans. The interaction between the reducing terminal of a *N*-Acetylglucosamine (GlcNAc) with the amine group of an asparagine via an asparylglycosylamine linkage constitutes the first step of the *N*-glycans biosynthesis (Magalhaes et al., 2010; Pinho and Reis, 2015; Eichler, 2019; Endo, 2019; Mereiter et al., 2019). The attachment of the reducing terminal of a *N*-Acetylgalactosamine (GalNAc) residue to the hydroxyl group of a serine or threonine initiates the protein *O*-glycosylation pathway (Schjoldager et al., 2020).

The biosynthesis of the abundant form of GalNAc-type *O*-glycosylation is initiated by a group of enzymes named polypeptide *N*-acetylgalactosamine transferases (ppGalNAc-Ts), which includes a family of 20 enzymes that catalyze the addition of a GalNAc residue (Bennett et al., 2012). The addition of a galactose residue in a β 1-3-linkage onto GalNAc-*O*-Ser/Thr originates the formation of the core-1 structure. Next, a second neutral sugar (GlcNAc) can be added onto a β 1-6 position to the core one structure, by core two synthases, forming the core-2 structure (Pinho and Reis, 2015; Symmes et al., 2018). These structures can be further elongated by the addition of Gal and GlcNAc residues. Additionally, these glycan structures can be terminally decorated with the addition of sialic acid and fucose residues, by sialyltransferases and fucosyltransferases (Pinho and Reis, 2015; Mereiter et al., 2019). This type of glycosylation is often called mucin type *O*-glycosylation because it is highly abundant in mucins. In addition to the *O*-GalNAc forms, *O*-glycosylation can also occur with fucose (Fuc), mannose (Man), GlcNAc or glucose (Glc) residues (Eichler, 2019).

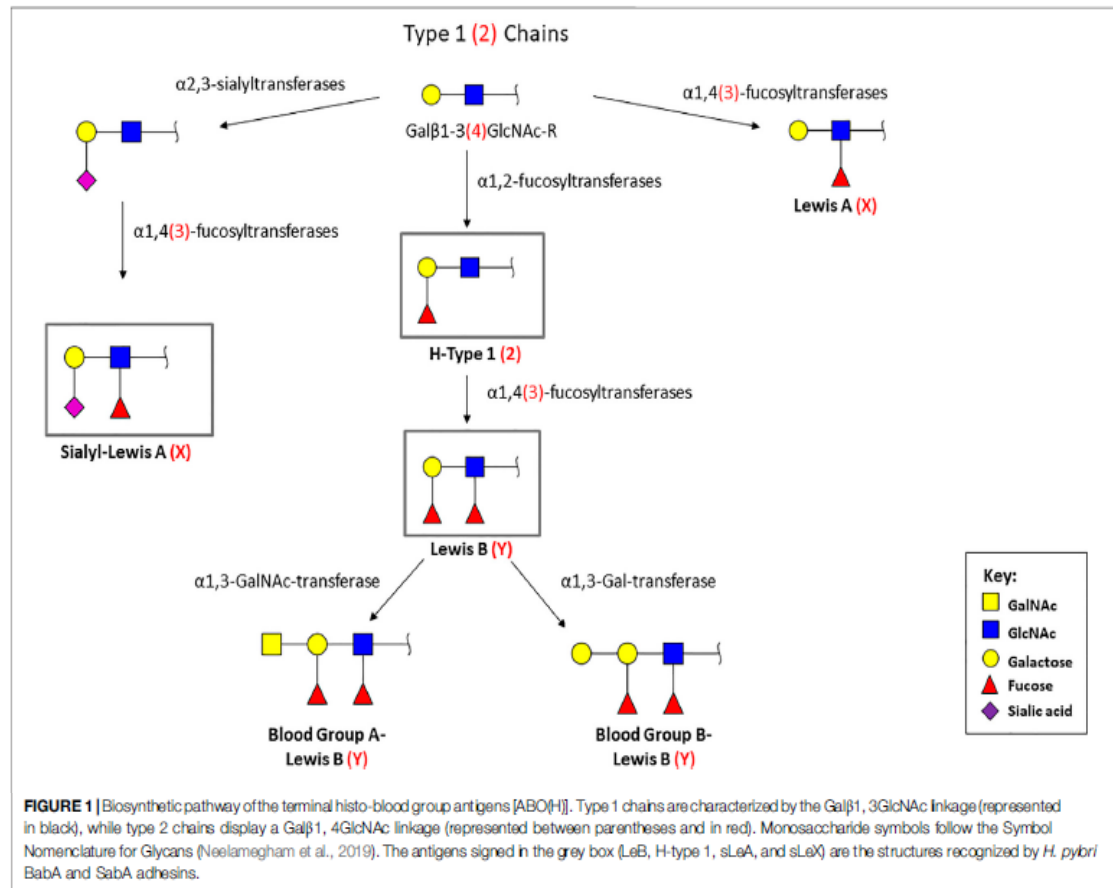
Mucins are high molecular weight glycoproteins, encoded by over 21 different genes, that can be expressed in a broad range of

epithelial tissues, in a tissue-specific manner (Hollingsworth and Swanson, 2004; Duarte et al., 2016). Mucins can be divided into three subgroups: the secreted-gel forming mucins, the cell-surface mucins and the secreted non-gel-forming mucins (Linden et al., 2008). Mucins are important players in the pathogen binding, regulation of antimicrobial responses and induction of tolerance mechanisms (Linden et al., 2008; Magalhaes and Reis, 2010). In fact, mucins are essential for cell protection against pathogens and chemical, enzymatic and mechanical damage, since this mucous layer is the first barrier that these agents need to cross (Linden et al., 2008; Heggelund et al., 2017). The main mucins present on the human healthy gastric mucosa are the membrane-bound MUC1, and the secreted MUC5AC and MUC6, which are the main constituents of the gastric mucous layer (Reis et al., 1997; Reis et al., 2000; Ferreira et al., 2006; Linden et al., 2008; Magalhaes and Reis 2010; Symmes et al., 2018). MUC1 is the most widely studied mucin and is expressed mainly by the foveolar epithelial cells. The secreted MUC5AC is expressed by the foveolar epithelium, while the MUC6 expression is limited to the glands (Reis et al., 1997; Reis et al., 1999; Reis et al., 2000; Linden et al., 2008; Magalhaes and Reis 2010). Occasionally, under pathological conditions, namely intestinal metaplasia, a different pattern of mucins expression can be observed, such as the *de novo* expression of MUC2 (Reis et al., 1999; Teixeira et al., 2002; Linden et al., 2008; Magalhaes and Reis 2010).

Besides these two main types of glycans on proteins, other classes of glycoconjugates are present on the cell, including the proteoglycans and the glycosphingolipids, and other glycosylation forms can also occur on specific types of proteins, namely the Notch receptor (Pinho and Reis, 2015).

BIOSYNTHESIS OF HISTO-BLOOD GROUP ANTIGENS

Glycosylation is a highly regulated process important for homeostasis, but alterations have been described to occur in pathological scenarios. The histo-blood group ABO (H) antigens are found in several extracellular fluids, such as saliva and tears, and at the surface of epithelial cells. They mediate the contact with multiple pathogens (De Oliveira et al., 2017; Le Pendu and Ruvoen-Clouet, 2020). The synthesis of the ABO (H) antigens occur by the addition of saccharide residues to both glycoproteins and glycolipids (Yamamoto et al., 1990) (Figure 1). The H phenotype results from the linkage of a fucose to the terminal galactose of the core chains, by an α 1,2-fucosyltransferase, resulting in the H antigens 1 and 2 (H-Type 1 and H-Type 2 (Le Pendu et al., 1982; Oriol et al., 1986; Clausen and Hakomori, 1989; Koda et al., 1996; Torrado et al., 2000). The blood-group A and B phenotypes result from the addition of terminal galactose derivatives to the H antigens (GalNAc α 1,3 or Gal α 1,3), by the action of a GalNAc or a D-galactosyltransferase, respectively (Le Pendu et al., 1982; Oriol et al., 1986; Clausen and Hakomori, 1989; Koda et al., 1996). The Lewis antigens are terminal fucosylated glycan structures that decorate a huge number of glycan chains, which can be classified as type 1 or type 2 Lewis antigens, based on their biosynthesis



(Amorim et al., 2014; Magalhaes et al., 2015). The type 1 structures (Lewis A, Lewis B and sialyl-Lewis A) are characterized by the Galβ1,3GlcNAc linkage, while type 2 antigens (Lewis X, Lewis Y and sialyl-Lewis X) display a Galβ1,4GlcNAc linkage (Le Pendu et al., 1982; Oriol et al., 1986; Pinho and Reis, 2015; Symmes et al., 2018). The Lewis A (LeA) and Lewis X (LeX) antigens are formed by the addition of a branched fucose residue to the type 1/2 chains (Watkins, 1980; Le Pendu et al., 1982; Oriol et al., 1986; Clausen and Hakomori, 1989; Torrado et al., 2000). On the other hand, Lewis B (LeB) and Lewis Y (LeY) are difucosylated structures, resulting from the attachment of a fucose to the monofucosylated H-Type 1 and H-Type 2 antigens, respectively (Watkins, 1980; Le Pendu et al., 1982; Oriol et al., 1986; Clausen and Hakomori, 1989). The synthesis of these antigens is mediated by several glycosyltransferases, including fucosyltransferases and sialyltransferases, among others (Harduin-Lepers et al., 2012; Pinho and Reis, 2015; De Oliveira et al., 2017).

The H phenotype is the most frequent worldwide (70%), particularly in Western Europe and North America (De Oliveira et al., 2017), and the expression of the ABO (H) blood-group antigens is dependent on the expression of the

FUT2 gene, encoding the fucosyltransferase 2 (Heneghan et al., 1998; De Oliveira et al., 2017). Individuals that express the *FUT2* gene are called secretors, due to the secretion of the corresponding blood-group antigens by mucin-secreting cells (Kelly et al., 1995; Serpa et al., 2004; Magalhaes et al., 2009; Silva et al., 2009; Magalhaes et al., 2016; De Oliveira et al., 2017).

For the secretor individuals, the ABO (H) antigens are expressed by epithelial cells present in many types of tissues (Ornftoft et al., 1991; Tsukazaki et al., 1991; Murata et al., 1992; Teixeira et al., 2002; Lee et al., 2006). Due to this localization, these antigens can act as ligands to specific pathogens, such as *H. pylori*, one of the most studied human pathogens.

ROLE OF GLYCANS IN HOST-PATHOGEN INTERACTION

The role of glycans in the recognition of pathogens has been widely studied for several types of microorganisms. Indeed, the interaction between the pathogen and the glycan receptors at the cell surface is the first step and is crucial for a successful colonization and infection (Kreisman and Cobb, 2012). The

majority of pathogens present glycan binding proteins on their surface that will recognize and bind to specific receptors on the host cell membrane or mucous layer. One of the major players are the mucin O-glycans, which are essential for bacterial adhesion, in a species-specific manner (Ringot-Destrez et al., 2017). This binding is crucial for survival of the pathogen in the host microenvironment, since it will help to avoid the elimination by clearance mechanisms and to escape from the immune response (Kreisman and Cobb, 2012; Belotserkovsky et al., 2018). Indeed, the majority of the pathogens are able to modulate the expression of their lectins, in order to adapt to alterations in the host glycosylation profile observed for several diseases (Ringot-Destrez et al., 2017). Glycans have been implicated in host-pathogen interactions of several microorganisms, including viruses such as Influenza virus (Bateman et al., 2010; Air, 2014; Cohen et al., 2016; Byrd-Leotis et al., 2017; Mayr et al., 2018), Calicivirus (Bhella et al., 2008), and recently SARS-CoV-2 (Casalino et al., 2020; Clausen et al., 2020; Watanabe et al., 2020), as well as bacteria, such as *Mycobacterium tuberculosis* (Barnes et al., 2017; Shukla et al., 2018; Fonseca et al., 2020; Matos et al., 2020), *Pseudomonas aeruginosa* (Linden et al., 2008), *Escherichia coli* (Krishnan et al., 2014; Bottomley et al., 2020), *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Pakharukova et al., 2016) and *H. pylori* (Borén et al., 1994; Aspholm-Hurtig et al., 2004; Benktander et al., 2012).

HELICOBACTER PYLORI ADHESION TO HUMAN GASTRIC MUCOSA: AN INTERACTION MEDIATED BY GLYCANS

Helicobacter pylori is a Gram-negative, microaerophilic, spiral-shaped bacterium, that colonizes the stomach of half of the worldwide human population (Altman et al., 2005; Magalhaes and Reis, 2010; Huang et al., 2016; Ansari and Yamaoka, 2017). A persistent infection with *H. pylori* results in chronic gastritis, which can evolve to peptic ulcers, precancerous lesions (atrophy, intestinal metaplasia and dysplasia) and gastric adenocarcinoma (Huang et al., 2016; Ansari and Yamaoka, 2017). Indeed, *H. pylori* is considered a group I carcinogen due to its strong association with gastric carcinoma, with a 2-8-fold increased risk vs. the non-infected population (Huang et al., 2016; Ansari and Yamaoka, 2017). Fortunately, in most cases, *H. pylori* infection remains asymptomatic, however 10% of the individuals develop severe gastric lesions and of these, 1-3% progresses to gastric carcinoma with a low 5-years survival rate (De Falco et al., 2015; Huang et al., 2016). Furthermore, *H. pylori* may also play a role in non-gastric diseases, such as Parkinson's disease, Alzheimer's disease, diabetes mellitus, hematologic disorders and asthma, which might be associated with changes in host immune response and human microbiome induced by an infection with this bacterium (Bravo et al., 2018; Menard and Smet, 2019; Gottesmann et al., 2020).

Infection with *H. pylori* is usually clustered in families, and individuals are normally infected during childhood and, if not eradicated, it can persist through individual's lifetime (Clyne and

May, 2019). The prevalence of this pathogen varies within the geographic region and depends on several factors, such as age, socio-economic status, educational level and hygiene (De Falco et al., 2015; Huang et al., 2016). The transmission mechanism is not completely known but it may occur *via* fecal-oral or oral-oral route (Huang et al., 2016; Ansari and Yamaoka, 2017).

H. pylori uses several virulence factors to colonize the human gastric mucosa (Ansari and Yamaoka, 2017). The virulence factors expressed by *H. pylori* include the CagA (cytotoxin-associated gene A) and the VacA (vacuolating cytotoxin A) molecules (Marcos et al., 2008; Ansari and Yamaoka, 2017). Besides these factors, the bacterial genome encodes a large number of outer membrane proteins (OMP), which are expressed at the bacterial cell envelope and can be divided into six families: the *H. pylori* OMP, the Hop-related, the *Helicobacter* OMP, the *Helicobacter* outer membrane (Hom), the iron-regulated OMPs and the efflux pump OMPs (Magalhaes and Reis, 2010; Ansari and Yamaoka, 2017; Bauwens et al., 2018). The majority of the OMPs belong to the Hop proteins, and can act as transport channels (porins) or adhesins such as the blood-group antigen binding adhesin (BabA) (Borén et al., 1993), the sialic acid-binding adhesin (SabA) (Borén et al., 1994), the lactiNAC-binding adhesin (LabA) (Rossez et al., 2014), the adherence-associated lipoprotein A/B (AlpA/B) and the outer inflammatory protein A (OipA), the HopQ and the HopZ (Benktander et al., 2012; Ansari and Yamaoka, 2017; Bauwens et al., 2018; Benktander et al., 2018). The attachment of the adhesins present on the bacterial surface to the gastric mucus layer is the initial step and is crucial for the colonization and subsequent infection by *H. pylori* (Rossez et al., 2014; Huang et al., 2016; Ansari and Yamaoka, 2017). The binding to the gastric epithelium is essential for a successful colonization because it will protect the bacteria from clearance mechanisms, such as liquid flow, peristaltic movement, washing and mucus shedding (Aspholm et al., 2006; Huang et al., 2016; Ansari and Yamaoka, 2017). Additionally, attachment provides a source of nutrients to the bacteria and promotes the delivery of bacterial toxins or other virulence factors to the host cells, contributing to the development of a persistent infection (Aspholm et al., 2006; Ansari and Yamaoka, 2017) (Figure 2).

H. pylori can be classified based on its ability to bind to the blood-group antigens: specialist strains bind to blood-group O antigens, whereas the generalist strains bind to group O, A and B antigens (Ansari and Yamaoka, 2017). Besides this, the triple positive strains (BabA-, VacA- and CagA-positive) are associated with more severe gastric diseases, namely peptic ulcers and gastric carcinoma (Benktander et al., 2012; Benktander et al., 2018).

A successful infection occurs in two steps: 1) the bacteria reach the surface of the gastric mucosa, which has a nearly neutral pH, after crossing the mucus layer; and 2) the bacteria adhere to the gastric epithelial cells via the OMPs (Borén et al., 1994; Borén et al., 1993; Huang et al., 2016). The first interaction is mediated by the BabA adhesin, which recognizes and binds to fucosylated blood-group antigens H-type 1 and LeB (Borén et al., 1993; Ilver et al., 1998; McGuckin et al., 2011; Benktander et al., 2012). The LeB antigen, is the main receptor for the BabA adhesin, and it can be expressed on MUC5AC mucin, on MUC1 and MUC2 mucins

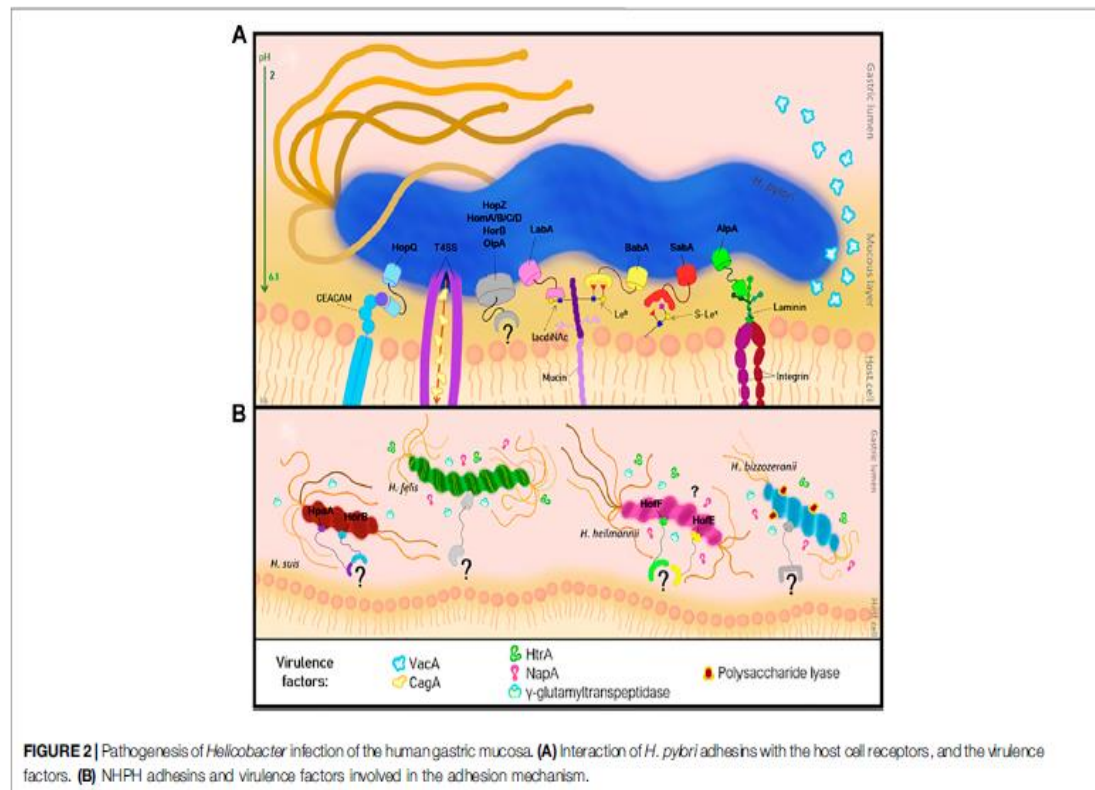


TABLE 1 | *Helicobacter pylori* adhesins and their described glycan ligands.

Adhesin	Receptors identified	Localization	Reference
BabA	Lewis b blood group antigen (LeB) and terminal fucose, H1-antigen, A-antigen and B-antigen	Gastric epithelium	Borén et al. (1993); Ilver et al. (1998); Benktander et al. (2012); McGuckin et al. (2011)
SabA	sialyl-Lewis A blood group antigen (sLeA)	Gastric epithelium	Madhavi et al. (2002)
	sialyl-Lewis X blood group antigen (sLeX)	Gastric epithelium	Madhavi et al. (2002)
LabA	lactfINAc motif	Gastric epithelium	Rossez et al. (2014); Benktander et al. (2018)

(Ansari and Yamaoka, 2017; Mahdavi et al., 2013) (Table 1). The MUC5AC mucin is co-expressed with the LeB and LeA antigens by the gastric epithelial cells (Linden et al., 2008; Magalhaes et al., 2010; Ansari and Yamaoka, 2017). On the other hand, MUC6 mucin is co-expressed with the type 2 Lewis antigens (LeX and LeY) (Reis et al., 2000).

The binding mechanism of the *H. pylori* BabA adhesin to the carbohydrate receptors, more specifically to LeB, has been studied in detail. Recently, Moonens *et al.*, revealed the X-ray structures of representative specialist and generalist BabA isoforms (Moonens et al., 2016). This analyses demonstrated that the preference shift results from a single amino acid substitution in the carbohydrate binding domain, which is constituted by one conserved loop (CL2) and two diversity loops (DL1 and DL2) (Moonens et al., 2016). In addition, it was showed that the DL1 is

responsible for the specialist or generalist BabA binding: the specialist binds to the blood group O through the CL2 and DL2 loops, while the binding of generalists involves the CL2 and both DL1 and DL2 (Moonens et al., 2016). Furthermore, the biophysical properties and the strength of the binding of the *H. pylori* BabA adhesin to the LeB antigen was evaluated by atomic force microscopy (Parreira et al., 2014). Detailed analysis showed the presence of two different bond populations, suggesting that the binding follows a two-state model, to improve the adhesion efficiency and stability (Parreira et al., 2014). Additionally, Hage *et al.*, also evaluated the molecular aspects of the BabA-LeB interaction, showing that the binding involves two fucose residues (Fuc1 and Fuc4), two galactose residues (Gal2 and Gal5), one N-Acetylglucosamine residue (GlcNAc3) and one glucose residue (Glc6) (Hage N 2015).

Furthermore, the amino acids in the BabA adhesin involved in the binding to each LeB glycan residue were also identified (Hage et al., 2015).

H. pylori is very sensitive to the low pH of the gastric fluid, and the bacteria use unique strategies to survive in this harsh microenvironment (Ansari and Yamaoka, 2017; Gottesmann et al., 2020). The helical shape and the flagella, along with the urease production, favors *H. pylori* entrance and penetration within the gastric mucus, following a pH gradient (Gottesmann et al., 2020). Recently, it has been described that the binding of the BabA adhesin to its ligands is also pH responsive (Bugaytsova et al., 2017). Given the acidic gastric environment, the adherence of *H. pylori* and its strength linkage to the ABO glycan antigens have been shown to be crucial for the survival in this unique environment (Bugaytsova et al., 2017). Under low pH, the linkage is suppressed, and the bacteria are released and relocated into a more neutral microenvironment, escaping to physiological events such as the shedding of epithelial cells and mucus turnover (Bugaytsova et al., 2017).

The non-secretor phenotype is characterized by the absence of ABO (H) antigens on gastric epithelial cells, which express only simple fucosylated Lewis structures (Heggelund et al., 2017). In these individuals, the colonization by *H. pylori* is mediated by the interaction between SabA and the sialylated glycans (Benktander et al., 2018), suggesting that the BabA-LeB interaction is important but not essential for colonization. However, it has been demonstrated that infection with *H. pylori* BabA-positive strains, as well the secretor status of the individual are associated with increased risk for development of peptic ulcer disease in Western countries, where the secretor phenotype prevails (Ansari and Yamaoka, 2017).

The majority of the bacteria are located in the gastric pits and in the mucus, and only a small amount colonizes deeper portions and are attached to gastric cells (Clyne and May, 2019). The specific localization of *H. pylori* has been widely investigated and some hypotheses have been formulated, based on the glycans expressed on this region. The deeper glands are characterized by the expression of MUC6 and α 1, 4-GlcNAc residues, which are attached to core 2-branched O-glycans (Kawakubo et al., 2004; Ferreira et al., 2006). *In vitro* studies have demonstrated that the biosynthesis of a major *H. pylori* cell wall component can be inhibited by these α 1, 4-GlcNAc-capped O-glycans, which can preclude bacterial growth, and function as a natural antibiotic (Kawakubo et al., 2004).

Colonization of the human gastric mucosa by *H. pylori* results in inflammation, which is accompanied by a remodeling of the glycosylation profile, with *de novo* expression of α 2, 3-sialylated glycans, namely sialyl-Lewis A (sLeA) and sialyl-Lewis X (sLeX) (Mahdavi et al., 2002). These sialylated structures are recognized by SabA adhesin, which constitutes a second point of attachment to the host gastric mucosa and is important for the colonization process (Mahdavi et al., 2002; Linden et al., 2008; Magalhaes et al., 2015; Benktander et al., 2018) (Table 1).

An additional adhesin was suggested to play a role in *H. pylori* interaction with the human gastric mucosa: the lacdiNAc-

binding adhesin (LabA) (Rossez et al., 2014; Benktander et al., 2018). This adhesin was first described as binding specifically to the lacdiNAc motif on MUC5AC mucins (Rossez et al., 2014). Recently, structural analysis of LabA suggested that the lacdiNAc motif might not be the ligand for this adhesin (Mthembu et al., 2020; Paraskevopoulou et al., 2021). Thus, the role of LabA and the binding specificity to lacdiNAc residues remains unclear and further studies are needed to elucidate this interaction (Mthembu et al., 2020; Paraskevopoulou et al., 2021).

The efficacy of the conventional *H. pylori* eradication therapy, usually a combination of 2-3 antibiotics with an acid-suppressive drug (Chey et al., 2017), has been declining in the last years, and the WHO considered priority the search for new drugs and alternative therapies (De et al., 2009; Blaecher et al., 2013). One of the major problems has been the acquisition of antimicrobial resistance, not only for *H. pylori*, but also for some NHPH such as *H. heilmannii*, *H. ailurogastricus*, *H. felis* and *H. suis* (Van den Bulck et al., 2005; Berlamont et al., 2019; Matos et al., 2020). The analysis of the antimicrobial susceptibility pattern is important to better understand the factors underneath the increasing resistance and to improve the available treatment.

On the other hand, since the search of new anti-adhesion drugs is urgent, the BabA mediated adhesion and the pH-responsive detachment and reattachment has been the aim of several anti-*H. pylori* adhesion studies. The first investigation were based on the anti-adhesive properties of the 3'-sialyllactose (Mysore et al., 1999; Parente et al., 2003), but this molecule was unable to prevent bacterial colonization. Additionally, several other molecules have been evaluated, including polyphenols (Shmueli et al., 2004; Hensel et al., 2007; Wittschier et al., 2007; Niehues et al., 2010), peptides (Niehues et al., 2010) and polysaccharides (Lengsfeld et al., 2007; Wittschier et al., 2009; Messing et al., 2014). The anti-adhesive properties of these molecules have been widely demonstrated however, it is not clear which bacterial OMPs are being targeted by each compound and which interactions are being blocked (Gottesmann et al., 2020). Besides these, another interesting approach is the use of glycans and their characteristics to produce antiadhesion microspheres, namely chitosan-microspheres (Gonçalves et al., 2013; Henriques et al., 2020) and glycan-coated microspheres (Gonçalves et al., 2016) to impair *H. pylori* colonization.

ALTERNATIVE PLAYERS IN HELICOBACTER PYLORI ADHESION

The lipopolysaccharide (LPS) is a structural component present in the outer cell wall of Gram-negative bacteria and may serve as a docking to the host cell (Gottesmann et al., 2020). The toxicity of the LPS from *H. pylori* is low compared to that of several other Gram-negative bacteria (Monteiro et al., 1998). One of the major components of the LPS is the O antigen composed of repeating units of sugars (Van Amersfoort et al., 2003). Biochemical analysis demonstrated that O antigen of *H.*

pylori is homologous to the Lewis blood group antigens H-type 1, LeA, LeX and LeY (Monteiro et al., 1998; Altman et al., 2005). The expression of these structures in the bacterial surface mimics the glycans expressed by the gastric epithelial cells, which might be helpful in colonization (Altman et al., 2005). Furthermore, it has been suggested that the *H. pylori* O-antigen sidechains can induce the expression of LeX antigen, which may promote the adhesion of the bacteria to the gastric epithelial cells (Edwards et al., 2000; Mahdavi et al., 2003). However, the exact role of this antigen in the colonization process remains unknown (Mahdavi et al., 2003).

H. pylori also possess a type IV secretion system, which is helpful for the adhesion and fixation on the host cell surface (Chmiela and Kupcinskas 2019). The BabA-LeB interaction can also help in the bacterial fixation to the cell membrane, which will allow the transfer of bacterial virulence factors into the host cells (Gottesmann et al., 2020).

The *H. pylori* adhesion to the human gastric mucosa through BabA and SabA adhesins is well-characterized (Jin et al., 2018). Detailed analysis of the glycoconjugates present in the human stomach, from individuals with different blood groups, showed a high degree of structural complexity. Furthermore, the composition of glycosphingolipids composition seems to be different according to the blood group, being the type 2 chains the dominant core chain (Jin et al., 2018). Interestingly, it was demonstrated that *H. pylori* did not bind to the O (Rh-) P blood group, whose major complex glycosphingolipids are the LeX, LeA and H-type 2 pentaosylceramides and LeY hexaosylceramides (Jin et al., 2018). In the other blood groups, glycosphingolipids present on the stomach of these individuals are able to bind to several *H. pylori* compounds (Jin et al., 2018).

NON-HELICOBACTER PYLORI HELICOBACTER (NHPH) ADHESION: WHAT IS THE MECHANISM AND WHICH MOLECULES ARE INVOLVED?

H. pylori is the most prevalent *Helicobacter* species in the human stomach, however other gastric, spiral-shaped NHPH have also been associated with the development of gastric disorders in humans (Baele et al., 2008; Ali et al., 2018; Matos et al., 2020). NHPH have been detected in 0.2–6% of the human gastric biopsies, usually accompanied by gastritis, antral erosions, duodenal ulcers and gastric MALT lymphoma (Morgner et al., 2000; Haesebrouck et al., 2009; Joosten et al., 2016; Ali et al., 2018). So far, *H. suis*, *H. felis*, *H. bizzozeronii*, *H. salomonis*, and *H. heilmannii* are the NHPH species detected in the human stomach (Boyanova et al., 2003; Šýkora et al., 2003; Haesebrouck et al., 2009; Yakoob et al., 2012). *H. suis* is mainly associated with pigs and non-human primates, whereas the other gastric NHPH preferentially colonize the canine and feline stomach. However, occasionally they can cross the host species barrier and infect humans (Haesebrouck et al., 2009; Amorim et al., 2014; Bento-Miranda and Figueiredo, 2014; Liu et al., 2015). Compared to the

natural hosts, NHPH colonization of the human stomach is lower and more focal (Haesebrouck et al., 2009).

Detailed analysis demonstrated that NHPH species lack major *H. pylori* adhesins described so far, such as BabA, SabA or AlpA/B (De Witte et al., 2016; Padra et al., 2018). The genome of *H. bizzozeronii*, *H. felis*, *H. salomonis* and *H. heilmannii* encodes several OMPs but lacks genes encoding the Bab and Sab adhesins (Arnold et al., 2011; Schott et al. 2011a; Smet et al., 2013). Furthermore, the *alpA* and *alpB* genes are not present in *H. felis*, *H. heilmannii* and *H. bizzozeronii* (Odenbreit et al., 1999; Schott et al., 2011b). Even without the main molecules used by *H. pylori* for colonization, the NHPH species are still able to colonize the stomach from both humans and animals. So the question is: which are the adhesion molecules used by NHPH? The answer is not clear, and the full mechanisms and the intervenients remain to be elucidated.

Biochemical studies on *H. heilmannii* showed the expression of the OMPs HofE and HofF, which were identified as adhesins (Cheng et al., 2016) (Figure 2). These OMPs are involved in binding of this bacterium to the gastric mucosa. They present higher affinity to gastric epithelial cells than mucins, and they are essential for the activation of IL-1 β which on its turn induces MUC13 expression on the gastric epithelium (Cheng et al., 2016; De Witte et al., 2016). *H. suis* is the most prevalent NHPH found in the human stomach (Haesebrouck et al., 2009; Padra et al., 2018) and a remarkable high prevalence of *H. suis* DNA (i.e. 27%) was detected in gastric biopsies from human patients with idiopathic parkinsonism (Blaeche et al., 2013). Like the other gastric NHPH, *H. suis* does not express major adhesins involved in colonization of the human stomach by *H. pylori*. However, it expresses some *H. pylori* similar OMPs, namely HofB and HpaA (*H. pylori* adhesin A) (Padra et al., 2018) (Figure 2). *In vitro* studies demonstrated that the porcine stomach expresses mucins at the surface epithelium, that resemble to human MUC5AC and MUC6 mucins and may constitute a binding site for *H. suis* (Padra et al., 2018). However, the identity of these mucins, the glycans and the specific *H. suis* adhesins are not known yet (Padra et al., 2018). Structural analysis of the porcine gastric mucins showed that these are large, oligomeric and highly glycosylated structures, similar to the human mucins (Aspholm et al., 2006). The major difference was at mucins sulfation level, that is very frequent among porcine mucins, but virtually absent in mucins of a healthy human stomach (Padra et al., 2018). This difference might help to explain the different colonization pattern of *H. suis* in the human and porcine stomach. Compared to the porcine stomach, colonization of the human stomach is indeed more limited and much more focal (Haesebrouck et al., 2009). Similar to the human stomach, a pH gradient across the mucus layer is observed in the porcine stomach, ranging from acidic in the lumen to neutral at the epithelial surface (Padra et al., 2018). Additionally, *in vitro* studies demonstrated that *H. suis* is able to bind to Gal β 3GlcNAc β 4Glc glycoconjugates at neutral and acidic pH and binds to negatively charged glycans at acidic pH (Padra et al., 2018). Furthermore, it was observed that Gal β 3GlcNAc β 3Gal β 4Glc structures can inhibit the binding of *H. suis* to mucins (Padra et al., 2018).

H. suis and *H. heilmannii* are two NHPH species that have been widely studied and recently their *in vitro* adhesion ability to human gastric cell lines was evaluated (Berlamont et al., 2020). Interestingly, both *Helicobacter* species were able to bind to the gastric epithelial cells *in vitro*, but detailed analysis showed that frequently two genes were up-regulated and four genes were down-regulated in both pathogens (Berlamont et al., 2020). BLASTp analysis demonstrated that the genes differentially expressed by *H. suis* and *H. heilmannii* belong to several functional classes, which suggests that the *in vitro* adhesion of both strains to human gastric cells demands pleiotropic adaptive responses (Berlamont et al., 2020). However, the clinical significance of these findings and the complete pathway involved in the adhesion process needs to be better elucidated (Berlamont et al., 2020).

The canine gastric mucosa is one of the preferential niches for *H. felis*, *H. bizzozeronii*, *H. heilmannii* and, less often, *H. salomonis* (Amorim et al., 2014). Analysis of the canine glycophenotype showed that the canine gastric mucosa is characterized by absence of LeB and sLeA antigens expression and a slight expression of LeA (Amorim et al., 2014). The minor expression of type 1 Lewis antigens could be explained by the lack of α 1, 4-fucosyltransferase activity in canine gastric epithelial cells (Kulhlmann et al., 1983; Amorim et al., 2014). On the other hand, type 2 Lewis antigens, mostly LeX and LeY are strongly expressed at the canine gastric superficial and foveolar epithelium (Amorim et al., 2014). Furthermore, it was demonstrated that the dog orthologous of human *FUT3* and *FUT5* genes present an arginine residue, which is compatible to the α 1, 3-fucosyltransferase activity, instead of the α 1, 4-fucosyltransferase observed in humans, further resulting in type 2 Lewis antigens biosynthesis by the dog gastric epithelial cells (Kulhlmann et al., 1983; Amorim et al., 2014). The different glycophenotype, together with the absence of BabA homologs, could explain the low infection rate by canine NHPH in humans, and concomitantly, the very rare colonization of dogs with *H. pylori*.

Both *H. pylori* and NHPH share virulence factors involved in epithelial cell death such as the γ -glutamyl transpeptidase (Haesebrouck et al., 2009). In addition, genes encoding homologues of other *H. pylori* virulence factors such as the secreted serine protease HtrA and the immunomodulator NapA are present in *H. felis*, *H. bizzozeronii* and *H. heilmannii* s.s. genomes. In *H. bizzozeronii* a polysaccharide lyase was regarded as a potential new virulence factor (Schott et al., 2011a). Later Kondadi et al. (Kondadi et al., 2012), characterized a novel lipopolysaccharide α 2, 3-sialyltransferase from *H. bizzozeronii* that showed a preference for N-acetyllactosamine as a substrate. The authors showed that the expression of a terminal 3'sialyl-LacNAc on LPS is a phase-variable characteristic of both human- and canine derived *H. bizzozeronii* strains. Moreover, the authors suggest that this sialylated structure can mimic the surface glycans of the host mammalian cells (Kondadi et al., 2012).

FINAL REMARKS

H. pylori infects half of the worldwide population, being associated with several gastric diseases. The interaction between *H. pylori* and the human gastric mucosa has been well characterized, and several intervenients have been described. The adhesion of *H. pylori* through the interaction of the BabA adhesin to both LeB and H-type 1 glycans, expressed by the gastric epithelial cells of healthy secretors, results in the initiation of an inflammatory response, with *de novo* synthesis of sialylated structures, such as sLeA and sLeX. These are carbohydrate antigens that are recognized by the SabA adhesin expressed at the bacterial surface. Gastric NHPH that are primary associated with pigs, dogs, and cats, may also colonize the human stomach resulting in gastric disorders. However, the precise adhesion mechanisms of NHPH, including the adhesins and ligands responsible for the colonization, are almost unknown. Further studies, including the screening of NHPH OMPs using glycan arrays will be very helpful in the identification of the players in adhesion, improving and contributing to a thorough knowledge about human NHPH infection.

AUTHOR CONTRIBUTIONS

Conceptualization, CR and RM; writing-original draft preparation, RM; writing-review and editing, RM, AM, IA, FH, FG, and CR; supervision—CR. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was supported by FEDER funds through the Operational Programme for Competitiveness Factors—COMPETE (POCI-01-0145-FEDER-016585; POCI-01-0145-FEDER-007274); National Funds through the Foundation for Science and Technology (FCT), under the projects: PTDC/BBB-EBI/0567/2014 (to CR) and UID/BIM/04293/2013; RM is funded by Fundação para a Ciência e Tecnologia/Ministério da Ciência, Tecnologia e Inovação (FCT) PhD scholarship SFRH/BD/131159/2017. We also acknowledge the COST Action CA18103 INNOGLY: INNOvation with Glycans new frontiers from synthesis to new biological targets.

ACKNOWLEDGMENTS

The authors appreciate the contribution of Mário Jorge Ribeiro (ICBAS-UP, Portugal), for the help on the illustration of Figure 2.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Antimicrobial Susceptibility
Pattern of gastric *Helicobacter*
species**

2 CHAPTER

Paper II

Antimicrobial Susceptibility Pattern of *Helicobacter heilmannii*
and *Helicobacter ailurogastricus* isolates

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Microorganisms

2020

doi:10.3390/microorganisms8060957



Article

Antimicrobial Susceptibility Pattern of *Helicobacter heilmannii* and *Helicobacter ailurogastricus* Isolates

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Received: 8 May 2020; Accepted: 23 June 2020; Published: 25 June 2020



Abstract: A combined agar and broth dilution method followed by qPCR was used to determine the antimicrobial susceptibility of feline *H. heilmannii* and *H. ailurogastricus* isolates. All *H. ailurogastricus* isolates showed a monomodal distribution of MICs for all the antimicrobial agents tested. For *H. heilmannii*, a bimodal distribution was observed for azithromycin, enrofloxacin, spectinomycin, and lincomycin. Single nucleotide polymorphisms (SNPs) were found in 50S ribosomal proteins L2 and L3 of the *H. heilmannii* isolate not belonging to the WT population for azithromycin, and in 30S ribosomal proteins S1, S7, and S12 of the isolate not belonging to the WT population for spectinomycin. The antimicrobial resistance mechanism to enrofloxacin and lincomycin remains unknown (2 and 1 *H. heilmannii* isolate(s), resp.). Furthermore, *H. heilmannii* isolates showed higher MICs for neomycin compared to *H. ailurogastricus* isolates which may be related to the presence of SNPs in several 30S and 50S ribosomal protein encoding genes and ribosomal RNA methyltransferase genes. This study shows that acquired resistance to azithromycin, spectinomycin, enrofloxacin, and lincomycin occasionally occurs in feline *H. heilmannii* isolates. As pets may constitute a source of infection for humans, this should be kept in mind when dealing with a human patient infected with *H. heilmannii*.

Keywords: *Helicobacter heilmannii*; *Helicobacter ailurogastricus*; antimicrobial susceptibility; zoonoses; gastric disease; resistance mechanisms

1. Introduction

Helicobacter species colonize the gastrointestinal tract of both humans and animals which may result in the development of gastrointestinal disorders such as inflammation, ulceration, and cancer. *Helicobacter pylori* is the best-studied and most prevalent *Helicobacter* species colonizing the stomach of human patients with gastric disorders [1–6]. Other gastric, spiral-shaped non-*H. pylori* helicobacters (NHPHs) have also been associated with the development of gastric disorders in humans [1,7–9]. So far, detected NHPHs in the human stomach are *H. suis*, *H. felis*, *H. bizzozeronii*, *H. salomonis*, and *H. heilmannii* [1,10–14]. *Helicobacter suis* naturally colonizes the stomach of pigs and non-human primates,

while the others are associated with a wide range of canine and feline species. Living in close proximity as well as intense contact with infected animals are potential risk factors for humans to contract NHPH infection. The zoonotic potential of other gastric NHPHs, such as *H. cynogastricus*, *H. baculiformis*, *H. ailurogastricus*, *H. acinonychis*, *H. cetorum* and *H. mustelae*, is currently unknown.

Helicobacter heilmannii naturally colonizes the gastric mucosa of dogs and cats, with prevalence rates varying from 20% up to 100% [15–17]. Although infection has been associated with chronic active gastritis, peptic ulceration and chronic vomiting in dogs and cats, its pathogenic significance for these animal species is unclear and may be strain dependent [5,9,10,18–24]. In humans, *H. heilmannii* has been detected in 8–19% of gastric biopsies with histological evidence of NHPH infection [1,12,13,25]. In general, NHPH infections in human patients have been associated with gastritis, peptic ulcers, and mucosa-associated lymphoid tissue lymphoma (MALT) [6,23,25].

More recently, a new feline gastric *Helicobacter* species closely related to *H. heilmannii* has been described, namely, *H. ailurogastricus* [6]. Both species are phenotypically identical and cannot be distinguished by means of 16S rRNA and *ureAB* gene sequencing [6]. Although both species are morphologically very similar, *H. ailurogastricus* bacteria may be slightly smaller (3–5.5 µm length; 0.5–0.7 µm width) compared to *H. heilmannii* (3–6.5 µm length; 0.6–0.7 µm width) [6]. Both species present bipolar flagella and absence of periplasmic fibrils [6]. Biochemical analysis showed that the two species present urease activity, nitrate production, and both are able to hydrolyze indoxyl acetate, but only *H. ailurogastricus* shows alkaline phosphatase activity [6]. In vitro binding assays have shown that *H. ailurogastricus* has a lower capacity for binding to human- and murine-derived gastric epithelial cells than *H. heilmannii* which may explain why its virulence is lower than that of *H. heilmannii* [6]. Nevertheless, due to the fact of its recent discovery, the prevalence and pathogenic significance of *H. ailurogastricus* for animals and humans remains to be investigated.

Antimicrobial treatment of gastric NHPH infections in humans is based on clinical experience and mostly, treatment schemes applied for eradicating *H. pylori* are used [19]. *Helicobacter pylori* eradication treatment consists of 2–3 antibiotics (e.g., clarithromycin, amoxicillin, metronidazole, tetracycline, and/or levofloxacin) combined with an acid-suppressive drug (e.g., proton pump inhibitor-PPI or H₂-receptor antagonists) [26]. However, not much is known on the antimicrobial susceptibility pattern of gastric NHPH species.

For some NHPH species, such as *H. suis*, *H. heilmannii*, and *H. ailurogastricus*, standard antimicrobial susceptibility assays are unsuitable since they only grow in an enriched biphasic medium with an acidic pH [6,8]. Therefore, Vermoote et al. [27] developed a combined agar and broth dilution method to analyze the antimicrobial susceptibility pattern of nine porcine *H. suis* isolates. More recently, the antimicrobial susceptibility of a larger collection of *H. suis* isolates obtained from pigs and non-human primates was investigated and acquired resistance to fluoroquinolones, spectinomycin, lincomycin, and tetracycline was shown [28]. So far, antimicrobial susceptibility testing has not been performed for *H. heilmannii* and *H. ailurogastricus*. These are very fastidious microorganisms which are difficult to cultivate. Therefore, only a limited number of isolates is available.

The aim of this study was to investigate the intrinsic susceptibility of *H. heilmannii* and *H. ailurogastricus* and the presence and mechanisms of acquired resistance in a collection of isolates obtained from the gastric mucosa of cats. This may eventually help to improve the management of infection with these bacteria in both humans and animals.

2. Materials and Methods

2.1. Minimal Inhibitory Concentration (MIC) Determination of *H. heilmannii* and *H. ailurogastricus* Isolates

Seven *H. heilmannii* (i.e., strains ASB1.4, ASB2.1, ASB3.2, ASB6.3, ASB14.1, ASB19, and ASB20) and six *H. ailurogastricus* (i.e., strains ASB7.1, ASB9.4, ASB11.2, ASB13.1, ASB21, and ASB23) isolates were included in this study. All strains were isolated by Smet et al. [17] and Joosten et al. [6] from the gastric mucosa of cats humanely euthanized at different animal shelters in Belgium or at the Faculty

of Veterinary Medicine (Merelbeke, Belgium). Strains ASB2.1 and ASB3.2 were isolated from cats positive for feline immunodeficiency virus. No information on the health status of the other cats could be obtained, as they were “street cats” (*Felix vulgaris*) originating from different animal shelters. Whole genome sequence typing already demonstrated that all isolates were genetically different [6]. *Helicobacter heilmannii* and *H. ailurogastricus* isolates were cultivated according to the method described for *H. suis* by Berlamont et al. [28]. In brief, bacteria were grown using a biphasic medium consisting of Brucella agar (BD, Franklin Lakes, New Jersey, USA) supplemented with 20% inactivated fetal calf serum (Hyclone, ThermoFisher Scientific, USA), Vitox supplement (Oxoid, UK), and *Campylobacter* selective supplement (Skirrow, Oxoid, UK), with Brucella broth (Oxoid) added on top. Both agar and broth were supplemented with 0.05% HCl to obtain a pH of 5. Bacteria were incubated under microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂) at 37 °C. The concentration of viable bacteria was determined using a Neubauer improved counting chamber (Sigma–Aldrich).

The susceptibility of *H. heilmannii* and *H. ailurogastricus* to aminoglycosides (i.e., gentamicin and neomycin), β -lactam antibiotics (i.e., ampicillin and ceftiofur), fluoroquinolones (i.e., enrofloxacin and levofloxacin), lincomycin, macrolides (i.e., clarithromycin, azithromycin, and tylosin), metronidazole, rifampicin, spectinomycin, and tetracyclines (i.e., oxytetracycline and doxycycline) was determined using a combined agar and broth dilution method, according to the method described by Berlamont et al. [28]. The antimicrobial agents were purchased as standards powders from Sigma–Aldrich and were dissolved and diluted according to the Clinical & Laboratory Standards Institute (CLSI) guidelines. Serial two-fold dilutions of each antimicrobial agent were freshly prepared, with concentrations ranging from 0.003 to 128 μ g/mL. *Helicobacter heilmannii* and *H. ailurogastricus* isolates were harvested and 150 μ L of broth containing 5×10^7 viable bacteria/mL was added to each corresponding well. Positive (i.e., wells containing agar, broth, and bacteria but without antimicrobial compound) and negative (i.e., wells without bacteria and antimicrobial compound) controls were included. All the concentrations and isolates were tested in duplicate. All the plates were incubated for 48 h under microaerophilic conditions at 37 °C. Broth containing *Helicobacter* spp. was collected from each well (i.e., approximately 100 μ L) and bacterial DNA was extracted using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, CA, USA), according to the manufacturer’s instructions. Minimal inhibitory concentrations (MICs) were determined by software-assisted calculation of bacterial growth as determined by quantitative real-time PCR based on the *ureA* gene [21]. Similar to *H. suis*, the MIC was determined as the lowest concentration of the antimicrobial agent for which Δ Ct was at least 1 Ct higher than Δ Ct (Ct after incubation – Ct before incubation of the antimicrobial agent exposed strains; Δ Ct = Ct after incubation – Ct before incubation of the controls; Ct = threshold cycle value) [20,25]. In other words, the MIC is the lowest concentration of the antimicrobial agent with at least 50% less bacterial growth [21,27,28].

The potency of the antimicrobials and the primers used for qPCR detection are listed in Tables S1 and S2, respectively.

2.2. Minimal Inhibitory Concentration Determination of Reference Strains

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as reference strains. Both species were grown overnight and fresh suspensions with a density of 0.5 McFarland standard were prepared. The distribution of MIC values for both strains was determined using two different MIC assays as described by Berlamont et al. [28]. In brief, antimicrobial susceptibility patterns of both reference strains were determined using the combined agar and broth dilution method with a pH of 5 (assay (1)) as well as the broth microdilution method according to the CLSI standards at pH 7 (assay (2)). For assay (1), *E. coli* and *S. aureus* were cultured in the biphasic conditions at pH 5, as described for *H. heilmannii* and *H. ailurogastricus*. For assay (2), unsupplemented Mueller–Hinton broth at pH 7 was used according to the CLSI guidelines. Additionally, positive (i.e., wells containing agar, broth, and bacteria but free of the tested antimicrobials) and negative (i.e., wells containing agar and broth free of the tested antimicrobials and reference bacteria) controls were included. All samples were tested

in duplicate. Plates were incubated for 16–20 h under aerobic conditions at 37 °C. The presence of bacterial growth was evaluated by the presence of turbidity, and the value was defined as the lowest concentration for which turbidity was visually absent.

2.3. *Helicobacter* Resistance Mechanisms to Antimicrobial Agents

Genome sequences of all tested *H. heilmannii* and *H. ailurogastricus* isolates are available from the ftp NCBI database (Table S3). As described by Berlamont et al. [28], multiple tools were used to screen these genomes for the presence of acquired antimicrobial resistance genes, namely: ABRicate [29] Resfinder [30] ARG-ANNOT [31], The Comprehensive Antibiotic Resistance Database (CARD) [32], NCBI Bacterial Antimicrobial Resistance Reference Gene Databases [33], EcoOH [34], PlasmidFinder [35], and Virulence Factors Database (VFDB) [36]. In addition, the presence of point mutations in specific gene sequences from *H. heilmannii* and *H. ailurogastricus* isolates (not) belonging to the wild-type population [37] were screened manually using Clustal Omega [38], PredictSNP [39], and ConSurf [28,40]. Single nucleotide polymorphisms (SNPs) were only considered relevant if they were detected in all isolates not belonging to the wild-type population for a certain antimicrobial agent, while absent in all isolates belonging to the wild-type population for that antimicrobial agent.

2.4. Ethical Statement

Procedures for strain isolation were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium (approval number EC2011/090).

2.5. Statistical Analysis

Average and standard deviation were calculated for each antimicrobial agent for all the *H. heilmannii* and *H. ailurogastricus* strains belonging to the wild-type population. The MIC pattern of *H. heilmannii* isolates was compared to the *H. ailurogastricus* isolates, using a two-way ANOVA test (Sidak's test). Differences were considered statistically significant at a *p*-value of 0.05. GraphPad Prism 7 software was used for the analysis.

3. Results

3.1. Antimicrobial Activity at Different pH

Two different MIC assays were performed to determine the impact of culture and pH conditions. When using the combined agar and broth dilution method at pH 5 on the *E. coli* and *S. aureus* reference strains, the MIC values of spectinomycin were two times, 2-fold dilutions; for azithromycin, clarithromycin, enrofloxacin, and lincomycin three times, 2-fold dilutions; for tylosin and levofloxacin four times, 2-fold dilutions; and for gentamicin and neomycin six times, 2-fold dilutions higher than the highest value of the acceptable quality ranges of the CLSI standards. On the other hand, MIC values of ceftiofur and ampicillin were one, 2-fold dilution lower than the lowest value of the acceptable quality ranges of the CLSI standards when using the combined agar and broth dilution method at pH 5 [41]. Minimal inhibitory concentration (MIC) endpoints of doxycycline, tetracycline, metronidazole, and rifampicin fell within the acceptable quality ranges when using the combined agar and broth method at pH 5.

When using the broth microdilution method according to the CLSI guidelines, at pH 7, on both reference strains, the MIC endpoints of all antimicrobial agents fell within the acceptable quality control ranges [41].

3.2. Minimal Inhibitory Concentration Pattern of *H. heilmannii* and *H. ailurogastricus*

All positive control plates showed bacterial growth and all negative controls did not show bacterial growth, indicating that all experiment conditions were performed correctly.

The distribution of MIC values for all *H. heilmannii* and *H. ailurogastricus* isolates is shown in Table 1 and Figure 1. Average and standard deviation of *H. heilmannii* and *H. ailurogastricus* isolates for each antimicrobial agent are presented in Table S4. All *H. ailurogastricus* isolates showed a monomodal distribution for all the antimicrobial agents tested. For the *H. heilmannii* isolates, a monomodal distribution of MICs was observed for azithromycin, β -lactam antibiotics (i.e., ampicillin and ceftiofur), clarithromycin, gentamicin, neomycin, nitroimidazole antibiotics (i.e., metronidazole), levofloxacin, rifamycins (i.e., rifampicin), tetracyclines (i.e., oxytetracycline and doxycycline) and tylosin. Interestingly, a bimodal distribution was seen for azithromycin (i.e., strain ASB19), enrofloxacin (i.e., strains ASB14.1 and ASB20), lincomycin (i.e., strain ASB6.3), and spectinomycin (i.e., strain ASB1.4). The presence of a bimodal distribution indicates that the isolates showing a higher MIC range did not belong to the wild-type population for these antimicrobial agents.

Differences in the distribution of MIC values between *H. heilmannii* and *H. ailurogastricus* were observed for lincomycin and neomycin. For lincomycin, higher MIC values were observed for *H. ailurogastricus* isolates compared to *H. heilmannii*. In more detail, for *H. ailurogastricus*, the MIC values for lincomycin varied between 4–16 $\mu\text{g/mL}$ ($9.33 \pm 5.47 \mu\text{g/mL}$), while for *H. heilmannii*, the MIC values varied between 0.5–2 $\mu\text{g/mL}$ ($1.25 \pm 0.61 \mu\text{g/mL}$) with the exception of *H. heilmannii* ASB6.3 showing a high MIC value of 32 $\mu\text{g/mL}$ (Table 1 and Table S4). Compared to *H. heilmannii* isolates, *H. ailurogastricus* isolates showed lower MIC values. More specifically, for *H. heilmannii*, the MIC values for neomycin varied between 2–32 $\mu\text{g/mL}$ ($13.43 \pm 10.11 \mu\text{g/mL}$), while for *H. ailurogastricus* the MIC values varied between 0.5–2 $\mu\text{g/mL}$ ($1.33 \pm 0.75 \mu\text{g/mL}$) (Table 1 and Table S4). For isolates belonging to the wild-type population, no significant differences were detected between both species for all the antimicrobial agents tested (Figure 1 and Table S4).

Table 1. Detailed overview of MIC distribution of seven *H. heilmannii* and six *H. ailurogastricus* isolates. Feline *H. heilmannii* isolates: ASB1.4, ASB2.1, ASB3.2, ASB6.3, ASB14.1, ASB19, and ASB20. Feline *H. ailurogastricus* isolates: ASB7.1, ASB9.4, ASB11.2, ASB13.1, ASB21, and ASB23. *H. heilmannii* isolates showing acquired antimicrobial resistance are indicated in bold and red.

Group	Antimicrobial Agent	Isolates with a MIC (µg/mL) of													
		≤0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
β-lactams	Ampicillin			ASB3.2 ASB13.1	ASB1.4 ASB6.3 ASB23	ASB2.1 ASB14.1 ASB20 ASB11.2	ASB19 ASB7.1 ASB9.4 ASB21								
	Ceftiofur					ASB3.2	ASB20	ASB2.1 ASB19	ASB1.4 ASB6.3 ASB7.1 ASB9.4 ASB11.2 ASB13.1	ASB14.1 ASB23	ASB21				
Macrolides	Clarithromycin	ASB2.1	ASB3.2 ASB6.3 ASB21	ASB1.4 ASB14.1 ASB19 ASB7.1 ASB11.2 ASB13.1 ASB23	ASB20 ASB9.4										
	Tylosin					ASB20	ASB19 ASB11.2	ASB3.2 ASB21	ASB2.1 ASB6.3 ASB14.1 ASB7.1 ASB9.4 ASB13.1 ASB23	ASB1.4					
	Azithromycin	ASB2.1 ASB3.2 ASB6.3 ASB7.1 ASB9.4 ASB11.2	ASB20 ASB13.1 ASB23	ASB1.4 ASB21	ASB14.1				ASB19						

Table 1. Cont.

Group	Antimicrobial Agent	Isolates with a MIC (µg/mL) of													
		≤0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Lincosamides	Lincomycin					ASB1.4	ASB2.1 ASB3.2 ASB19	ASB14.1 ASB20	ASB7.1 ASB23	ASB13.1 ASB21	ASB9.4 ASB11.2	ASB6.3			
Quinolones	Enrofloxacin	ASB2.1 ASB23	ASB13.1	ASB1.4 ASB3.2 ASB19 ASB7.1 ASB11.2	ASB6.3 ASB9.4 ASB21				ASB14.1			ASB20			
	Levofloxacin	ASB1.4 ASB2.1 ASB3.2 ASB20 ASB7.1 ASB13.1 ASB21 ASB23	ASB19 ASB9.4 ASB11.2	ASB14.1	ASB6.3										
Rifamycins	Rifampicin	ASB2.1 ASB3.2 ASB6.3 ASB7.1 ASB11.2 ASB13.1	ASB1.4 ASB19 ASB20 ASB9.4 ASB21 ASB23	ASB14.1											
Aminoglycosides	Gentamicin							ASB2.1 ASB3.2 ASB14.1	ASB19 ASB7.1 ASB13.1 ASB21	ASB6.3 ASB20 ASB11.2 ASB23	ASB9.4	ASB1.4			
	Neomycin					ASB7.1 ASB9.4	ASB11.2	ASB2.1 ASB13.1 ASB21 ASB23	ASB14.1	ASB1.4	ASB3.2 ASB6.3 ASB20	ASB19			

Table 1. Cont.

Group	Antimicrobial Agent	Isolates with a MIC (µg/mL) of													
		≤0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Aminocyclitol	Spectinomycin	ASB2.1 ASB3.2	ASB7.1 ASB11.2 ASB13.1 ASB23	ASB6.3 ASB9.4	ASB20 ASB21	ASB19	ASB14.1				ASB1.4				
	Oxytetracycline	ASB3.2 ASB20 ASB7.1 ASB9.4 ASB13.1 ASB23	ASB1.4 ASB11.2	ASB2.1 ASB19 ASB21	ASB6.3 ASB14.1										
Tetracyclines	Doxycycline		ASB19 ASB21	ASB2.1 ASB3.2 ASB20 ASB7.1	ASB1.4 ASB6.3 ASB14.1 ASB9.4 ASB11.2 ASB13.1 ASB23										
	Metronidazole			ASB3.2 ASB23	ASB1.4 ASB2.1 ASB20 ASB7.1 ASB9.4 ASB13.1	ASB11.2 ASB21	ASB14.1	ASB6.3 ASB19							

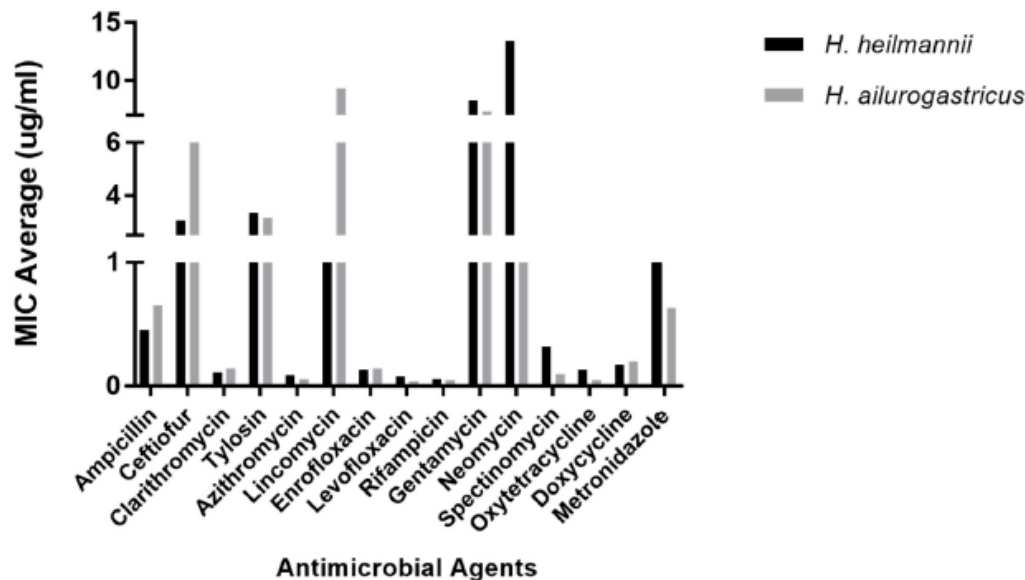


Figure 1. Graphical representation of distribution of MIC values of *H. heilmannii* and *H. ailurogastricus* isolates to the different tested antimicrobial agents. Statistical analysis was performed using the GraphPad software, but no significant differences were found between both species.

3.3. Antimicrobial Resistance Mechanisms Presented by *Helicobacter* species

No known acquired antimicrobial resistance genes were detected in the *H. heilmannii* and *H. ailurogastricus* isolates. Although point mutations in the 23S rRNA genes were associated with resistance of *H. pylori* and other pathogens to macrolides, none were found in the *H. heilmannii* isolate ASB19 not belonging to the wild-type population for azithromycin.

Specific point mutations in the *gyrA* and *gyrB* genes were associated with resistance of *H. pylori* and *Enterobacteriaceae* to fluoroquinolones. Nevertheless, no known point mutations were detected in *H. heilmannii* isolates ASB14.1 and ASB20 not belonging to the wild-type population for enrofloxacin.

Single nucleotide polymorphisms were found in two 50S ribosomal protein genes of the *H. heilmannii* isolate ASB19 not belonging to the wild-type population for azithromycin (i.e., MIC value of 4 µg/mL) which are shown in Table S5. For example, SNPs were found in the 50S ribosomal protein L3 (*RplC*) at codon 259 (CCT (proline) → TCT (serine)) and at codon 277 (CGC (arginine) → TGC (cysteine)). Using BLAST, various amino acids were present at these positions in other bacterial species and the ConSurf Server gave conservation scores below five. Nevertheless, cysteine was never present at codon 277 in other bacterial species. None of the PredictSNP tools showed that the amino acid substitution at codon 259 may affect *RplC* activity. Three PredictSNP tools—MAPP, PhD-SNP, and PolyPhen-1—indicated that the amino acid substitution at codon 277 may affect *RplC* activity with an average accuracy of 56%, while the other tools indicated a neutral effect with an average accuracy of 60%. Using the I-Mutant 3.0 tool, the amino acid substitution at both codon 259 and 277 were predicted to decrease protein stability (DDG: −1.31, RI: 8; DDG: −0.64, RI:0, resp.). Analysis of the SNP present in 50S ribosomal protein L2 gene (*RplB*) indicated that this amino acid substitution most likely did not affect protein activity and stability (Table S5).

Single nucleotide polymorphisms unique for *H. heilmannii* isolate ASB1.4, not belonging to the wild-type population for spectinomycin, were also found in four different genes which are shown in Table S5. For 30S ribosomal protein S1 gene (*RpsA*), a SNP was present at codon 523 (GAG (glutamate) → AAG (lysine)). Using BLAST and ConSurf, various amino acids were present at this position in other bacterial species, but never lysine. The Sorting Intolerant From Tolerant (SIFT) tool indicated that this amino acid substitution may affect the activity of *RpsA* with an accuracy of 46%, while the other tools predicted a neutral effect with an average accuracy of 76%. Using the I-Mutant 3.0 tool, the amino acid

substitution did not seem to affect protein stability (DDG: -0.25 , RI: 4). In addition, an SNP was found at codon 304 of the Ribosomal RNA small subunit methyltransferase D gene (*RsmD*) (ACC (threonine) $\rightarrow$ GCG (alanine)). The BLAST analysis showed the presence of various amino acids at this position in other bacterial species and the ConSurf Server gave a conservation score below five. The MAPP tool indicated that this amino acid substitution may affect the activity of *RsmD* with an accuracy of 41%, while the other tools predicted a neutral effect with an average accuracy of 75%. The I-Mutant 3.0 tool showed that this amino acid substitution may decrease protein stability (DDG: -1.13 , RI:9). Analysis of SNPs present in 30S ribosomal protein S12 (*RpsL*) and S7 (*RpsG*) genes indicated that these amino acid substitutions most likely did not affect protein activity and stability, Table S5).

Single nucleotide polymorphisms in ribosomal protein genes unique for *H. heilmannii* isolate ASB6.3, not belonging to the wild-type population for lincomycin, were not found.

3.4. Decreased Antimicrobial Susceptibility Mechanisms

Several SNPs were found in the 30S ribosomal protein genes of all *H. heilmannii* isolates showing high MIC values for neomycin, but not in *H. ailurogastricus* isolates showing low MIC values for this antimicrobial. The potential impact of these SNPs on protein activity and stability are shown in Table S5.

For example, an SNP was present at codon 1393 of the 30S ribosomal protein S1 gene (*RpsA*) (GAC (aspartate) $\rightarrow$ GGC (glycine)). Using BLAST, various amino acids were present at the same position in other bacterial species, and the ConSurf Server gave a conservation score of five. The PredictSNP, PhD-SNP, PolyPhen-1, PolyPhen-2, and SNAP tools indicated that this amino acid substitution may affect the activity of *RpsA* with an average accuracy of 52%, while the other tools predicted a neutral effect with an average accuracy of 68%. Using the I-Mutant 3.0 tool, this substitution was predicted to decrease protein stability (DDG: -0.94 , RI:2). Analysis of other SNPs present in the *RpsA* gene indicated that these amino acid substitutions could affect protein activity, although this was only observed for 1–3 PredictSNP tools, while the other tools indicated a neutral effect (Table S5).

Helicobacter heilmannii isolates also showed presence of a SNP in the 30S ribosomal protein S2 gene (*RpsB*) at codon 250, leading to an amino acid substitution from arginine (CGA) to glutamine (CAA). Using BLAST, various amino acids were present at these positions in other bacterial species and the ConSurf Server gave conservation score of 6. The PredictSNP, PolyPhen-1, PolyPhen-2, PhD-SNP, SIFT, SNAP, and PANTHER tools indicated that this amino acid substitution may affect the activity of *RpsB* with an average accuracy of 72%, while the MAPP tool predicted a neutral effect with an accuracy of 70%. Using the I-Mutant 3.0 tool, this substitution was predicted to decrease protein stability (DDG: -1.24 , RI: 9). Other SNPs were also found, but most likely did not impact protein activity and stability of *RpsB*.

Several SNPs were present in the ribosomal RNA small subunit methyltransferase D (*RsmD*), for example at codon 217 (TTT (phenylalanine) $\rightarrow$ GTG (valine)/ATT (isoleucine)), at codon 430 (CTA (leucine) $\rightarrow$ GCA (alanine)) and at codon 527 (AAG (lysine) $\rightarrow$ ACA (threonine)). Using BLAST, various amino acids were present at these position in other bacterial species, while the ConSurf Server gave conservation scores of at least seven. All the PredictSNP tools indicated that the amino acid substitution at codon 217 may affect the activity of *RsmD* with an average accuracy of 87%, while this was only indicated by 4 PredictSNP tools for the amino acid substitutions at codon 430 and 527. Using the I-Mutant 3.0 tool, the amino acid substitutions at codon 217, 430, and 527 were predicted to decrease the protein stability (DDG: -0.63 , RI: 5; DDG: -1.48 , RI: 8; DDG: -0.62 , RI: 5, resp.). Analysis of other SNPs present in the *RsmD* gene indicated that these amino acid substitutions most likely did not affect protein activity and stability (Table S5).

Helicobacter heilmannii isolates also showed presence of a SNP in the ribosomal RNA small subunit methyltransferase H (*RsmH*) at codon 265, resulting in an amino acid substitution from isoleucine (ATT) to proline (CCA). Using BLAST, various amino acids were present at this position in other bacterial species and the ConSurf Server gave a conservation score of six. The PredictSNP, MAPP, PhD-SNP,

SIFT, and PANTHER tools indicated that this amino acid substitution may affect the activity of *RsmH* with an average accuracy of 51%, while the other tools predicted a neutral effect with an average accuracy of 66%. Using the I-Mutant 3.0 tool, this amino acid substitution was predicted to decrease protein stability (DDG: −1.60, RI:6). Analysis of other SNPs present in the *RsmH* gene indicated that these amino acid substitutions could affect protein activity, although this was only observed for 1–3 PredictSNP tools, while the other tools indicated a neutral effect (Table S5).

An SNP was also present in the Ribosomal silencing factor (*RsfS*) gene at codon 25 (CTA (leucine) → ATG (methionine)). Using BLAST, various amino acids were present at this position in other bacterial species and the ConSurf Server gave a conservation score below five. The PredictSNP, MAPP, PolyPhen-1, PolyPhen-2, and SIFT tools indicated that this amino acid substitution may affect *RsfS* activity, with an average accuracy of 52%, while the other tools indicated a neutral effect with an average accuracy of 75%. Using the I-Mutant 3.0 tool, this amino acid substitution was predicted to decrease protein stability (DDG: −1.01, RI:9). Analysis of other SNPs present in the *RsfS* gene indicated that these amino acid substitutions most likely did not affect protein activity and stability (Table S5).

Finally, several SNPs ($n = 85$) were found in the 30S ribosomal protein S15 (*RpsO*), S16 (*RpsP*), S21 (*RpsU*), S6 (*RpsF*), S9 (*RpsI*), ribosomal protein S12 methyltransferase (*RimO*), ribosomal RNA large subunit methyltransferase H (*RlmH* group_1616) and ribosomal RNA small subunit methyltransferase I (*RsmI*) genes of all *H. heilmannii* isolates showing high MIC values for neomycin, but analysis indicated that these amino acid substitutions most likely did not impact protein activity and stability (Table S5). No SNPs were detected in the 30S ribosomal protein genes S10 (*RpsJ*), S11 (*RpsK*), S12 (*RpsL*), S13 (*RpsM*), S14 type Z (*RpsZ*), S17 (*RpsQ*), S18 (*RpsR*), S19 (*RpsS*), S20 (*RpsT*), S3 (*RpsC*), S4 (*RpsD*), S5 (*RpsE*), S7 (*RpsG*), and S8 (*RpsH*), nor in ribosomal RNA small subunit methyltransferase genes E (*RsmE*), G (*RsmG*), and A (*RsmA* group 2011) of the *H. heilmannii* isolates (Table S6).

No unique SNPs in ribosomal protein genes were found for the all *H. ailurogastricus* strains (ASB7.1, ASB9.4, ASB11.2, ASB13.1, ASB21 and ASB23) showing higher wild-type MIC values for lincomycin compared to the *H. heilmannii* isolates (ASB1.4, ASB2.1, ASB3.2, ASB14.1, ASB19, and ASB20). Therefore, it cannot be concluded that there is a clear distinction between *H. heilmannii* and *H. ailurogastricus*, in contrast with neomycin where we can find a clear distinct distribution.

4. Discussion

Antimicrobial susceptibility testing of *H. pylori* was performed using the agar dilution method according to the CLSI guidelines [41]. However, since *H. heilmannii* and *H. ailurogastricus* can only be cultivated in a biphasic medium with acidic pH, we used an alternative method which has been shown feasible to determine the antimicrobial susceptibility of *H. suis* [21,27]. The results from this study demonstrate that the combined agar and broth dilution method followed by qPCR can be used for antimicrobial susceptibility testing of *H. heilmannii* and *H. ailurogastricus* as well. Nevertheless, this technique is most likely not feasible for clinical applications since the cultivation of *Helicobacter* spp. is laborious.

Compared to the CLSI standards, the MIC endpoints for both reference strains *E. coli* and *S. aureus* were significantly higher for aminoglycosides, fluoroquinolones, lincosamides, and macrolides when using the combined agar and broth dilution method at pH 5. For ceftiofur and ampicillin, antimicrobial activity was increased with acidic pH, as already described by CLSI [41]. These findings are in line with those of by Berlamont et al. [28], further indicating that medium composition and pH may influence antimicrobial activity in vitro [42]. Interpretation of MIC values of antimicrobial agents obtained in vitro may thus not always reflect their activity in vivo. In addition, the complexity of the gastric environment further complicates prediction of activity of these antimicrobials.

As described by Berlamont et al. [28], we mainly used the epidemiological criterion for interpretation of MICs to determine which isolates showed potential presence of acquired resistance [28,37]. Microorganisms without (i.e., wild type) and with acquired resistance mechanisms (i.e., non-wild type) to the antimicrobial agent in question are defined by this criterion, regardless of

the clinical context (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2019). The presence of monomodal MIC distributions indicated that all *H. ailurogastricus* and the majority of *H. heilmannii* isolates fell within the wild-type range, whereas the presence of bimodal MIC distributions indicated that some *H. heilmannii* isolates ($n = 5$) that fell in the higher MIC range did not belong to the wild-type population. Using this epidemiological criterion does not guarantee the outcome of treatment of an infection with non-wild-type isolates and in vitro activity can still differ from in vivo activity. Still, by using this criterion, this may highlight that certain isolates have acquired mechanisms rendering them potentially less susceptible than the normal bacterial population to the antimicrobial agent tested. In this study, feline *H. heilmannii* isolates not belonging to the wild-type population were detected for azithromycin (one isolate), enrofloxacin (two isolates), lincomycin (one isolate), and spectinomycin (one isolate). Furthermore, *H. heilmannii* isolates showed higher MIC values for neomycin compared to *H. ailurogastricus* isolates. This indicates that acquired resistance and/or decreased susceptibility occasionally occur in *H. heilmannii* isolates. As pets may constitute a source of infection for humans, this should be kept in mind when dealing with a human patient infected with *H. heilmannii*.

Identifying presence of antimicrobial resistance mechanisms in *H. heilmannii* and *H. ailurogastricus* isolates not belonging to the wild type population is difficult due to the lack of available antimicrobial gene resistance databases for *Helicobacter* spp. As such, gene sequence comparison between isolates showing higher MIC values and the wild-type population was performed manually to detect potential presence of single nucleotide polymorphisms (SNPs). Several software tools were used to predict the impact of these SNPs on protein activity and stability (i.e., Jalview 2.10.5, ConSurf Server, I-Mutant 3.0 and PredictSNP) [39,40]. Further investigation remains warranted however, as the results from this study do not necessarily imply a causal relationship between the presence of antimicrobial resistance and the presence of SNPs.

Helicobacter heilmannii isolate ASB19 did not belong to the wild type population of azithromycin, indicating acquired resistance to this antimicrobial agent. This may be associated with the presence of SNPs in the *RplB* and *RplC* genes belonging to the 50S ribosomal protein family, as indicated by the different software tools for protein stability and activity (Table S5). Similarly, point mutations in these ribosomal protein encoding genes have also been associated with acquired resistance to macrolides in *Streptococcus criceti*, *S. pneumoniae*, *E. coli*, and *Brachyspira* spp. [43–46]. Cross-resistance to more than one macrolide has been described in several bacterial species, such as *Mycobacterium* sp. [47,48], but in this study, isolate ASB19 did not show cross-resistance to clarithromycin and tylosin.

Previous studies have shown presence of acquired resistance in two porcine *H. suis* isolates to fluoroquinolones (i.e., enrofloxacin, levofloxacin, and moxifloxacin) [27,28]. Similarly, in this study, two feline *H. heilmannii* isolates (i.e., ASB14.1 and ASB20) did not belong to the wild-type population for enrofloxacin, indicating acquired resistance. In contrast with *H. suis*, however, this was not correlated with co-resistance to levofloxacin. For both *H. pylori* and *H. suis*, acquired resistance to fluoroquinolones has been associated with the presence of a point mutation at position 78 in the QRDR region of *gyrA* [27,49]. Conversely, we could not find presence of SNPs in the *gyrA* or *gyrB* genes of the *H. heilmannii* isolates. The mechanism involved in the acquired resistance of the two *H. heilmannii* isolates to enrofloxacin, therefore, remains unclear.

Helicobacter heilmannii isolate ASB1.4 did not belong to the wild-type population for spectinomycin, indicating presence of acquired resistance to this antimicrobial agent. Spectinomycin is an aminocyclitol, which, like aminoglycosides, inhibits protein synthesis via a 30S ribosomal target. In addition, ASB1.4 also showed higher MIC values for gentamicin (MIC value 32 µg/mL) and neomycin (MIC value 8 µg/mL) compared to other *Helicobacter* spp. isolates, indicating decreased susceptibility to aminoglycosides (Table 1). Unique SNPs were found in the *RpsA*, *RpsL*, *RpsG*, and *RsmD* genes belonging to the 30S ribosomal protein gene family of *H. heilmannii* isolate ASB1.4. These SNPs may impact protein activity and stability, indicated by the different tools, and may be associated with the decreased susceptibility to the aminoglycosides and to spectinomycin. Similarly, point mutations in the *RpsA* gene have been

associated with acquired resistance to aminoglycosides in *H. suis* [28], *M. tuberculosis* [50–53], and *E. coli* [54].

Compared to *H. ailurogastricus* isolates, *H. heilmannii* isolates showed higher wild-type MIC values for neomycin. Single nucleotide polymorphisms unique for *H. heilmannii* isolates were found in several 30S and 50S ribosomal protein encoding genes as well as in ribosomal RNA methyltransferase genes and might be associated with this decreased susceptibility, as indicated by the software tools. A different pattern of MIC distribution between the two *Helicobacter* species was also observed for lincomycin. For this antimicrobial agent, the *H. ailurogastricus* isolates showed higher wild type MIC values compared to the *H. heilmannii* isolates. No SNPs in ribosomal protein genes unique for *H. ailurogastricus* isolates were however found. In any case, due to the limited number of isolates tested, further studies are necessary to confirm differences in intrinsic susceptibility between *H. heilmannii* and *H. ailurogastricus* for neomycin and lincomycin.

The wild-type MIC values of ampicillin, clarithromycin, enrofloxacin, spectinomycin, neomycin, and oxytetracycline for *H. heilmannii* and *H. ailurogastricus* isolates were similar to those described for *H. pylori*, *H. bizzozeronii*, *H. felis* and *H. salomonis* [9,55], while higher wild-type MIC values were obtained for tylosin, lincomycin, and gentamicin and lower wild-type MIC values for metronidazole (Table S7). Nevertheless, antimicrobial susceptibility patterns of different gastric *Helicobacter* spp. are difficult to compare, as medium composition and pH variations can affect antimicrobial activity in vitro [41]. The only relevant species for comparison is *H. suis*, as we used the same enriched biphasic medium with acidic pH for *H. heilmannii* and *H. ailurogastricus*. Compared to *H. suis*, *H. heilmannii*, and *H. ailurogastricus* showed lower wild-type MIC values for β -lactams, gentamicin, rifamycins, tetracyclines, neomycin, and nitroimidazole (Table S7) [27,28] with similar wild-type MIC values for macrolides [28]. In general, *H. heilmannii* and *H. ailurogastricus* are more susceptible to antimicrobial agents compared to *H. suis* which may affect in vivo treatment.

In conclusion, acquired resistance to azithromycin, enrofloxacin, lincomycin, and spectinomycin seems to occasionally occur in feline *H. heilmannii* isolates. As pets may constitute a source of infection for humans, this should be taken into consideration when dealing with a human patient infected with *H. heilmannii*.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-2607/8/6/957/s1>. Table S1: Antimicrobials agents used on the minimal inhibitory concentration determination in *H. heilmannii* and *H. ailurogastricus* species. Table S2: Primers used for the qPCR standards for *H. heilmannii* and *H. ailurogastricus*. Table S3: Detailed information of *Helicobacter* species used in the study. Table S4: Minimal inhibitory concentrations of *H. heilmannii* and *H. ailurogastricus* isolates (MIC range, average and standard deviation, and statistical analysis). Table S5: Feline *H. heilmannii* isolates included in analysis: ASB1.4, ASB2.1, ASB3.2, ASB6.3, ASB14.1, ASB19 and ASB20. Feline *H. ailurogastricus* isolates included in analysis: ASB7.1, ASB9.4, ASB11.2, ASB13.1, ASB21 and ASB23; Amino acid nomenclature: A: alanine, C: cysteine, D: aspartate, E: glutamate, F: phenylalanine, G: glycine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, S: serine, T: threonine, V: valine; DDG: predicted free energy change; RI: relative index; CS: conservation score. Table S6: Genes investigated for the antimicrobial resistance mechanism with no SNPs founded. Table S7: Comparison of MIC values of *H. heilmannii* and *H. ailurogastricus* with others gastric *Helicobacter* sp.

Author Contributions: F.H. and C.D.W. participated in the design of the study. R.M., C.D.W., S.D.B., and H.B. carried out the experiments. R.M. and C.D.W. analyzed the data and drafted the manuscript. F.H. coordinated the study. F.H., F.G., I.A., and A.S. analyzed the data and revised critically the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Research Fund of Ghent University, Belgium (BOF GOA 01G01014). R.M. (SFRH/BD/131159/2017) acknowledges FCT, the Portuguese Foundation for Science and Technology, for financial support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary Material

Supplementary Table S1. Antimicrobials agents used on the Minimal Inhibitory Concentration determination in *H. heilmannii* and *H. ailurogastricus* species.

Antimicrobial Agent	Code Sigma-Aldrich	Potency (µg/ml)
Ampicillin sodium salt	A9518	931,64
Azithromycin	75199	952,00
Ceftiofur hydrochloride (Pfizer)		
Clarithromycin	C9742	984,00
Doxycycline hyclate	D9891	987,04
Enrofloxacin	17849	998,00
Gentamycin sulphate	G1914	655,00
Levofloxacin	28266	999,00
Lincomycin hydrochloride	62143	919,68
Metronidazole	M1547	1000,00
Neomycin trisulfate salt hydrate	N1876	655,00
Oxytetracycline hydrochloride	O5875	950,37
Rifampicin	R3501	970,00
Spectinomycin dihydrochloride pentahydrate	S9007	667,00
Tylosin tartrate	T6271	936,00

Supplementary Table S2. Primers used for the qPCR standards for *H. heilmannii* and *H. ailurogastricus*.

Specie	Primer Sequence	
<i>H. heilmannii</i>	solU430F	5'- GCK GAW TTG ATG CAA GAA GG -3'
	sol1735R	5'- CTT CGT GRA TTT TAA RCC CAA T -3'
<i>H. ailurogastricus</i>	LpsA-ASB7-Fw	5'- CGA TCA AAG ATC GGG TGA AT -3'
	LpsA-ASB7-Rv	5'- CCA TTA AGG GGT GCT TGA AA -3'

Supplementary Table S3. Detailed information of *Helicobacter* species used in the study.

<i>Helicobacter</i> specie	Strain name	Strain ID	Host origin	Accession Number
<i>H. heilmannii</i>	ASB1.4	35817_16	Cat	CDMK00000000
<i>H. heilmannii</i>	ASB2.1	35817_7	Cat	CDMP00000000
<i>H. heilmannii</i>	ASB3.2	35817_8	Cat	CDMJ00000000
<i>H. heilmannii</i>	ASB6.3	35817_5	Cat	CDMM00000000
<i>H. heilmannii</i>	ASB14.1	35817_6	Cat	CDMI00000000
<i>H. heilmannii</i>	ASB19	35817_11	Cat	FZMG00000000
<i>H. heilmannii</i>	ASB20	35817_15	Cat	FZME00000000
<i>H. ailurogastricus</i>	ASB7.1	1578720_9	Cat	CDMG00000000
<i>H. ailurogastricus</i>	ASB9.4	1578720_10	Cat	CDMN00000000
<i>H. ailurogastricus</i>	ASB11.2	1578720_11	Cat	CDML00000000
<i>H. ailurogastricus</i>	ASB13.1	1578720_12	Cat	CDMH00000000
<i>H. ailurogastricus</i>	ASB21	1578720_3	Cat	FZLU00000000
<i>H. ailurogastricus</i>	ASB23	1578720_6	Cat	FZMH00000000

Supplementary Table S4. Minimal Inhibitory Concentrations of *H. heilmannii* and *H. ailurogastricus* isolates.

Group	Antimicrobial Agent	Species	MIC range (µg/ml)	Median MIC value (µg/ml)
β-lactams	Ampicillin	<i>H. heilmannii</i>	0.125 - 1	0.375
		<i>H. ailurogastricus</i>	0.125 - 1	0.375
	Ceftiofur	<i>H. heilmannii</i>	0.5 - 8	2
		<i>H. ailurogastricus</i>	4 - 16	8
Macrolides	Clarithromycin	<i>H. heilmannii</i>	0.03125 – 0.25	0.09375
		<i>H. ailurogastricus</i>	0.0625 – 0.25	0.125
	Tylosin	<i>H. heilmannii</i>	0.5 - 8	2
		<i>H. ailurogastricus</i>	1 - 4	2
	Azithromycin	<i>H. heilmannii</i>	0.03125 – 0.25 (exception of ASB19– MIC=4)	0.09375
		<i>H. ailurogastricus</i>	0.03125 – 0.125	0.0625
Lincosamides	Lincomycin	<i>H. heilmannii</i>	0.5 – 2 (exception of ASB6.3 – MIC=32)	1
		<i>H. ailurogastricus</i>	4 - 16	8
		<i>H. heilmannii</i>	0.03125 - 32	1
Quinolones	Enrofloxacin	<i>H. heilmannii</i>	0.03125 – 0.25	0.09375
		<i>H. ailurogastricus</i>	0.03125 – 0.25	0.09375
	Levofloxacin	<i>H. heilmannii</i>	0.03125 – 0.25	0.09375
		<i>H. ailurogastricus</i>	0.03125 – 0.0625	0.04686
Rifamycins	Rifampicin	<i>H. heilmannii</i>	0.03125 – 0.125	0.0625
		<i>H. ailurogastricus</i>	0.03125 – 0.0625	0.04686
		<i>H. heilmannii</i>	2 - 32	8
Aminoglycosides	Gentamicin	<i>H. heilmannii</i>	4 - 16	8
		<i>H. ailurogastricus</i>	4 - 16	8
	Neomycin	<i>H. heilmannii</i>	2 - 32	8
		<i>H. ailurogastricus</i>	0.5 - 2	1
Aminocyclitol	Spectinomycin	<i>H. heilmannii</i>	0.03125 – 1 (exception of ASB1.4 – MIC=16)	0.1875
		<i>H. ailurogastricus</i>	0.0625 – 0.25	0.125
Tetracyclines	Oxytetracycline	<i>H. heilmannii</i>	0.03125 – 0.25	0.09375
		<i>H. ailurogastricus</i>	0.03125 – 0.125	0.0625
	Doxycycline	<i>H. heilmannii</i>	0.0625 – 0.25	0.125
		<i>H. ailurogastricus</i>	0.0625 – 0.25	0.125
Nitroimidazole	Metronidazole	<i>H. heilmannii</i>	0.25 - 4	1
		<i>H. ailurogastricus</i>	0.25 – 0.5	0.375

Supplementary Table S5. Feline *H. heilmannii* isolates included in analysis: ASB1.4, ASB2.1, ASB3.2, ASB6.3, ASB14.1, ASB19 and ASB20. Feline *H. ailurogastricus* isolates included in analysis: ASB7.1, ASB9.4, ASB11.2, ASB13.1, ASB21 and ASB23; Amino acid nomenclature: A: alanine, C: cysteine, D: aspartate, E: glutamate, F: phenylalanine, G: glycine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, S: serine, T: threonine, V: valine; DDG: predicted free energy change; RI: relative index; CS: conservation score.

SNPs related to	Location		Codon and corresponding amino acid		Impact SNPs on protein activity/stability			
	Gene	Codon	non-wild type isolate(s)	wild type isolate(s)	amino acid characteristics	PredictSNP (accuracy %)	I-Mutant 3.0	ConSurf
acquired azithromycin resistance (macrolide) in ASB19 (<i>H. heilmannii</i>)	<i>50S ribosomal protein L2 (RplB)</i>	613	AGC -> S	AAC -> N	S: polar N: neutral	all tools: neutral (83%)	DDG: -0.44, RI: 3	other species: various amino acids CS: 1
	<i>50S ribosomal protein L3 (RplC)</i>	259	TCT -> S	CCT -> P	S: polar P: aromatic side chain	all tools: neutral (83%)	DDG: -1.31, RI: 8	other species: various amino acids CS: 1
		277	TGC -> C	CGC -> R	C: polar, S-group R: + charge	MAPP tool: deleterious (41%) PhD-SNP tool: deleterious (68%) PolyPhen-1: deleterious (59%) other tools: neutral (60%)	DDG: -0.64, RI: 0	other species: various amino acids, but never C CS: 1
acquired spectinomycin (aminoglycoside) resistance in ABS1.4	<i>30S ribosomal protein S1 (RpsA)</i>	523	AAG -> K	GAG -> E	K: + charge E: - charge	SIFT tool: deleterious (46%) other tools: neutral (74%)	DDG: -0.25, RI: 4	other species: E, A, G, Q, S, T, L, D, N, R, but never K CS: 1
	<i>30S ribosomal protein S12 (RpsL)</i>	391	GAC -> D	GAA -> E	D: - charge E: - charge	all tools: neutral (83%)	DDG: 0.02, RI: 1	other species: E, K, G, S, A, but never D CS: 1
	<i>30S ribosomal protein S7 (RpsG)</i>	64	GTA -> V	ATC -> I	V: no S-group I: neutral	all tools: neutral (83%)	DDG: -1.05, RI: 7	other species: V, L, I, T, A, F CS: 7
	<i>Ribosomal RNA small subunit methyltransferase D (RsmD)</i>	304	GCG -> A	ACC -> T	A: neutral, hydrophobic side chain, non-polar T: polar	MAPP tool: deleterious (41%) all tools: neutral (75%)	DDG: -1.13, RI: 9	other species: T, M, S, I, F, P, A, L, C, V CS: 4
higher MICs for neomycin in all <i>H. heilmannii</i>	<i>30S ribosomal protein S1 (RpsA)</i>	31	CAT-> H	CAA-> Q	H: + charge Q: neutral, non-polar	PolyPhen-1: deleterious (74%)	DDG: -0.54, RI: 2	other species: various amino acids CS: 1

strain isolates						PolyPhen-2: deleterious (54%) other tools: neutral (63%)		
	73	GAG -> E	GGG-> G	E: - charge G: neutral		all tools: neutral (83%)	DDG: -0.07, RI: 3	other species: various amino acids CS: 5
	118	GAG-> D	GGG-> E	D: - charge E: - charge		all tools: neutral (83%)	DDG: -0.23, RI: 5	other species: various amino acids CS: 1
	226	CAG-> E	GAC-> Q	E: - charge Q: neutral		all tools: neutral (83%)	DDG: -0.38, RI: 4	other species: various amino acids CS: 1
	238	GGA-> G	GCA-> A	G: neutral A: neutral, hydrophobic side chain, non-polar		all tools: neutral (83%)	DDG: -1.33, RI: 9	other species: various amino acids CS: 2
	241	CTC-> V	GTA-> L	L/ V: neutral, hydrophobic side chain		PANTHER tool: deleterious (57%) other tools: neutral (83%)	DDG: -1.79, RI: 9	other species: various amino acids CS: 1
	250	CCC-> T	ACG-> P	T: polar P: aromatic side chain		PANTHER tool: deleterious (61%) other tools: neutral (83%)	DDG: -1.43, RI: 9	other species: various amino acids CS: 1
	271	TTA-> Q	CAA-> L	Q: neutral L: neutral, hydrophobic side chain		PhD-SNP tool: deleterious (59%) PANTHER tool: deleterious (65%) other tools: neutral (74%)	DDG: -2.31, RI: 9	other species: various amino acids CS: 1
	274	AGA-> K	AAG-> R	K/R: + charge		all tools: neutral (83%)	DDG: -1.04, RI: 9	other species: various amino acids CS: 4
	343	ACT-> S	TCT-> T	S/T: polar		all tools: neutral (83%)	DDG: -0.62, RI: 6	other species: various amino acids CS: 1
	352	GCG-> G	GGA-> A	G: neutral		SIFT tool: deleterious (53%)	DDG: -1.21, RI: 8	other species: various amino acids CS: 1

					A: neutral, hydrophobic side chain, non-polar	other tools: neutral (74%)		
		364	GAC-> E	GAA-> D	E/D: - charge	all tools: neutral (83%)	DDG: -1.02, RI: 5	other species: various amino acids CS: 2
		370	TTT-> I	ATC-> F	I: neutral F: non-polar	PANTHER tool: deleterious (65%) other tools: neutral (83%)	DDG: -0.90, RI: 9	other species: various amino acids CS: 1
		385	ATC-> V	GTC-> I	V: neutral, hydrophobic side chain I: neutral	all tools: neutral (83%)	DDG: -0.90, RI: 4	other species: V, I CS: 6
		388	GTT-> A	GTC-> V	A: neutral, hydrophobic side chain, non-polar V: neutral, hydrophobic side chain	SIFT tool: deleterious (53%) other tools: neutral (74%)	DDG: -1.54, RI: 9	other species: various amino acids CS: 3
		412	GTT-> I	ATC-> V	I: neutral V: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.36, RI: 6	other species: various amino acids CS: 4
		418	ATC-> V	GTG-> I	V: neutral, hydrophobic side chain I: neutral	all tools: neutral (83%)	DDG: -0.94, RI: 6	other species: various amino acids CS: 1
		424	CAT-> N	AAT-> H	N: neutral, polar H: + charge	PhD-SNP tool: deleterious (58%) Other tools: neutral (74%)	DDG: -0.74, RI: 4	other species: various amino acids CS: 1
		427	CAG-> E	GAG-> Q	E: - charge Q: neutral	all tools: neutral (83%)	DDG: -0.18, RI: 2	other species: various amino acids CS: 2
		532	AGC-> N	AAC-> S	N: neutral, polar S: polar	all tools: neutral (83%)	DDG: -0.67, RI: 1	other species: various amino acids CS: 2

		543	TCT-> A	GCG-> S	A: neutral, hydrophobic side chain, non-polar S: polar	MAPP tool: deleterious (62%) PhD-SNP tool: deleterious (58%) Other tools: neutral (63%)	DDG: -0.94, RI: 8	other species: various amino acids CS: 5
		547	GTT-> I	ATC-> V	I: neutral V: neutral, hydrophobic side chain, non-polar	Poly-Phen-2 tool: deleterious (47%) PANTHER tool: deleterious (61%) Other tools: neutral (74%)	DDG: -0.56, RI: 4	other species: various amino acids CS: 5
		568	GAG-> D	GAT-> E	D/E: - charge	PhD-SNP tool: deleterious (86%) Poly-Phen-2 tool: deleterious (43%) PANTHER tool: deleterious (57%) Other tools: neutral (63%)	DDG: -0.45, RI: 2	other species: various amino acids CS: 5
		571	GTC-> I	ATT-> V	I: neutral V: neutral, hydrophobic side chain, non-polar	PANTHER tool: deleterious (61%) Other tools: neutral (81%)	DDG: -0.26, RI: 0	other species: various amino acids CS: 2
		580	AAA-> K	CGA-> R	K/R: + charge	all tools: neutral (83%)	DDG: -0.79, RI: 8	other species: various amino acids CS: 1
		1111	GAA-> E	GAT-> D	E/D: - charge	all tools: neutral (83%)	DDG: -0.48, RI: 0	other species: various amino acids CS: 1
		1129	CGC-> R	CAC-> H	R/H: + charge	PANTHER tool: deleterious (61%) other tools: neutral (83%)	DDG: -0.05, RI: 2	other species: various amino acids CS: 5
		1171	AAC-> N	GAT-> D	N: neutral, polar D: - charge	PhD-SNP tool: deleterious (59%) Poly-Phen-2 tool: deleterious (50%)	DDG: -0.72, RI: 3	other species: various amino acids CS: 6

						nsSNPAnalyzer tool: deleterious (63%) Other tools: neutral (65%)		
		1246	CAA-> Q	AAA-> K	Q: neutral K: + charge	Poly-Phen-2 tool: deleterious (47%) PANTHER tool: deleterious (57%) Other tools: neutral (74%)	DDG: -0.23, RI: 1	other species: various amino acids CS: 1
		1258	GAC-> D	GAA-> E	D/E: - charge	all tools: neutral (83%)	DDG: -0.29, RI: 4	other species: various amino acids CS: 3
		1285	AAA-> K	CAA-> Q	K: + charge Q: neutral	all tools: neutral (83%)	DDG: -0.29, RI: 2	other species: various amino acids CS: 6
		1288	ACC-> T	GTC-> V	T: polar V: neutral, hydrophobic side chain, non-polar	MAPP tool: deleterious (51%) SIFT tool: deleterious (45%) PANTHER tool: deleterious (71%) Other tools: neutral (65%)	DDG: -0.91, RI: 9	other species: V, A, F, T CS: 8
		1306	GAC-> D	AAC-> N	D: - charge N: neutral, polar	PANTHER tool: deleterious (68%) Other tools: neutral (83%)	DDG: 0.08, RI: 1	other species: various amino acids CS: 8
		1312	GAC-> D	GAA-> E	D/E: - charge	all tools: neutral (83%)	DDG: -0.03, RI: 2	other species: various amino acids CS: 1
		1315	AAT-> N	GAT-> D	N: neutral, polar D: - charge	all tools: neutral (83%)	DDG: -0.78, RI: 5	other species: various amino acids CS: 3
		1342	TAC-> Y	TTT-> F	Y: polar F: non-polar, no S-group	MAPP tool: deleterious (59%) Other tools: neutral (74%)	DDG: -0.76, RI: 0	other species: various amino acids CS: 1

		1351	GCC-> A	CCT-> P	A: neutral, hydrophobic side chain, non-polar P: aromatic side chain	all tools: neutral (83%)	DDG: -1.16, RI: 8	other species: various amino acids CS: 1
		1360	ACC-> T	GTC-> V	T: polar V: neutral, hydrophobic side chain, non-polar	all tools: neutral (83%)	DDG: -1.03, RI: 9	other species: various amino acids CS: 5
		1384	AAA-> K	CGC-> R	R/K: + charge	all tools: neutral (83%)	DDG: -0.53, RI: 7	other species: various amino acids CS: 1
		1393	GGC-> G	GAC-> D	G: neutral D: -charge	PredictSNP tool: deleterious (52%) PhD-SNP tool: deleterious (59%) PolyPhen-1 tool: deleterious (43%) PolyPhen-2 tool: deleterious (53%) SNAP tool: deleterious (56%) Other tools: neutral (67%)	DDG: -0.94, RI: 2	other species: various amino acids CS: 5
		1399	ATT-> I	GTT-> V	V/I: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.29, RI: 5	other species: M, I, A, V, L CS: 7
		1408	AAG-> K	ACA-> T	K: + charge T: polar	PhD-SNP tool: deleterious (59%) other tools: neutral (74%)	DDG: -0.66, RI: 5	other species: various amino acids CS: 1
		1435	ATT-> I	GTC-> V	V/I: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.68, RI: 8	other species: M, I, A, L, V CS: 4
		1444	AGT-> S	GGC-> G	S: polar G: neutral	PhD-SNP tool: deleterious (73%) other tools: neutral (75%)	DDG: -1.10, RI: 6	other species: various amino acids CS: 2

		1456	GTT-> V	ATT-> I	I/V: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.73, RI: 4	other species: Y, L, V, M, I CS: 6
		1474	GCC-> A	TCT-> S	A: neutral, hydrophobic side chain, non-polar S: polar	MAPP tool: deleterious (41%) other tools: neutral (74%)	DDG: -0.39, RI: 9	other species: various amino acids CS: 4
		1483	ATC-> I	CTC-> L	L/I: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.72, RI: 6	other species: M, I, Y, F, V, L CS: 6
		1486	TAC-> Y	CAC-> H	Y: polar H: + charge	all tools: neutral (83%)	DDG: 0.29, RI: 7	other species: various amino acids CS: 1
		1522	ATG-> M	GTC-> V	M: non-polar V: neutral, hydrophobic side chain	SIFT tool: deleterious (53%) other tools: neutral (74%)	DDG: -0.83, RI: 7	other species: various amino acids CS: 1
		1525	GTG-> V	ATT-> I	I/V: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.69, RI: 4	other species: I, V, L, F CS: 7
		1531	AGC-> S	GGT-> G	S: polar G: neutral	PhD-SNP tool: deleterious (61%) other tools: neutral (74%)	DDG: -1.19, RI: 9	other species: S, C, A, G CS: 5
		1555	GGC-> G	GCC-> A	G: neutral A: neutral, hydrophobic side chain, non-polar	SIFT tool: deleterious (46%) other tools: neutral (74%)	DDG: -1.29, RI: 7	other species: various amino acids CS: 1
		1594	CAC-> H	CGC-> R	R/H: + charge	all tools: neutral (83%)	DDG: -1.17, RI: 9	other species: various amino acids CS: 3
		1630	GCC-> A	TCG-> S	A: neutral, hydrophobic side chain, non-polar S: polar	all tools: neutral (83%)	DDG: -0.22, RI: 5	other species: various amino acids CS: 1
		1633	AAA-> K	AGC-> S	K: + charge S: polar	PhD-SNP tool: deleterious (61%)	DDG: -0.07, RI: 5	other species: various amino acids CS: 1

						Other tools: neutral (74%)		
		1639	AGC-> S	ACC-> T	T/S: polar	all tools: neutral (83%)	DDG: -0.61, RI: 4	other species: various amino acids CS: 1
		1666	GGC-> G	AGC-> S	G: neutral S: polar	all tools: neutral (83%)	DDG: -0.74, RI: 7	other species: various amino acids CS: 3
	<i>30S ribosomal protein S15 (RpsO)</i>	73	AGC -> S	TGT -> C	S: polar C: polar, S-group	PhD-SNP tool: deleterious (59%) Other tools: neutral (74%)	DDG: -0.70, RI: 6	other species: various amino acids CS: 3
		121	GCC -> A	GTC -> V	A/V: neutral, hydrophobic side chain, non-polar	MAPP tool: deleterious (51%) Other tools: neutral (74%)	DDG: -1.43, RI: 7	other species: various amino acids CS: 4
		133	AAC -> N	GAC -> D	N: neutral, polar D: - charge	all tools: neutral (83%)	DDG: -0.88, RI: 6	other species: various amino acids CS: 1
		214	GAC -> D	AAC-> N	D: - charge N: neutral, polar	MAPP tool: deleterious (59%) Other tools: neutral (74%)	DDG: -0.19, RI: 1	other species: various amino acids CS: 1
		238	ACA -> T	GAG -> E	T: polar E: - charge	all tools: neutral (83%)	DDG: 0.07, RI: 1	other species: various amino acids CS: 1
	<i>30S ribosomal protein S16 (RpsP)</i>	127	GCA -> A	AGC -> S	A: hydrophobic side chain S: polar	all tools: neutral (83%)	DDG: -0.90, RI: 8	other species: various amino acids CS: 2
		130	AGC -> S	AAC -> N	S: polar N: neutral, polar	MAPP tool: deleterious (62%) PANTHER tool: deleterious (57%) Other tools: neutral (73%)	DDG: -0.40, RI: 5	other species: various amino acids CS: 1
		157	GCG -> A	GAG -> E	A: hydrophobic side chain E: polar, - charge	MAPP tool: deleterious (57%) PhD-SNP tool: deleterious (73%)	DDG: -0.40, RI: 5	other species: various amino acids CS: 5

						PANTHER tool: deleterious (68%) Other tools: neutral (63%)		
		166	GAC -> D	ACG -> S	D: - charge S: polar	all tools: neutral (83%)	DDG: -0.14, RI: 2	other species: various amino acids CS: 2
	<i>30S ribosomal protein S2 (RpsB)</i>	250	CAA -> Q	CGA -> R	Q: non-polar, neutral R: + charge	Other tools: deleterious (72%) MAPP tool: neutral (70%)	DDG: -1.24, RI: 9	other species: various amino acids CS: 6
		694	ACG -> T	GCG -> A	T: polar A: hydrophobic side chain, non-polar	all tools: neutral (83%)	DDG: -0.55, RI: 4	other species: various amino acids CS: 1
		697	ATC -> I	GTT -> V	V/I: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.35, RI: 5	other species: various amino acids CS: 1
		700	AAA -> K	GAG -> E	K: polar, + charge E: polar, - charge	all tools: neutral (83%)	DDG: -0.66, RI: 8	other species: various amino acids CS: 1
		709	GAG -> E	CAG-> Q	E: polar, - charge Q: non-polar, neutral	all tools: neutral (83%)	DDG: -0.05, RI: 6	other species: various amino acids CS: 5
		739	ATC -> I	GTC -> V	V/I: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.03, RI: 2	other species: various amino acids CS: 1
		772	GTG -> V	GCA-> A	V/A: neutral, hydrophobic	MAPP tool: deleterious (46%) other tools: neutral (83%)	DDG: 0.05, RI: 3	other species: K, V, A, T, E CS: 6
		775	GAA -> E	GAC -> D	E: polar, - charge D: - charge	all tools: neutral (83%)	DDG: 0.73, RI: 8	other species: E, D CS: 7
	<i>30S ribosomal protein S21 (RpsU)</i>	202	TCT -> S	GCG -> A	S: polar A: hydrophobic side chain, non-polar	all tools: neutral (85%)	DDG: -0.71, RI: 8	other species: E, S, A CS: 5
	<i>30S ribosomal protein S6 (RpsF)</i>	61	AGC -> S	AAC-> N	S: polar N: neutral, polar	all tools: neutral (83%)	DDG: 0.05, RI: 3	other species: various amino acids CS: 4
		94	AAT-> N	GGA -> G	N: neutral, polar	all tools: neutral (83%)	DDG: -0.87, RI: 6	other species: various amino acids

					G: non-polar, no S-group			CS: 1
		175	TAC -> Y	CAC -> H	Y: polar H: + charge	all tools: neutral (83%)	DDG: 0.51, RI: 7	other species: various amino acids CS: 5
		208	CAG -> Q GCA -> A	AAA -> K	Q: neutral A: hydrophobic side chain, non-polar K: + charge	MAPP tool: deleterious (41%) other tools: neutral (74%)	DDG: -0.34, RI: 4	other species: various amino acids CS: 1
	<i>30S ribosomal protein S9 (RpsI)</i>	4	GCA -> A	ACA -> T	A: hydrophobic side chain, non-polar T: polar	all tools: neutral (83%)	DDG: -1.00, RI: 9	other species: T, A, V, S, E CS: 6
		271	ACG -> T	AGT -> S	T/S: polar	PhD-SNP tool: deleterious (59%) SIFT tool: deleterious (46%) other tools: neutral (65%)	DDG: -0.01, RI: 5	other species: various amino acids CS: 1
	<i>Ribosomal protein S12 methylthiotransferase (Rimbara E)</i>	808	TCC -> S	GCA -> A ACC -> T	S: polar A: hydrophobic side chain, non-polar T: polar	PANTHER tool: deleterious (66%) other tools: neutral (83%)	DDG: -0.18, RI: 4	other species: various amino acids CS: 2
		901	AGC -> S	AGG -> R	S: polar R: + charge	PANTHER tool: deleterious (66%) other tools: neutral (83%)	DDG: -0.69, RI: 6	other species: various amino acids CS: 4
		919	GAG -> E	CAA -> Q	E: polar, + charge Q: neutral	MAPP tool: deleterious (43%) SIFT tool: deleterious (43%) PANTHER tool: deleterious (69%) other tools: neutral (64%)	DDG: -0.05, RI: 3	other species: various amino acids CS: 1
		922	GCG -> A	CAG -> E	A: hydrophobic side chain, non-polar E: polar, + charge	MAPP tool: deleterious (48%) other tools: neutral (74%)	DDG: -0.29, RI: 6	other species: various amino acids CS: 1
		931	CAA -> Q	GAG -> E	Q: neutral E: polar, + charge	PANTHER tool: deleterious (66%)	DDG: -0.40, RI: 4	other species: various amino acids

					other tools: neutral (83%)		CS: 1
	934	GAG -> E	GGC -> G	E: polar, + charge G: non-polar, no S-group	PANTHER tool: deleterious (76%) other tools: neutral (83%)	DDG: -0.33, RI: 1	other species: various amino acids CS: 4
	940	CAA -> Q AGA -> R	ACA -> T	Q: neutral R: + charge T: polar	PolyPhen-2 tool: deleterious (67%) PANTHER tool: deleterious (74%) other tools: neutral (74%)	DDG: -0.52, RI: 4	other species: various amino acids CS: 3
	988	AAA -> K	AGG -> R	K/R: + charge	PANTHER tool: deleterious (61%) other tools: neutral (83%)	DDG: -0.38, RI: 5	other species: various amino acids CS: 1
	1015	GAC -> D AGC -> S AAC -> N	CCT -> P	D: - charge S: polar N: neutral, polar P: aromatic side chain	PhD-SNP tool: deleterious (68%) other tools: neutral (75%)	DDG: -1.35, RI: 8	other species: various amino acids CS: 4
	1030	GAG -> E	GCC -> A	E: polar, + charge A: hydrophobic side chain, non-polar	PANTHER tool: deleterious (69%) other tools: neutral (83%)	DDG: -0.60, RI: 6	other species: various amino acids CS: 1
	1036	CTT -> L	ATC -> I	L/I: neutral, hydrophobic side chain	PANTHER tool: deleterious (71%) other tools: neutral (83%)	DDG: 0.03, RI: 1	other species: various amino acids CS: 3
	1063	GAG-> E	AAA -> K	E: polar, + charge K: + charge	PANTHER tool: deleterious (61%) other tools: neutral (83%)	DDG: -0.76, RI: 7	other species: various amino acids CS: 1
	1075	GCT -> A ACT -> V	ATG -> M	A: hydrophobic side chain, non-polar V: neutral, hydrophobic M: non-polar	MAPP tool: deleterious (48%) PANTHER tool: deleterious (74%) other tools: neutral (83%)	DDG: -0.57, RI: 3	other species: various amino acids CS: 2

		1087	AGT -> S AAC -> N	GCT -> A	S: polar N: neutral, polar A: hydrophobic side chain, non-polar	PANTHER tool: deleterious (74%) other tools: neutral (83%)	DDG: -0.34, RI: 7	other species: various amino acids CS: 1
		1210	ATG -> M GTG -> V	TCT -> S	M: non-polar V: neutral, hydrophobic S: polar	all tools: neutral (83%)	DDG: 0.20, RI: 1	other species: various amino acids CS: 1
		1246	CAC -> H	CTT -> L	H: + charge L: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -1.50, RI: 6	other species: various amino acids CS: 1
		1252	CAC -> H	ACC -> T	H: + charge T: polar	PhD-SNP tool: deleterious (59%) PolyPhen-1 tool: deleterious (74%) PolyPhen-2 tool: deleterious (40%) other tools: neutral (60%)	DDG: -0.82, RI: 5	other species: T various amino acids CS: 7
		1255	AGC -> S	ACT -> T	S/T: polar	all tools: neutral (83%)	DDG: -0.21, RI: 1	other species: various amino acids CS: 1
		1258	TTT -> F	TGC -> C	F: non-polar, no S-group C: polar, S-group	PhD-SNP tool: deleterious (59%) other tools: neutral (74%)	DDG: 0.43, RI: 2	other species: C, S, I, F, A CS: 5
		1261	GAA -> E	CAA -> Q	A: hydrophobic side chain, non-polar T: polar	all tools: neutral (83%)	DDG: 0.07, RI: 4	other species: various amino acids CS: 1
		1270	ATA -> I	ATG -> M	I: neutral, hydrophobic side chain M: non-polar	all tools: neutral (83%)	DDG: -0.17, RI: 5	other species: M, F, I, E, D, S CS: 6
		1276	CTG -> L	GTG -> V	L: neutral, hydrophobic side chain V: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.56, RI: 5	other species: Y, F, I, L, V CS: 5

		1282	AGG -> R	AAG -> K	R/K: + charge	all tools: neutral (83%)	DDG: 0.20, RI: 1	other species: E, R, K CS: 5
	<i>Ribosomal RNA large subunit methyltransferase H (RlmH group_1616)</i>	25	TCT -> S	GCC -> A	S: polar A: hydrophobic side chain, non-polar	all tools: neutral (83%)	DDG: -0.75, RI: 8	other species: various amino acids CS: 5
		34	ACC -> T	AAC -> N	T: polar N: neutral, polar	MAPP tool: deleterious (46%) other tools: neutral (74%)	DDG: 0.32, RI: 2	other species: various amino acids CS: 3
		40	GAG -> E	GAT -> D	E: polar, + charge D: - charge	all tools: neutral (83%)	DDG: 0.35, RI: 4	other species: various amino acids CS: 1
		88	AGA -> R	AAG -> K	R/K: + charge	all tools: neutral (83%)	DDG: 0.22, RI: 2	other species: K, G, R, N CS: 5
		106	ATA -> I	GTC -> V	V/I: neutral, hydrophobic	all tools: neutral (83%)	DDG: 0.03, RI: 2	other species: various amino acids CS: 1
		115	GCG -> A	TTT -> F	A: hydrophobic side chain, non-polar F: non-polar, no S- group	MAPP tool: deleterious (72%) SIFT tool: deleterious (79%) other tools: neutral (63%)	DDG: -2.28, RI: 8	other species: various amino acids CS: 6
		127	TCA -> S	TTG -> L	S: polar L: neutral, hydrophobic side chain	PolyPhen-2 tool: deleterious (40%) SIFT tool: deleterious (53%) other tools: neutral (68%)	DDG: -2.29, RI: 9	other species: L, I, S, V CS: 8
		130	AAC -> N	ACA -> T	N: neutral, polar T: polar	MAPP tool: deleterious (75%) other tools: neutral (75%)	DDG: -1.31, RI: 7	other species: various amino acids CS: 2
		139	ACA -> T	TCC -> S	T/S: polar	MAPP tool: deleterious (59%) other tools: neutral (74%)	DDG: -0.12, RI: 5	other species: various amino acids CS: 1

		142	GAG -> E	AAT -> N	E: polar, + charge N: neutral, polar	MAPP tool: deleterious (51%) other tools: neutral (74%)	DDG: 0.25, RI: 7	other species: various amino acids CS: 4
		148	CAA -> Q	AAG -> K	Q: neutral K: + charge	all tools: neutral (83%)	DDG: -0.47, RI: 3	other species: various amino acids CS: 6
		154	TTT -> F	CTT -> L	F: non-polar, no S- group L: neutral, hydrophobic side chain	MAPP tool: deleterious (51%) PolyPhen-1 tool: deleterious (59%) PolyPhen-2 tool: deleterious (50%) other tools: neutral (60%)	DDG: -1.05, RI: 5	other species: various amino acids CS: 4
		160	ACT -> T	TCT -> S	T/S: polar	all tools: neutral (83%)	DDG: -0.25, RI: 3	other species: various amino acids CS: 4
		181	ATC -> I	TTA -> L	I/L: neutral, hydrophobic side chain	PolyPhen-2 tool: deleterious (40%) other tools: neutral (75%)	DDG: -0.96, RI: 5	other species: various amino acids CS: 3
		193	AGG -> T	ATC -> I	T: polar I: neutral, hydrophobic side chain	MAPP tool: deleterious (48%) other tools: neutral (74%)	DDG: -1.80, RI: 8	other species: various amino acids CS: 5
		199	GGC -> G	GCC -> A	G: non-polar, no S- group A: hydrophobic side chain, non-polar	MAPP tool: deleterious (57%) other tools: neutral (74%)	DDG: -1.11, RI: 8	other species: various amino acids, but never G CS: 5
		226	CTC -> L	ACT -> T	L: neutral, hydrophobic side chain T: polar	all tools: neutral (83%)	DDG: -0.09, RI: 2	other species: various amino acids CS: 1
		229	ATA -> I	TAT -> Y	I: neutral, hydrophobic side chain Y: polar	MAPP tool: deleterious (57%) other tools: neutral (74%)	DDG: 0.02, RI: 3	other species: various amino acids CS: 1
		259	AAT -> N	AGC -> S	N: neutral, polar S: polar	Deleterious (61%) in all tools, except	DDG: -0.15, RI: 5	other species: A, S CS: 7

						for PhD-SNP (neutral 68%) and PolyPhen-1 (neutral 67%)		
		298	TAT -> Y	TTT -> F	Y: polar F: non-polar, no S-group	all tools: neutral (83%)	DDG: -0.54, RI: 1	other species: various amino acids CS: 5
		307	GCC -> A	GAA -> E	A: hydrophobic side chain, non-polar E: polar, + charge	MAPP tool: deleterious (84%) SIFT tool: deleterious (43%) other tools: neutral (63%)	DDG: -0.40, RI: 9	other species: various amino acids CS: 5
		322	AAA -> K	ACG -> T	K: + charge T: polar	all tools: neutral (83%)	DDG: -0.65, RI: 4	other species: various amino acids CS: 1
		325	CAA -> Q	CGT -> R	Q: neutral R: + charge	PolyPhen-2 tool: deleterious (43%) other tools: neutral (74%)	DDG: -0.59, RI: 6	other species: various amino acids CS: 5
		331	ATC -> I	ACC -> T	I: neutral, hydrophobic side chain T: polar	MAPP tool: deleterious (51%) other tools: neutral (74%)	DDG: -0.07, RI: 1	other species: various amino acids CS: 1
		367	GCC -> A	GAA -> E	A: hydrophobic side chain, non-polar E: polar, + charge	Deleterious (51%) in all tools, except for PhD-SNP (neutral 78%) and PolyPhen-1 (neutral 67%)	DDG: -0.34, RI: 6	other species: various amino acids CS: 8
		370	TTA -> L	ATG -> M	L: neutral, hydrophobic side chain M: non-polar	MAPP tool: deleterious (43%) other tools: neutral (74%)	DDG: -0.61, RI: 6	other species: M, F, L, I, V CS: 7
		382	ATT -> I	GTT -> V	I: neutral, hydrophobic side chain V: neutral, hydrophobic	MAPP tool: deleterious (77%) PolyPhen-2 tool: deleterious (43%) SIFT tool: deleterious (79%)	DDG: -0.30, RI: 5	other species: M, V, F, I, L CS: 6

						other tools: neutral (60%)		
		406	GGT -> G	GCC -> A	G: non-polar, no S-group A: hydrophobic side chain, non-polar	PolyPhen-2 tool: deleterious (43%) other tools: neutral (74%)	DDG: -1.14, RI: 6	other species: A, T, G, S CS: 7
	<i>Ribosomal silencing factor RsfS</i>	25	ATG -> M	CTA -> L	M: non-polar L: neutral, hydrophobic side chain	Deleterious (52%) in all tools, except for PhD-SNP tool (neutral 83%) and SNAP tool (neutral 67%)	DDG: -1.01, RI: 9	other species: various amino acids CS: 3
		49	AAT -> N	GAC -> D	N: neutral, polar D: + charge	PolyPhen-2 tool: deleterious (43%) other tools: neutral (74%)	DDG: 0.03, RI: 5	other species: L, F, D, A, E, N, S, G CS: 5
		52	GTC -> V	ATC -> I	V: neutral, hydrophobic I: neutral, hydrophobic side chain	MAPP tool: deleterious (51%) other tools: neutral (74%)	DDG: -0.62, RI: 6	other species: I, L, V CS: 8
		112	TCC -> S	ACC -> T	S: polar T: polar	all tools: neutral (83%)	DDG: -0.67, RI: 8	other species: V, G, T, A, S CS: 7
		181	GTG -> V	GAG -> E	V: neutral, hydrophobic E: polar, + charge	PolyPhen-2 tool: deleterious (43%) other tools: neutral (74%)	DDG: 0.51, RI: 3	other species: various amino acids CS: 1
		193	GTG -> V	ATA-> I	V: neutral, hydrophobic I: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.83, RI: 7	other species: various amino acids CS: 4
		199	GAG -> E	GAT -> D	E: polar, + charge D: + charge	all tools: neutral (83%)	DDG: 0.11, RI: 5	other species: I, G, R, T, D, K, A, E CS: 7
		202	AGC -> S	GGC -> G	S: polar G: non-polar, no S-group	all tools: neutral (83%)	DDG: -0.99, RI: 8	other species: various amino acids CS: 3

		220	ATT-> I	GTT -> V	I: neutral, hydrophobic side chain V: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.04, RI: 1	other species: I, L, A, V CS: 7
		244	GTG -> V	ATT -> I	V: neutral, hydrophobic I: neutral, hydrophobic side chain	MAPP tool: deleterious (48%) other tools: neutral (74%)	DDG: -0.90, RI: 8	other species: V, I, L CS: 7
		250	ATT -> I	CTT -> L	I/L: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -1.23, RI: 7	other species: L, I, M, V CS: 7
		271	GAA -> E	GAC -> D	E: polar, + charge D: + charge	all tools: neutral (83%)	DDG: -0.34, RI: 0	other species: various amino acids CS: 1
		307	CAC -> H	AAT -> N	H: + charge N: neutral, polar	all tools: neutral (83%)	DDG: -0.47, RI: 7	other species: various amino acids CS: 1
		316	CAT -> H	AAT -> N	H: + charge N: neutral, polar	PolyPhen-1 tool: deleterious (74%) PolyPhen-2 tool: deleterious (55%) SIFT tool: deleterious (43%) other tools: neutral (60%)	DDG: -0.34, RI: 6	other species: N, H CS: 6
	<i>Ribosomal RNA small subunit methyltransferase D (RsmD)</i>	124	AGC -> S AAC -> N	GAC -> D	S: polar N: neutral, polar D: + charge	all tools: neutral (83%)	DDG: -0.53, RI: 2 DDG: -0.89, RI: 2	other species: various amino acids CS: 1
		205	TTT -> F CAC -> H	TAT -> Y	F: non-polar, no S- group H: + charge Y: polar	all tools: neutral (83%)	DDG: -0.12, RI: 1 DDG: -1.06, RI: 5	other species: various amino acids CS: 4
		217	GTG -> V ATT -> I	TTT -> F	V/I: neutral, hydrophobic F: non-polar, no S- group	all tools: deleterious (87%)	DDG: -0.63, RI: 5	other species: various amino acids CS: 8

		232	GAA -> Q AAA -> K	GCG -> A	Q: neutral A: hydrophobic side chain, non-polar	PolyPhen-1 tool: deleterious (59%) PolyPhen-2 tool: deleterious (54%) other tools: neutral (63%)	DDG: -0.46, RI: 6	other species: various amino acids CS: 3
		235	GAA -> E	CAG -> Q	E: polar, + charge Q: neutral	all tools: neutral (83%)	DDG: 0.01, RI: 4	other species: various amino acids CS: 1
		244	GGG -> G TTG -> L	AAG -> K	G: non-polar, no S-group L: neutral, hydrophobic side chain K: + charge	all tools: neutral (83%)	DDG: -0.51, RI: 4 DDG: 0.72, RI: 7	other species: various amino acids CS: 2
		271	TTT -> F TTA -> L	ATC -> I	F: non-polar, no S-group L/I: neutral, hydrophobic side chain	PhD-SNP tool: deleterious (59%) other tools: neutral (75%)	DDG: -1.01, RI: 5 DDG: -0.42, RI: 4	other species: C, A, L, I, Y, T, V, F CS: 5
		289	ACC -> T	CAA -> Q	T: polar Q: neutral	PolyPhen-2 tool: deleterious (40%) other tools: neutral (74%)	DDG: -0.22, RI: 6	other species: various amino acids CS: 1
		301	AGA -> R	AAG -> K	R/K: + charge	MAPP tool: deleterious (48%) other tools: neutral (74%)	DDG: 0.03, RI: 2	other species: various amino acids CS: 2
		310	CAC -> H	TGC -> C	H: + charge C: polar, S-group	MAPP tool: deleterious (76%) other tools: neutral (75%)	DDG: -0.91, RI: 6	other species: various amino acids CS: 1
		319	ATA -> I	GTG -> V	I: neutral, hydrophobic side chain V: neutral, hydrophobic	SIFT tool: deleterious (53%) other tools: neutral (74%)	DDG: -0.41, RI: 6	other species: various amino acids CS: 7
		322	TTT -> F	TTG -> L	F: non-polar, no S-group	all tools: neutral (83%)	DDG: -1.09, RI: 8	other species: A various amino acids

					L: neutral, hydrophobic side chain			CS: 7
		328	ACC -> T	GCG -> A	T: polar A: hydrophobic side chain, non-polar	MAPP tool: deleterious (51%) PolyPhen-2 tool: deleterious (43%) other tools: neutral (68%)	DDG: -0.45, RI: 2	other species: various amino acids CS: 2
		331	CTA -> L	TTT -> F	L: neutral, hydrophobic side chain F: non-polar, no S-group	all tools: neutral (83%)	DDG: -1.04, RI: 7	other species: various amino acids CS: 7
		334	GCG -> A	AGC -> S	A: hydrophobic side chain, non-polar S: polar	all tools: neutral (83%)	DDG: -0.60, RI: 9	other species: various amino acids CS: 1
		340	TAT -> Y	CAT -> H	Y: polar H: + charge	MAPP tool: deleterious (41%) other tools: neutral (75%)	DDG: 0.31, RI: 7	other species: various amino acids CS: 4
		346	GAA -> E	AAG -> K	E: polar, + charge K: + charge	PhD-SNP tool: deleterious (59%) other tools: neutral (75%)	DDG: 0.02, RI: 3	other species: various amino acids CS: 1
		355	AAG -> K	GAG -> E	K: + charge E: polar, + charge	all tools: neutral (83%)	DDG: -0.79, RI: 8	other species: various amino acids CS: 1
		430	GTG -> V	CTA -> L	V: neutral, hydrophobic L: neutral, hydrophobic side chain	Deleterious (51%) in all tools, except for PolyPhen-1 tool (neutral 67%), SIFT tool (neutral 68%) and SNAP tool (neutral 61%)	DDG: -1.48, RI: 8	other species: M, V, L, I, F CS: 7
		433	GCA -> A	GAA -> E	A: hydrophobic side chain, non-polar E: polar, + charge	MAPP tool: deleterious (63%) other tools: neutral (75%)	DDG: -0.23, RI: 4	other species: various amino acids CS: 3

		442	AGG -> R	AAT -> N	R: + charge N: neutral, polar	all tools: neutral (83%)	DDG: 0.39, RI: 4	other species: various amino acids CS: 2
		445	GGT -> G	CCT -> P	G: non-polar, no S-group P: aromatic side chain	all tools: neutral (83%)	DDG: -1.62, RI: 9	other species: G, P, I CS: 1
		463	CAA -> Q	AAG -> K	Q: neutral K: + charge	PolyPhen-2 tool: deleterious (43%) other tools: neutral (74%)	DDG: -0.26, RI: 2	other species: various amino acids CS: 1
		466	TAT -> Y	CAT -> H	Y: polar H: + charge	PolyPhen-1 tool: deleterious (59%) PolyPhen-2 tool: deleterious (50%) other tools: neutral (74%)	DDG: 0.50, RI: 8	other species: various amino acids CS: 1
		472	CTT -> L	TTT -> F	L: neutral, hydrophobic side chain F: non-polar, no S-group	all tools: neutral (83%)	DDG: -1.04, RI: 8	other species: various amino acids CS: 1
		505	GTG -> C	AGT -> S	C: polar, S-group S: polar	PolyPhen-1 tool: deleterious (74%) PolyPhen-2 tool: deleterious (81%) SIFT tool: deleterious (46%) other tools: neutral (60%)	DDG: -0.50, RI: 3	other species: various amino acids CS: 2
		514	AAG -> K	AAT -> N	K: + charge N: neutral, polar	PolyPhen-2 tool: deleterious (41%) other tools: neutral (74%)	DDG: -0.27, RI: 2	other species: various amino acids CS: 1
		517	ACG -> T	GCG -> A	T: polar A: hydrophobic side chain, non-polar	MAPP tool: deleterious (51%) PolyPhen-2 tool: deleterious (40%) SIFT tool: deleterious (45%)	DDG: -0.74, RI: 6	other species: various amino acids CS: 5

						other tools: neutral (60%)		
		524	ACA -> T	GAA -> E	T: polar E: polar, + charge	PolyPhen-2 tool: deleterious (43%) other tools: neutral (74%)	DDG: -0.38, RI: 2	other species: various amino acids CS: 5
		527	ACA > T	AAG -> K	T: polar K: + charge	Deleterious (51%) in all tools, except for PhD-SNP tool (neutral 72%), PolyPhen-1 tool (neutral 67%), and SNAP tool (neutral 50%)	DDG: -0.62, RI: 5	other species: various amino acids CS: 8
	<i>Ribosomal RNA small subunit methyltransferase I (RsmI)</i>	73	AAC -> N AGC -> S	GGC -> G	N: neutral, polar S: polar G: non-polar, no S- group	all tools: neutral (83%)	DDG: -0.76, RI: 6 DDG: -0.84, RI: 8	other species: various amino acids CS: 2
		79	GGC -> A GAT -> D	GGT -> G	A: hydrophobic side chain, non-polar D: + charge G: non-polar, no S- group	G27A -> MAPP tool: deleterious (48%) other tools: neutral (74%) G27D -> all tools: neutral (83%)	DDG: -0.57, RI: 8 DDG: -0.89, RI: 8	other species: various amino acids CS: 7
		136	AGT -> Q CAA -> S	GAG -> E	Q: neutral S: polar E: polar, + charge	all tools: neutral (83%)	DDG: 0.02, RI: 0 DDG: -0.29, RI:	other species: various amino acids CS: 7
		169	GCC-> A CAA -> Q	AAA -> K	A: hydrophobic side chain, non-polar Q: neutral K: + charge	all tools: neutral (83%)	DDG: -0.22, RI: 6 DDG: -0.16, RI: 0	other species: various amino acids CS: 4
		178	CAG -> Q	TGT -> C	Q: neutral C: polar. S-group	all tools: neutral (83%)	DDG: -0.46, RI: 4	other species: various amino acids CS: 4
		211	GCG -> A	ACC -> T	T: polar A: hydrophobic side chain, non-polar	all tools: neutral (83%)	DDG: -0.81, RI: 5	other species: various amino acids CS: 1
		334	AAC -> N AAG -> K	AGC -> S	N: neutral, polar K: + charge	S112N -> all tools: neutral (83%)	DDG: -0.19, RI: 3	other species: various amino acids

				S: polar	S112K -> PhD-SNP tool: deleterious (73%) other tools: neutral (75%)	DDG: -0.17, RI: 0	CS: 3
	445	CTA -> L CAA -> Q	AAG -> K	L: neutral, hydrophobic side chain Q: neutral K: + charge	K149L -> MAPP tool: deleterious (43%), PhD-SNP tool: deleterious (59%), PANTHER tool: deleterious (57%) other tools: neutral (65%) K149Q -> PhD-SNP tool: deleterious (58%) other tools: neutral (74%)	DDG: 0.36, RI: 1 DDG: -0.10, RI: 0	other species: V, A, G, T, Q, R, N, S, K but never L CS: 4
	454	CGC -> R	TGC -> C	R: + charge C: polar, S-group	PANTHER tool: deleterious (74%) other tools: neutral (83%)	DDG: 0.07, RI: 2	other species: various amino acids CS: 4
	484	GGG -> G	ACG -> T	A: hydrophobic side chain, non-polar T: polar	all tools: neutral (83%)	DDG: -1.50, RI: 9	other species: various amino acids CS: 1
	535	GAA -> E	GCA -> A	E: polar, + charge A: hydrophobic side chain, non-polar	PhD-SNP tool: deleterious (59%) other tools: neutral (74%)	DDG: 0.0, RI: 4	other species: various amino acids CS: 1
	565	AGT -> S	TAT -> Y	S: polar Y: polar	all tools: neutral (83%)	DDG: -1.05, RI: 5	other species: various amino acids CS: 1
	577	ATA -> I	ACA -> T	I: neutral, hydrophobic side chain T: polar	all tools: neutral (83%)	DDG: -0.08, RI: 2	other species: various amino acids CS: 6
	625	CTT -> L	CCA -> P	L: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -0.02, RI: 1	other species: various amino acids CS: 1

					P: aromatic side chain			
			-> E -> K	-> Q	E: - charge K: + charge Q: neutral	Q210E -> all tools: neutral (83%) Q210K -> SIFT tool: deleterious (46%), other tools: neutral (74%)	DDG: 0.10, RI: 4 DDG: -0.23, RI: 1	other species: various amino acids CS: 6
		637	ACA -> T	GCC -> A	T: polar A: hydrophobic side chain, non-polar	PolyPhen-2 tool: deleterious (40%) other tools: neutral (74%)	DDG: -0.36, RI: 4	other species: various amino acids CS: 2
		655	CAG -> Q	CAC -> H	Q: neutral H: + charge	all tools: neutral (83%)	DDG: 0.26, RI: 1	other species: various amino acids CS: 1
			-> L -> A	-> P	L: neutral, hydrophobic side chain A: hydrophobic side chain, non-polar P: aromatic side chain	P240L -> all tools: neutral (83%) P240A -> PolyPhen-2 tool: deleterious (40%), other tools: neutral (75%)	DDG: -0.01, RI: 1 DDG: -1.01, RI: 6	other species: various amino acids CS: 1
		742	AGC -> S	ATG -> M	S: polar M: non-polar	MAPP tool: deleterious (41%) other tools: neutral (74%)	DDG: -0.96, RI: 3	other species: various amino acids CS: 7
		796	AAC -> N	CAC -> H	N: neutral, polar H: + charge	all tools: neutral (83%)	DDG: -0.12, RI: 1	other species: N, E, D, G, Q, H CS: 5
	<i>Ribosomal RNA small subunit methyltransferase H (RsmH)</i>	37	CTT -> L	TTT -> F	L: neutral, hydrophobic side chain F: non-polar, no S-group	all tools: neutral (83%)	DDG: -0.89, RI: 8	other species: L, F CS: 3
		43	AAA -> K	GAG -> E	K: + charge E: polar, + charge	all tools: neutral (83%)	DDG: -0.68, RI: 8	other species: various amino acids CS: 4
		58	AGG -> R	AAG -> K	R/K: + charge	all tools: neutral (83%)	DDG: 0.04, RI: 1	other species: various amino acids CS: 3

		70	ATT -> I	GTT -> V	I: neutral, hydrophobic side chain V: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.33, RI: 4	other species: various amino acids CS: 3
		76	ATT -> I	GTG -> V	I: neutral, hydrophobic side chain V: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.36, RI: 4	other species: C, V, A, M, L, I, F CS: 6
		121	CAA -> Q	GAA -> E	Q: neutral E: polar, + charge	all tools: neutral (83%)	DDG: -0.41, RI: 5	other species: various amino acids CS: 1
		133	AAT -> N	CGC -> R	L: neutral, hydrophobic side chain F: non-polar, no S- group	all tools: neutral (83%)	DDG: -1.12, RI: 8	other species: various amino acids CS: 1
		139	CAC -> H	CGT -> R	H/R: + charge	PANTHER tool: deleterious (69%) other tools: neutral (83%)	DDG: -1.23, RI: 9	other species: various amino acids CS: 4
		142	ATT -> I	GTC -> V	I: neutral, hydrophobic side chain V: neutral, hydrophobic	all tools: neutral (83%)	DDG: -0.32, RI: 4	other species: F, I, L, Y, A, V CS: 7
		145	GTC > V	ATA -> I	V: neutral, hydrophobic I: neutral, hydrophobic side chain	all tools: neutral (83%)	DDG: -1.04, RI: 8	other species: various amino acids CS: 5
		163	AAA -> K	CGC -> R	K/R: + charge	all tools: neutral (83%)	DDG: -0.92, RI: 8	other species: various amino acids CS: 1
		166	GAA -> E	CAA -> Q	E: polar, + charge Q: neutral	all tools: neutral (83%)	DDG: -0.32, RI: 4	other species: various amino acids CS: 1

		172	CAA -> Q	AGC -> S	Q: neutral S: polar	PhD-SNP tool: deleterious (61%) other tools: neutral (74%)	DDG: -0.12, RI: 3	other species: various amino acids CS: 4
		184	CTT -> L	TCA -> S	L: neutral, hydrophobic side chain S: polar	PhD-SNP tool: deleterious (61%) other tools: neutral (74%)	DDG: 0.41, RI: 7	other species: various amino acids CS: 1
		187	GAC -> D	GAA -> E	D: + charge E: polar, + charge	PhD-SNP tool: deleterious (61%) SIFT tool: deleterious (45%) other tools: neutral (65%)	DDG: -0.61, RI: 5	other species: various amino acids CS: 2
		190	AAA -> K	AGA -> R	K/R: + charge	PhD-SNP tool: deleterious (59%) other tools: neutral (74%)	DDG: -0.71, RI: 6	other species: various amino acids CS: 5
		196	ACC -> T	GCC -> A	T: polar A: hydrophobic side chain, non-polar	MAPP tool: deleterious (46%) PhD-SNP tool: deleterious (77%) other tools: neutral (63%)	DDG: -0.83, RI: 8	other species: various amino acids CS: 1
		199	CCC -> P	GCC -> A	P: aromatic side chain A: hydrophobic side chain, non-polar	PhD-SNP tool: deleterious (61%) other tools: neutral (74%)	DDG: -0.09, RI: 3	other species: various amino acids CS: 1
		205	AAA -> K	ACG -> T	K: + charge T: polar	all tools: neutral (83%)	DDG: -1.05, RI: 8	other species: various amino acids CS: 1
		209	GAG -> E	GAC -> D	E: polar, + charge D: + charge	all tools: neutral (83%)	DDG: 0.01, RI: 0	other species: various amino acids CS: 1
		217	ACC -> T	AAC -> N	T: polar N: neutral, polar	all tools: neutral (83%)	DDG: -0.23, RI: 3	other species: various amino acids CS: 1
		220	CAC -> H	TAT -> Y	H: + charge Y: polar	MAPP tool: deleterious (76%) PhD-SNP tool:	DDG: -1.32, RI: 4	other species: various amino acids CS: 3

						deleterious (59%) other tools: neutral (63%)		
		223	AAA -> K	TGT -> C	K: + charge C: polar, S-group	PhD-SNP tool: deleterious (61%) other tools: neutral (75%)	DDG: -0.76, RI: 6	other species: various amino acids CS: 2
		227	CAG -> Q	GCG -> A	Q: neutral A: hydrophobic side chain, non-polar	PhD-SNP tool: deleterious (68%) other tools: neutral (75%)	DDG: -0.67, RI: 8	other species: various amino acids CS: 3
		241	CAA -> Q	ACA -> T	Q: neutral T: polar	MAPP tool: deleterious (59%) other tools: neutral (74%)	DDG: -0.99, RI: 7	other species: various amino acids CS: 1
		262	TCC -> S	CCC -> P	S: polar P: aromatic side chain	PhD-SNP tool: deleterious (59%) PANTHER tool: deleterious (70%) other tools: neutral (74%)	DDG: -1.29, RI: 8	other species: various amino acids CS: 1
		265	CCA -> P	ATT -> I	P: aromatic side chain I: neutral, hydrophobic side chain	Deleterious (51%) in all tools, except for PolyPhen-1 tool (neutral 67%), PolyPhen-2 tool (neutral 69%) and SNAP tool (neutral 61%)	DDG: -1.60, RI: 6	other species: various amino acids CS: 6
		268	CAG -> Q	GAT -> D	Q: neutral D: + charge	MAPP tool: deleterious (46%) PhD-SNP tool: deleterious (86%) PolyPhen-2 tool: deleterious (43%) PANTHER tool: deleterious (57%) other tools: neutral (60%)	DDG: -0.36, RI: 3	other species: various amino acids CS: 7

		313	AAC -> N	AGC -> S	N: neutral, polar S: polar	all tools: neutral (83%)	DDG: -0.47, RI: 2	other species: various amino acids CS: 1
		319	AAT -> N	CAT -> H	N: neutral, polar H: + charge	all tools: neutral (83%)	DDG: -0.54, RI: 1	other species: various amino acids CS: 4
		337	AAC -> N	CAC -> H	N: neutral, polar H: + charge	all tools: neutral (83%)	DDG: -0.67, RI: 2	other species: various amino acids CS: 4
		340	GCC -> A	TCT -> S	A: hydrophobic side chain, non-polar S: polar	MAPP tool: deleterious (62%) SIFT tool: deleterious (45%) other tools: neutral (65%)	DDG: -0.77, RI: 8	other species: various amino acids CS: 7
		346	AGC -> S	AAT -> N	S: polar N: neutral, polar	all tools: neutral (83%)	DDG: -0.45, RI: 4	other species: various amino acids CS: 6
		352	ACG -> T	ATG -> M	T: polar M: non-polar	PANTHER tool: deleterious (61%) other tools: neutral (83%)	DDG: -1.09, RI: 7	other species: various amino acids CS: 1
		355	GGG -> G	GAG -> E	G: non-polar, no S- group E: polar, + charge	all tools: neutral (83%)	DDG: -1.09, RI: 6	other species: various amino acids CS: 1
		403	GGC -> G	CAT -> H	G: non-polar, no S- group H: + charge	PhD-SNP tool: deleterious (58%) other tools: neutral (74%)	DDG: -0.68, RI: 3	other species: various amino acids CS: 1
		439	ACG -> T	GCG -> A	T: polar A: hydrophobic side chain, non-polar	all tools: neutral (83%)	DDG: -0.52, RI: 4	other species: various amino acids CS: 6
		484	CAA -> Q	CGG -> R	Q: neutral R: + charge	all tools: neutral (83%)	DDG: -0.92, RI: 8	other species: various amino acids CS: 1

Supplementary Table S6. Genes investigated for the antimicrobial resistance mechanism with no SNPs founded.

	Gene	Protein	SNPs
Acquired resistance to azithromycin (macrolide) in ASB19	<i>rplA</i>	50S ribosomal protein L1	No
	<i>rplJ</i>	50S ribosomal protein L10	No
	<i>rplK</i>	50S ribosomal protein L11	No
	<i>rplM</i>	50S ribosomal protein L13	No
	<i>rplN</i>	50S ribosomal protein L14	No
	<i>rplO</i>	50S ribosomal protein L15	No
	<i>rplP</i>	50S ribosomal protein L16	No
	<i>rplQ</i>	50S ribosomal protein L17	No
	<i>rplR</i>	50S ribosomal protein L18	No
	<i>rplS</i>	50S ribosomal protein L19	No
	<i>rplT</i>	50S ribosomal protein L20	No
	<i>rplU</i>	50S ribosomal protein L21	No
	<i>rplV</i>	50S ribosomal protein L22	No
	<i>rplW</i>	50S ribosomal protein L23	No
	<i>rplX</i>	50S ribosomal protein L24	No
	<i>rplY</i>	50S ribosomal protein L25	No
	<i>rpmA</i>	50S ribosomal protein L27	No
	<i>rpmB</i>	50S ribosomal protein L28	No
	<i>rpmC</i>	50S ribosomal protein L29	No
	<i>rpmE</i>	50S ribosomal protein L31	No
	<i>rpmF</i>	50S ribosomal protein L32	No
	<i>rpmG2</i>	50S ribosomal protein L33 2	No
	<i>rpmH</i>	50S ribosomal protein L34	No
	<i>group_744</i>	50S ribosomal protein L35	No
	<i>rplD</i>	50S ribosomal protein L4	No
	<i>rplE</i>	50S ribosomal protein L5	No
	<i>rplF</i>	50S ribosomal protein L6	No
	<i>rplL</i>	50S ribosomal protein L7/L12	No
	<i>rplI</i>	50S ribosomal protein L9	No

	<i>rluA</i>	Ribosomal large subunit pseudouridine synthase A	No
	<i>rluB</i>	Ribosomal large subunit pseudouridine synthase B	No
	<i>rluD_2</i>	Ribosomal large subunit pseudouridine synthase D	No
	<i>rluD_1</i>	Ribosomal large subunit pseudouridine synthase D	No
	<i>prmA</i>	Ribosomal protein L11 methyltransferase	No
Acquired resistance to spectinomycin (aminoglycoside) in ASB1.4	<i>rpsJ</i>	30S ribosomal protein S10	No
	<i>rpsK</i>	30S ribosomal protein S11	No
	<i>rpsM</i>	30S ribosomal protein S13	No
	<i>rpsZ</i>	30S ribosomal protein S14 type Z	No
	<i>rpsO</i>	30S ribosomal protein S15	No
	<i>rpsP</i>	30S ribosomal protein S16	No
	<i>rpsQ</i>	30S ribosomal protein S17	No
	<i>rpsR</i>	30S ribosomal protein S18	No
	<i>rpsS</i>	30S ribosomal protein S19	No
	<i>rpsB</i>	30S ribosomal protein S2	No
	<i>rpsT</i>	30S ribosomal protein S20	No
	<i>rpsU</i>	30S ribosomal protein S21	No
	<i>rpsC</i>	30S ribosomal protein S3	No
	<i>rpsD</i>	30S ribosomal protein S4	No
	<i>rpsE</i>	30S ribosomal protein S5	No
	<i>rpsF</i>	30S ribosomal protein S6	No
	<i>rpsH</i>	30S ribosomal protein S8	No
	<i>rpsI</i>	30S ribosomal protein S9	No
	<i>rimO</i>	Ribosomal protein S12 methylthiotransferase RimO	No
	<i>rlmH group_1616</i>	Ribosomal RNA large subunit methyltransferase H	No
	<i>rsmA group_2011</i>	Ribosomal RNA small subunit methyltransferase A	No
	<i>rsmE group_892</i>	Ribosomal RNA small subunit methyltransferase E	No
	<i>rsmG</i>	Ribosomal RNA small subunit methyltransferase G	No
	<i>rsmH</i>	Ribosomal RNA small subunit methyltransferase H	No
	<i>rsmI</i>	Ribosomal RNA small subunit methyltransferase I	No
	<i>rsfS</i>	Ribosomal silencing factor RsfS	No
	<i>rpsJ</i>	30S ribosomal protein S10	No

Aminoglycoside decreased susceptibility in <i>H. heilmannii</i> isolates	<i>rpsK</i>	30S ribosomal protein S11	No
	<i>rpsL</i>	30S ribosomal protein S12	No
	<i>rpsM</i>	30S ribosomal protein S13	No
	<i>rpsZ</i>	30S ribosomal protein S14 type Z	No
	<i>rpsQ</i>	30S ribosomal protein S17	No
	<i>rpsR</i>	30S ribosomal protein S18	No
	<i>rpsS</i>	30S ribosomal protein S19	No
	<i>rpsT</i>	30S ribosomal protein S20	No
	<i>rpsC</i>	30S ribosomal protein S3	No
	<i>rpsD</i>	30S ribosomal protein S4	No
	<i>rpsE</i>	30S ribosomal protein S5	No
	<i>rpsG</i>	30S ribosomal protein S7	No
	<i>rpsH</i>	30S ribosomal protein S8	No
	<i>rsmE</i> group_892	Ribosomal RNA small subunit methyltransferase E	No
	<i>rsmG</i>	Ribosomal RNA small subunit methyltransferase G	No
	<i>rsmA</i> group_2011	Ribosomal RNA small subunit methyltransferase A	No

Supplementary Table S7. Comparison of MIC values of *H. heilmannii* and *H. ailurogastricus* with others gastric *Helicobacter* spp.

	MICs <i>H. heilmannii</i> (µg/ml)	MICs <i>H. ailurogastricus</i> (µg/ml)	MICs other gastric <i>Helicobacter</i> sp. (µg/ml)	References
Ampicillin	0.125 - 1	0.125 - 1	<i>H. pylori</i> : 0.015-0.125 <i>H. suis</i> : 0.125-8 <i>H. bizzozeronii</i> : 0.06-0.125 <i>H. felis</i> : 0.03-0.25 <i>H. salomonis</i> : 0.03-0.125	Loo <i>et al.</i> , 1997 Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019
Ceftiofur	0.5 - 8	4 - 16	<i>H. pylori</i> : no data <i>H. suis</i> : 1-32 <i>H. bizzozeronii</i> : no data <i>H. felis</i> : no data <i>H. salomonis</i> : no data	Berlamont <i>et al.</i> , 2019
Clarithromycin	≤0.03125 - 0.25	0.0625 – 0.25	<i>H. pylori</i> : 0.015-0.125 <i>H. suis</i> : 0.03-0.5 <i>H. bizzozeronii</i> : 0.06-0.125 <i>H. felis</i> : 0.03-0.25 <i>H. salomonis</i> : 0.03	Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019
Tylosin	0.5 - 8	1 - 4	<i>H. pylori</i> : no data	Van den Bulck <i>et al.</i> , 2005

			<i>H. suis</i> : 0.5-16 <i>H. bizzozeronii</i> : 0.125-0.5 <i>H. felis</i> : 0.03-0.25 <i>H. salomonis</i> : 0.03-0.125	Berlamont <i>et al.</i> , 2019
Azithromycin	≤0.03125 - 0.25	≤0.03125 - 0.125	<i>H. pylori</i> : no data <i>H. suis</i> : no data <i>H. bizzozeronii</i> : no data <i>H. felis</i> : no data <i>H. salomonis</i> : no data	
Lincomycin	0.5 - 32	4 - 16	<i>H. pylori</i> : no data <i>H. suis</i> : 2-128 <i>H. bizzozeronii</i> : 0.5-8 <i>H. felis</i> : 0.25-8 <i>H. salomonis</i> : 0.25-1	Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019
Enrofloxacin	≤0.03125 - 0.25	≤0.03125 - 0.25	<i>H. pylori</i> : no data <i>H. suis</i> : 0.03-8 <i>H. bizzozeronii</i> : 0.03-0.125 <i>H. felis</i> : 0.03-0.06 <i>H. salomonis</i> : 0.03-0.125	Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019
Levofloxacin	≤0.03125 - 0.25	≤0.03125 - 0.0625	<i>H. pylori</i> : no data	Berlamont <i>et al.</i> , 2019

			<i>H. suis</i> : 0.03-32 <i>H. bizzozeronii</i> : no data <i>H. felis</i> : no data <i>H. salomonis</i> : no data	
Rifampicin	≤0.03125 - 0.125	≤0.03125 - 0.25	<i>H. pylori</i> : no data <i>H. suis</i> : 0.03-4 <i>H. bizzozeronii</i> : no data <i>H. felis</i> : no data <i>H. salomonis</i> : no data	Berlamont <i>et al.</i> , 2019
Gentamicin	2 - 32	4 - 16	<i>H. pylori</i> : no data <i>H. suis</i> : 1-16 <i>H. bizzozeronii</i> : 0.06-0.25 <i>H. felis</i> : 0.06-0.25 <i>H. salomonis</i> : 0.125	Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019
Spectinomycin	≤0.03125 - 32	0.0625 - 0.25	<i>H. pylori</i> : no data <i>H. suis</i> : 0.25-32 <i>H. bizzozeronii</i> : 0.5-2 <i>H. felis</i> : 0.5-4 <i>H. salomonis</i> : 2	Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019
Neomycin	2 – 32	0.5 - 2	<i>H. pylori</i> : no data	Van den Bulck <i>et al.</i> , 2005

			<i>H. suis</i> : 2-128 <i>H. bizzozeronii</i> : 0.25-0.5 <i>H. felis</i> : 0.125-0.5 <i>H. salomonis</i> : 0.25-0.5	Berlamont <i>et al.</i> , 2019
Oxytetracycline	≤0.03125 – 0.25	≤0.03125 - 0.125	<i>H. pylori</i> : 0.125-1 <i>H. suis</i> : no data <i>H. bizzozeronii</i> : 0.25-1 <i>H. felis</i> : 0.125-1 <i>H. salomonis</i> : 0.125-0.5	Loo <i>et al.</i> , 1997 Van den Bulck <i>et al.</i> , 2005
Doxycycline	0.0625 – 0.25	0.0625 - 0.25	<i>H. pylori</i> : no data <i>H. suis</i> : 0.06-32 <i>H. bizzozeronii</i> : no data <i>H. felis</i> : no data <i>H. salomonis</i> : no data	Berlamont <i>et al.</i> , 2019
Metronidazole	0.25 - 4	0.025 - 0.5	<i>H. pylori</i> : 64-256 <i>H. suis</i> : 0.5-64 <i>H. bizzozeronii</i> : 1-8 <i>H. felis</i> : 0.5-16 <i>H. salomonis</i> : 0.5	Loo <i>et al.</i> , 1997 Van den Bulck <i>et al.</i> , 2005 Berlamont <i>et al.</i> , 2019

**Epidemiological and clinical
implication of gastric
Helicobacter spp. occurrence
in human patients**

3 CHAPTER

Paper III

Epidemiological and clinical implications of gastric
Helicobacter spp. occurrence in human patients

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2021

Manuscript in preparation

Epidemiological and clinical implications of gastric *Helicobacter* spp. occurrence in human patients

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Abstract

The genus *Helicobacter* is composed of bacteria that colonize both the human and animal gastrointestinal tract. *Helicobacter pylori* infects half of the world's population, causing several disorders, such as gastritis, duodenitis and gastric cancer. Additionally, other *Helicobacter* species are commonly found in the stomach of pigs, dogs and cats. Most of these non-*Helicobacter pylori* *Helicobacter* species (NHPH) have zoonotic potential and prevalence rates of 0.2-6% have been described in human patients suffering from gastric disorders. The aim of this study was to determine the occurrence of *Helicobacter* spp. in the stomach of two distinct groups of patients, such as gastric cancer ($n=17$) and obese ($n=63$) patients. For that, histology and PCR with sequencing of the amplicon, were used. Furthermore, in order to evaluate a possible relation between gastric *Helicobacter*

infection and eventual animal contact or previously performed *Helicobacter* eradication therapy, each patient answered a questionnaire where this information was addressed.

Overall, *Helicobacter* spp. were detected in 46.3% of the patients, including single infection by *H. pylori* in 43.8% of the patients (35 out of 80) and a mixed infection by *H. pylori* and canine- or feline-associated *H. felis* in 2.5% (2 out of 80). About 32.5% of the patients were subjected to previous *Helicobacter* eradication therapy and the triple standard therapy was the most frequent scheme (42.3%).

In 48.0% of the patients submitted to the eradication treatment, bacteria were still detected, including one mixed infection. Within the 26 patients (32.5%) that reported having performed a subsequent test to confirm the elimination of the bacteria, in 23.1% of them bacteria were still detected.

In conclusion, although in a smaller percentage, NHPH are also present in the human stomach and these seem to use different methods of colonization, infection and even resistance mechanisms from *H. pylori*. Thus specific NHPH screening should be included on the diagnostic routine along with proper and specific treatment. The still *H. pylori* detection in the stomach of patients recently subjected to eradication schemes, raise questions about the effectiveness of the current *Helicobacter* treatments.

Keywords: *Helicobacter*; NHPH; Human stomach; Gastric disease; Eradication treatment

Introduction

The *Helicobacter* genus consists of a group of Gram-negative, motile bacteria, colonizing the stomach and intestinal tract of humans and animals (Ali B 2018). *Helicobacter pylori* (*H. pylori*) is the most common gastric species, with an average worldwide prevalence of 58%, and is considered a major public health issue (Doulberis M 2020; Hooi JKY 2017; van Amsterdam K 2006). Infection is usually acquired during childhood and although the majority of the individuals remain asymptomatic, some develop gastric disorders, of which chronic gastritis is the most frequently described pathology (Ali B 2018; Blaser N 2019; Hooi JKY 2017; van Amsterdam K 2006). In the

majority of the cases, the gastritis is superficial, but may progress into gastric or duodenal ulcers (10-20%) and/or gastric (adeno)carcinoma (1-2%) (Blaser N 2019; van Amsterdam K 2006). As such, *H. pylori* is considered a Group I carcinogen by the International Agency for Research on Cancer (Blaecher C 2013; Rodrigues MF 2019; van Amsterdam K 2006). Multiple studies have shown that *H. pylori* eradication reduces gastric inflammation, thereby i) preventing the progression towards pre-malignant lesions, ii) decreasing the incidence of gastric carcinoma (Cai X 2005; Rodrigues MF 2019; Yang-Ou YB 2018) and iii) improving corpus gastritis and dysplasia lesions (De Briyne N 2014). Nevertheless, treatment efficacy has been declining in the last decade due to several factors such as side effects, including increasing antimicrobial resistance and the high costs of the antibiotic treatments (De R 2009).

Besides *H. pylori*, other gastric helicobacters, named non-*Helicobacter pylori* helicobacters (NHPH), have been described colonizing both the human and animal stomach (Bento-Miranda M 2014; Berlamont H 2019; De Witte C 2018; Matos R 2020). So far, *H. suis*, *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. salomonis* and *H. bizzozeronii* were described in human patients (Ali B 2018; Boyanova L 2003; Haesebrouck F 2009; Sýkora J 2003; Yakoob J 2012). However, the infection rate is significantly lower than for *H. pylori*, with reported rates of 0.2-6.0% (Haesebrouck F 2009). Nevertheless, these values can be an underestimation of the true prevalence, considering the lack of proper diagnostic tools (Haesebrouck F 2009).

Human gastric NHPH infection is usually accompanied by gastritis, antral erosions and duodenal ulcers (Ali B 2018; Haesebrouck F 2009; Joosten M 2016). Furthermore, NHPH infection has also been associated with gastric low-grade MALT lymphoma, and the risk of developing this disease is higher than that with *H. pylori* infection (Morgner A 2000). Human infections with NHPH most likely originate from animals. Thus, living in close proximity with animals is considered a major risk factor. Besides frequency, the intensity of animal contact is also important, since a higher incidence of these infections has been noted in farmers, staff of slaughterhouses, pet owners and children having intense contact with pets. Other transmission routes might be consumption of contaminated water and for *H. suis* consumption of contaminated pork. Recent studies also demonstrated that patients infected with NHPH may be co-

infected with *H. pylori* and/or with more than two *Helicobacter* species (Bento-Miranda M 2014; Liu J 2015).

H. pylori infection rate is related to socioeconomic status, level of urbanization, sanitation, access to clean water and hygiene levels. This may explain why higher numbers of cases have been reported in developing countries (almost 80.0% on Africa), contrasting with low levels in the developed ones (p.e. 24.4% on Oceania) (Hooi JKY 2017). In Europe, the overall prevalence is around 47.0%, but in Portugal can be as higher as 86.4% (Hooi JKY 2017). At individual level, several risk factors for *H. pylori* infection have been proposed like older age, smoking, untreated water intake, alcohol and salty food consumption and family history of gastric disorders (Borges SS 2019). It can be concluded that the disease outcome of *Helicobacter* spp. infection is a result of a complex interaction between host, environmental and bacterial factors (van Amsterdam K 2006).

The aim of the current research was therefore to determine the occurrence of *Helicobacter* spp. in human gastric mucosa samples collected in different clinical settings and using several diagnostic approaches. Additionally, further considerations regarding the efficacy/ efficiency of the eradication treatment were evaluated.

Materials and Methods

Patients samples

Stomach samples were collected from patients submitted to stomach resection surgery to treat gastric cancer (GC) ($n=17$) or to surgical treatment of morbid obesity through laparoscopic sleeve gastrectomy ($n=63$). In patients diagnosed with gastric cancer, two samples were collected from tumor and adjacent tumor tissue. In obese patients, three samples were collected from body/fundus region. Regardless of the origin, each specimen was divided in two fragments: one was fixed in 10% formalin for histological evaluation and the other was immediately frozen at -80°C for further molecular studies. Samples were collected between March 2017 and July 2020, from surgeries taken place at Centro Hospitalar Universitário de São João (CHUSJ), Porto, Portugal. All the patients included in the study were informed about the project and their written consent was requested. During the pre-surgical consultation, the medical doctor performed a small and anonymous inquiry to the patients, concerning personal

details (age and gender), information regarding putative animal contact (frequency and type of animals), and evidence of a possible *Helicobacter* eradication treatment carried out in the 18 months prior to the gastric surgery. None of the participants were considered vulnerable individuals. All the procedures were approved by the Ethics Committee from the CHSJ/FMUP (CES 127-15).

Histological analysis of Helicobacter species in human gastric samples

Samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Serial sections of 3 µm were made, one being stained with haematoxylin-eosin (H&E) for histopathological evaluation and the others were used in the immunohistochemical study.

Sections stained with H&E were examined by a pathologist in order to confirm the gastric anatomical region. Additionally, a modified-Giemsa stain was performed to assist in the identification of *Helicobacter* spp. The bacterial density colonization was quantified according to the following classification: -, absence of organisms; +, few organisms (<10 organisms/400x); ++, moderate number of organisms (10 to 50 organisms/400x); +++, large number of organisms (>50 organisms/400x), as previously described (Prachasilpchai W 2007).

Immunohistochemical detection of Helicobacter species in human gastric samples

For the immunohistochemical study, sections were deparaffinized, hydrated and antigen retrieval was performed in 10 mmol/L sodium citrate buffer, pH 6.0, for 10 minutes. Slides were cooled for 20 minutes at room temperature and rinsed twice in phosphate buffered saline with 0.05% Tween 20 (PBS-T 0.05%) for 5 minutes. After blocking endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 minutes, slides were blocked for 30 minutes with normal goat serum (Dako, California, USA), diluted 1:5 in 10% bovine serum albumin (BSA 10%). Thereafter, slides were incubated overnight at 4 °C with a primary polyclonal antiserum against *H. pylori* (RBK012; Zytomed, Berlin, Germany), diluted 1:150 in BSA 5%, known to present immunoreactivity with a wide range of bacteria belonging to the *Helicobacter* genus (Amorim I 2015). Sections were rinsed with PBS-T 0.05% and incubated with secondary

antibody goat anti-rabbit diluted 1:200 in BSA 5%, for 30 minutes (Dako, California, USA). Slides were rinsed again and incubated with avidin-biotin-peroxidase complex solution for 30 minutes (Vectastain® Elite ABC HRP Kit, PK-6100, Vector Laboratories, USA). Colour was developed with 3,3 α -diamino-benzidine (DAB; Sigma, St. Louis, MO, USA) for 5 to 7 minutes at room temperature. Sections were then counterstained with haematoxylin, dehydrated and mounted. Slides were observed at light microscopy and positive immunoreactivity was recorded as a distinct golden-brown labelling of the bacteria located on mucosal surface, in gastric pits or glands and in parietal cells. Again, the amount of bacteria was evaluated according to the same classification described on the section above (Prachasilpchai W 2007).

DNA extraction and Helicobacter species identification through PCR analysis

Cryopreserved gastric samples were homogenized, and DNA was extracted using the ExtractMe DNA Tissue Plus Kit (EM04, Blirt, Poland), according to the manufacturer instructions.

The primers *Hcom1* and *Hcom2* (Table S1) were used to amplify a 389-bp fragment of the 16S rRNA gene sequence to determine the presence of *Helicobacter*-genus bacteria (Choi YK 2001; Farshad S 2004, 2006). Reaction mixtures contained 0.2 μ l *Taq* DNA Polymerase (Supreme NZY*Taq* II DNA Polymerase, NZYTech, Portugal), 1.2 μ l MgCl₂ (NZYTech, Portugal), 2.5 μ l 5x Reaction Buffer (NZYTech, Portugal), 4 μ l dNTPs (ThermoFisher Scientific, Massachusetts, USA), 0.2 μ l of forward and reverse primers and 9.7 μ l HPLC water. PCR amplification was performed under the following conditions: 5 min of preincubation at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 63 °C, and 1 min at 72 °C. A final extension was performed for 5 min at 72 °C. As a positive control, DNA extracted from a pure culture of *H. suis* (HS1) was used (Baele M 2008).

The primers used for *Helicobacter* species-specific PCR and the respective amplification conditions are listed in Table S1. Reaction mixtures contained 0.2 μ l *Taq* DNA Polymerase Supreme NZY*Taq* II DNA Polymerase, (NZYTech, Portugal), 1.2 μ l MgCl₂ (NZYTech, Portugal), 2.5 μ l 5x Reaction Buffer (NZYTech, Portugal), 4 μ l dNTPs (ThermoFisher Scientific, Massachusetts, USA), 0.1 μ l of forward and reverse primers

and 9.9 µl HPLC water. DNA extracted from a pure culture of *H. pylori* (26695) was used as positive control.

Ten µl of each PCR amplification product was analysed through gel electrophoresis in 1.5% agarose (SeaKem® LE Agarose, Lonza, Switzerland) in TBE buffer (Tris-Borate-EDTA Buffer 10x, GRiSP, Portugal). 1Kb Plus DNA Ladder (Invitrogen™, Massachusetts, USA) was used as a weight marker. Images were acquired on a GelDoc Imaging system (Bio-Rad, USA) and analysis was performed using the ImageLab™ software (Bio-Rad, USA).

Positive samples (on genus and species-specific PCR) were then used for sequencing analysis of the obtained amplicon in order to confirm the presence of *Helicobacter* species.

Results

Patients Data

A total of 221 gastric samples belonging to 80 patients were evaluated in this study, comprising 34 samples obtained from 17 gastric cancer (GC) patients and 187 samples from 63 obese (OB) patients. The mean age was 50.9 ± 14.8 years (72.5 ± 10.0 for the GC patients and 45.1 ± 9.6 for the OB patient). Female:male ratio for the overall patients evaluated was 1.8:1.

Histological and Immunohistochemical detection of Helicobacter species in human gastric samples

Histological analysis using haematoxylin-eosin stain (H&E) confirmed the presence of malignant epithelial neoplasia in tissue fragments from GC patients, while gastric samples from OB patients showed no signs of neoplastic transformation.

Based on the modified-Giemsa (MG) stain, bacteria were detected in 43 out of 80 patients (53.8%). In more detail, *Helicobacter* spp.-like organisms were visualized in 7 out of 17 GC patients (41.2%) and in 36 out of 63 OB patients (57.1%) (Table 1). With routine staining, organisms were mainly identified within the mucus layer and close to the superficial gastric epithelium (Figure 1 A-D).

Using immunohistochemistry (IHC), curve- or spiral-shaped bacteria were identified in 53 out of 80 patients (66.3%). In the cases of GC, *Helicobacter* spp. immunopositivity was detected in 9 of the 17 patients (52.9%), while in the remaining 8 no specific nor compatible immunoreactivity was identified (47.1%). Among the samples of OB patients, *Helicobacter* spp. immunopositivity was detected in 44 out of 63 patients (69.8%), whereas 19 individuals were considered negative (30.2%). Both MG stain and IHC technique allowed the identification of bacteria, not only when they were present in smaller amounts (+/++), but also when present in more discrete locations such as the lumen of deeper glands and intracellularly, in the cytoplasm of parietal cells (Figure 1 E-F, Table 1).

The results of histological and immunohistochemical analysis are presented on Figure 1 and Table 1.

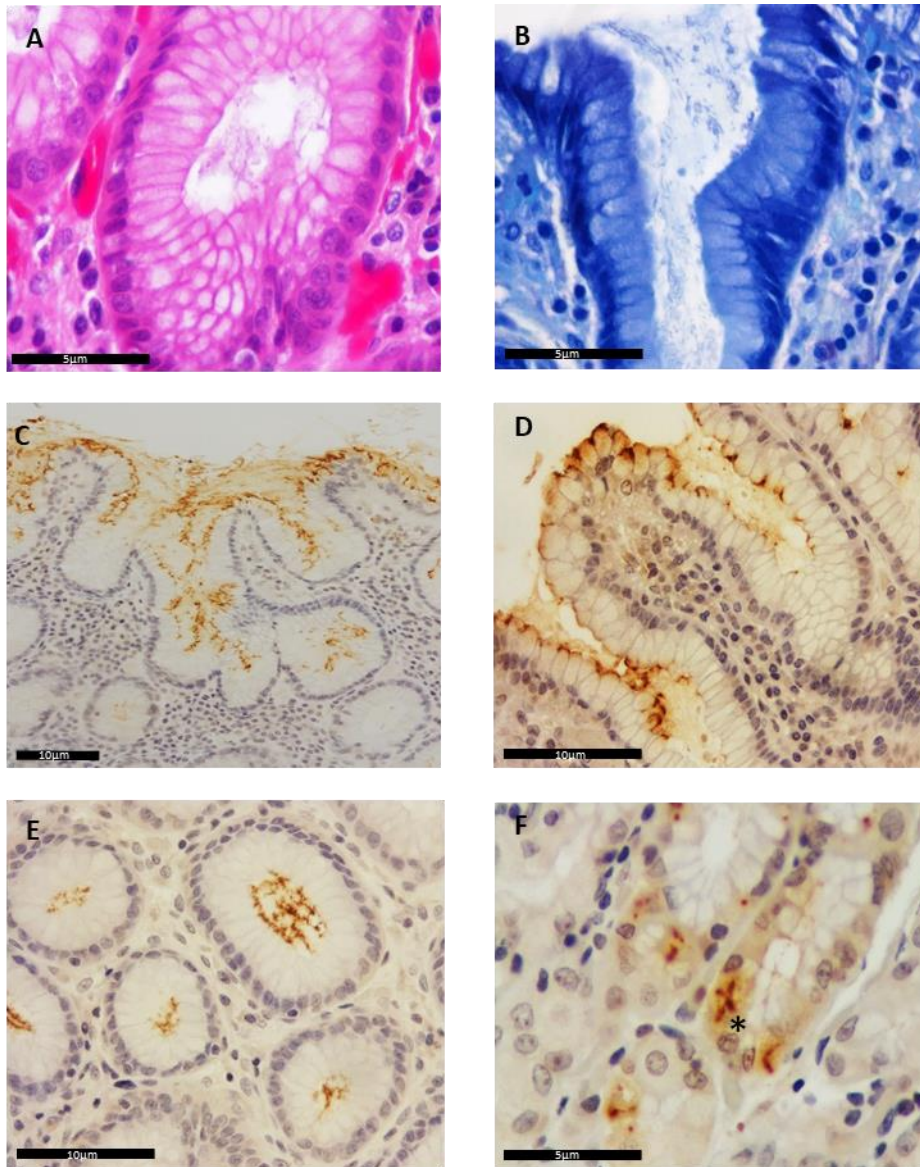


Figure 1. Detection of *Helicobacter* species in human gastric mucosa sections.

(A) H&E stain, 600x and (B) modified-Giemsa stain, highlighting bacteria at the epithelium surface and gastric crypts, 600x. (C-F) *Helicobacter* spp. immunopositivity within the superficial mucus, 200x (C), in the lumen of gastric superficial and deeper glands, 400x (D-E), and inside parietal cells, 600x (F) (*).

Table 1. Occurrence of *Helicobacter* species in human gastric samples, based on different technical approaches.

Gastric Samples	Detection methods		
	Positive (percent & number)		
	MG	IHC	Genus specific-PCR
Total (n =80)	53.8% (43/80)	66.3% (53/80)	46.3% (37/80)
Gastric cancer patients (n =17)	41.2% (7/17)	52.9% (9/17)	11.8% (2/17)
Obese patients (n =63)	57.1% (36/63)	69.8% (44/63)	55.5% (35/63)

Detection of Helicobacter species

PCR for detection of *Helicobacter*-genus was performed in all human gastric samples. Overall analysis showed that 37 out of 80 (46.3%) patients were positive for *Helicobacter* spp. (Table 1). Two out of 17 GC patients were positive for the *Helicobacter* genus (11.8%) while in the OB patient's group, 35 out of 63 presented positivity (55.5%).

Detection of *H. pylori* and NHPH species was also determined using a species-specific PCR. The results indicated that 18 out of 80 (22.5%) individuals were positive for *H. pylori*, while two individuals were positive on the *H. felis* species-specific PCR (2.5%; 2 out of 80). Amongst the positive patients, the majority belonged to the OB group (17 patients; 26.9%), and only two positive patients to the GC group.

The positive amplicons obtained on the genus and species-specific PCR were used for sequencing analysis to confirm the species identified. Overall, 35 out of 80 patients (43.8%) were positive for single infection by *H. pylori*, and a mixed infection with *H. pylori* and *H. felis* was observed in two patients (2 out of 80; 2.5%), both belonging to the OB group.

The results are listed in Table 1. Representative electrophoresis of the PCR products is depicted in Figure S1.

Correlation between Helicobacter spp. presence and animal contact

Among the 80 patients included in the study, only 68 answered the inquiry, of which 13 belong to the GC group and 55 to the OB group. Close contact with animals was referred by 55 patients (80.9%), comprising 11 out of 13 GC (84.6%) and 44 out of 55 OB patients (80.0%). The great majority of the respondents classified the contact with pets as a regular and daily basis (51/55; 92.7%). Close contact with dogs and cats was

mentioned by 8 out of 11 GC patients (72.7%) and 43 out of 44 OB patients (97.7%). Contact with other animals, such as chickens, rabbits, parakeets, sheeps, *etc.*, was also mentioned by 34.5% of the patients, embracing 6 out of 11 GC (54.5%) and 13 out of 44 OB patients (29.5%).

All the information obtained from the inquiries is resumed on Table 2.

Table 2. Overall results of the animal contact based on the patients inquiries.

Inquiry Participation	Yes	No
Overall (<i>n</i> =80)	68 (85.0%)	12 (15.0%)
Gastric cancer patients (<i>n</i> =17)	13 (76.5%)	4 (23.5%)
Obesity patients (<i>n</i> =63)	55 (87.3%)	8 (12.7%)
Animal Contact	Yes	No
Overall (<i>n</i> =68)	55 (80.9%)	13 (19.1%)
Gastric cancer patients (<i>n</i> =13)	11 (84.6%)	2 (15.4%)
Obesity patients (<i>n</i> =55)	44 (80.0%)	11 (20%)
Type of animals	Dogs and cats	Others (chicken, rabbit, birds, etc.)
Overall (<i>n</i> =55)	51 (92.7%)	19 (34.5%)
Gastric cancer patients (<i>n</i> =11)	8 (72.7%)	6 (54.5%)
Obesity patients (<i>n</i> =44)	43 (97.7%)	13 (29.5%)

Helicobacter spp. presence in patients with previous history of eradication treatment

Information regarding previous *Helicobacter* eradication therapy in the last 18 months was collected, either from the inquiries and from the clinical report. Results indicated that 26 out of 80 patients (32.5%) were subjected to the eradication treatment, 51 out of 80 (63.8%) did not receive any treatment, and for 3 patients (3.7%) this information was not available (Figure 2, Table 3).

Regarding the *Helicobacter* eradication scheme, the triple standard therapy (PPI, metronidazole, clarithromycin/amoxicillin) was the most common scheme performed (11 out of 26; 42.3%). Amoxicillin and levofloxacin were used in 4 out of 26 patients (15.4%), and 3 patients (11.5%) were subjected to two different *Helicobacter* eradication protocols (first with the triple standard therapy and later with amoxicillin and levofloxacin). For the remaining 8 patients there was no information available regarding the treatment performed (Figure 2).

The patients submitted to the eradication treatment belong predominantly to the OB group (25 out of 26 patients; 96.2%). Amongst these 25 patients, results indicated that *Helicobacter* organisms were still detected in 12 individuals (48.0%). Based on species-specific PCR and sequencing of the amplicon, a mixed infection with two *Helicobacter* species (*H. pylori* + *H. felis*) was found in one patient (Figure 2, Table 3). The other OB patient presenting a mixed infection with *H. pylori* and *H. felis* was not submitted to any *Helicobacter* eradication scheme in the 18 months prior to the gastric surgery.

In the GC group, only one patient out of 26 performed the eradication scheme prior to surgery (3.8%), and in this particular individual no *Helicobacter* DNA was detected.

For 26 out of 80 patients (32.5%), a confirmatory test was also performed after the eradication treatment, either by urea breath test (UBT) (6 out of 26; 23.1%) or using other methods (upper digestive endoscopy (UDE) (13 out of 26; 50.0%)). For those evaluated through UBT, 5 individuals (19.2%) were negative for urease-producing organisms and only 1 out of 26 was positive (3.9%) (Figure 2). The UDE screening demonstrated that 8 out of 26 (30.8%) patients were negative for *Helicobacter*, while in 5 out of 26 individuals (19.2%) the bacteria were still detected by species-specific PCR followed sequencing of the amplicon. For 7 individuals (26.9%) there was information that a confirmatory test was performed but the result was not mentioned.

All the information is presented in Figure 2 and Table 3.

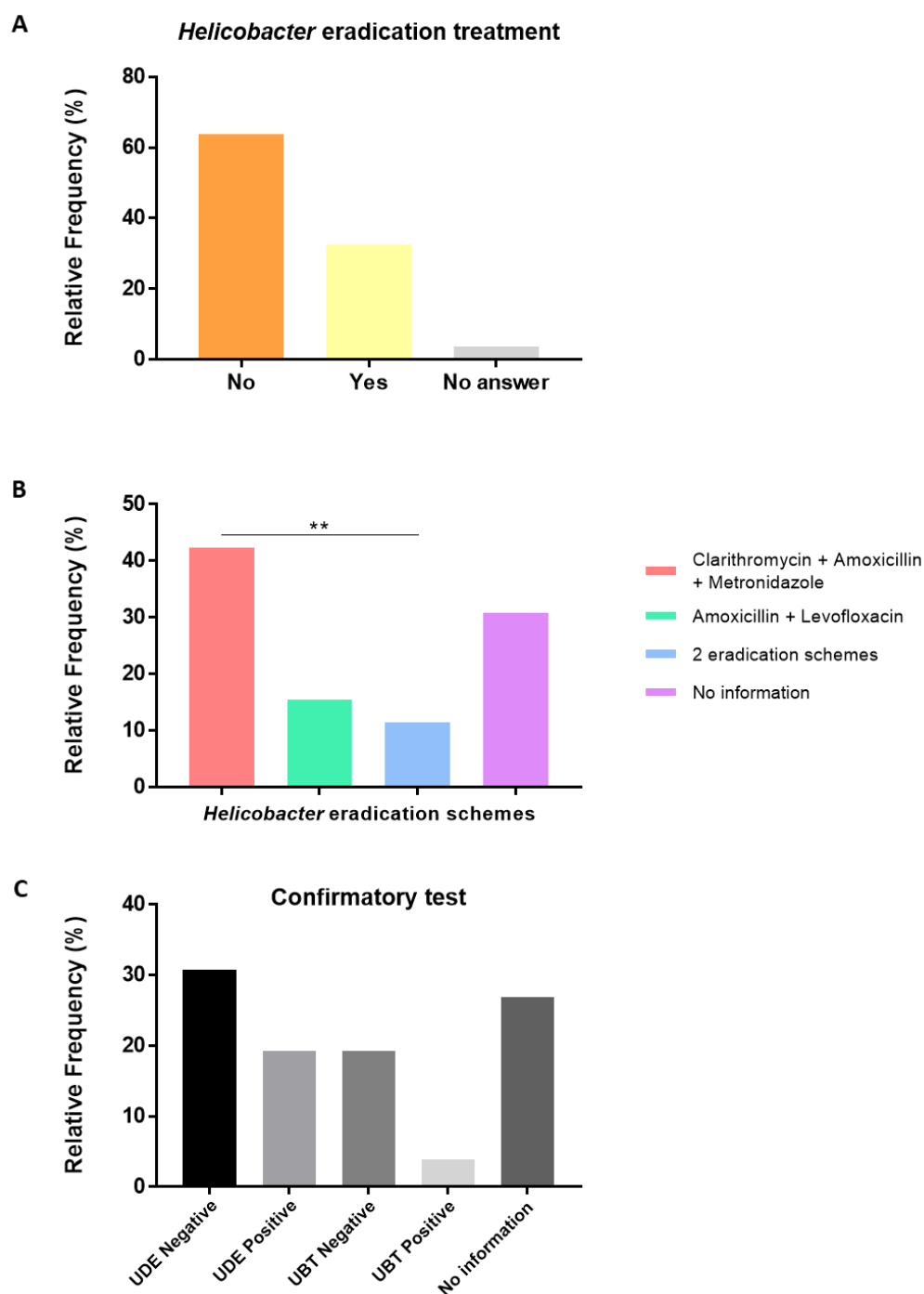


Figure 2. *Helicobacter* eradication treatment. (A) Relative frequency of patients submitted to the *Helicobacter* eradication treatment. (B) Detailed information about the therapeutical schemes used. (C) Confirmatory test performed to evaluate the presence of *Helicobacter* organisms after eradication treatment. UDE: upper digestive endoscopy; UBT: urea breath test.

Table 3. Information about the *Helicobacter* eradication treatment and the current infectious status.

<i>Helicobacter</i> eradication treatment	Yes	No	Unknown/ No answer
Overall (n =80)	32.5% (26/80)	63.8% (51/80)	3.7% (3/80)
Gastric cancer patients (n =17)	5.9% (1/17)	88.2% (15/17)	5.9% (1/17)
Obesity patients (n =63)	39.7% (25/63)	57.1% (36/63)	3.2% (2/63)

Actual infection status	Subjected to <i>Helicobacter</i> eradication treatment	Currently <i>Helicobacter</i> positive
Overall (n =80)	32.5% (26/80)	46.2% (12/26)
Gastric cancer patients (n =17)	5.9% (1/17)	-
Obesity patients (n =63)	39.7% (25/63)	48.0% (12/25)

Discussion

In this study, human gastric samples were collected from 17 GC and 63 OB patients and screened for the presence of *Helicobacter* species. Typically, *Helicobacter* organisms are not easily visualized with H&E, reason why a more specialized staining technique, such as MG, is used to highlight their presence (Amorim I 2015; Prachasilpchai W 2007). Additionally, IHC can also be applied for a more precise bacterial identification (Amorim I 2015). Higher sensitivity for this detection using molecular techniques, such as PCR, is recognized (Chung TH 2014), allowing the identification of the bacteria at genus or species level (Prachasilpchai W 2007). In this regard, herein a conventional *Helicobacter*-genus and a species-specific PCR analysis were performed in order to differentiate between *H. pylori*, *H. suis* and the group of canine/feline NHPH.

The percentage of *Helicobacter*-positive patients based solely on MG and IHC analysis was 53.8% and 66.3%, respectively. IHC also allowed the identification of bacteria in the cytoplasm of the gastric parietal cells. It has been suggested that *H. pylori* may present an inter-epithelial location representing one of the few bacteria able to invade the host cell membrane, and being internalized in gastric epithelial or immune cells (Dubois A 2007). Interestingly, the presence of NHPH inside parietal cells was already described in the canine and feline gastric mucosa and it was thought that this phenomena was an exclusive feature of NHPH (Amorim I 2015; Scanziani E 2001; Wiinberg B 2005). Nevertheless, in the present investigation it was not possible to associate this subcellular location with a specific species of *Helicobacter*.

In the genus specific PCR 46.3% of the samples were positive, while in the species-specific PCR only 23.8% of the samples were positive, with *H. pylori* being detected in 18 patients, while *H. felis* species-specific PCR was detected in two patients. Sequencing of the amplicon obtained confirm that single *H. pylori* infection in 43.8% of the patients, and a mixed infection by *H. pylori* and *H. felis* in two patients (2.5%). This low rate of NHPH occurrence goes towards the values described in the literature (Haesebrouck F 2009). However, it should be noted that in the present study, NHPH have been identified in association with *H. pylori*.

The interaction between *H. pylori* and the human gastric mucosa has been extensively studied (Bento-Miranda M 2014; van Amsterdam K 2006). Infection with *H. pylori* leads to chronic inflammation, characterized by infiltration of neutrophils and macrophages (Lee YC 2016; Rodrigues MF 2019). The induced-inflammation will favour the accumulation of pro-inflammatory cytokines and reactive oxygen/nitrogen species, and eventually may lead to epigenetic changes (Lee YC 2016; Rodrigues MF 2019; Valenzuela MA 2005). Furthermore, human gastric *H. pylori* infection may result in achlorhydria and decreased acid secretion, which affects the composition of gastric microbiome, with steady decrease of bacterial diversity (Mentis AA 2019; Noto JM 2017). Indeed, the density of bacterial colonization by *H. pylori* is reported to be high in the initial steps of infection, but it decreases during the disease progression, being almost devoid of organisms in late stages of cancer transformation that are characterised by marked increase of *Lactobacillus* composition (Dicksved J 2009; Noto JM 2017). However, even in these extreme conditions, *H. pylori* was able to survive and persist, being detected in 11.8% of the GC patients. Regarding the OB group, *H. pylori* was found in 55.5% of the patients. Furthermore, based on species-specific PCR followed by sequencing of the amplicon, two patients exhibited mixed infections with two *Helicobacter* species, namely *H. pylori* and the canine- and feline-associated *H. felis* (2.5%). Although these patients did not present any clinical alteration besides the obesity condition, histopathological analysis demonstrated that the majority presented signs of hyperplasia of the superficial epithelium, gastritis and presence of multiple lymphoid follicles (data not shown). These findings could be related with the presence of *H. pylori* (Dixon MF 1996b). Recently, our group studied the *in vitro* binding capacity of several gastric *Helicobacter* species to the

superficial human gastric mucosa and it was concluded that, as expected, *H. pylori* was the species that adhered the most. Amongst the NHPH group, *H. heilmannii* presented the highest adhesion value, followed by *H. salomonis*, *H. felis*, *H. bizzozeronii* and *H. ailurogastricus* (data under publication). However, no bacterial combinations were tested in this assay. Nevertheless, it would be interesting to investigate the adhesion and colonization potential of these bacterial associations, as it may constitute a key strategy to increase pathogenicity.

Previous studies showed that patients treated with an acid inhibitor (such as proton pump inhibitors), present an increased NHPH burden, and this finding was correlated with inflammatory responses (Noto 2017, Mowat 2000, Sanduleanu 2001). It was hypothesized that NHPH are capable of colonizing an achlorhydric environment promoting inflammation, which can eventually favour cancer progression (Mowat C 2000; Noto JM 2017; Sanduleanu S 2001). This may highlight the versatility and resilience of the various *Helicobacter* species that are able to adapt effectively to different gastric microenvironments and thus, survive efficiently in altered tissues, even when considered disadvantageous or adverse.

Most gastric NHPH associated with dogs, cats or pigs are considered zoonotic agents (Baele M 2008; Haesebrouck 2011; Haesebrouck F 2009). Amongst the patients who answered the inquiry, the great majority reported a close and frequent contact with different animal species (80.9%). Interestingly, one of the patients presenting mixed infection with *H. pylori* and *H. felis*, referred to a close contact with a cat as a pet while the other patient reported no contact with animals. More interesting was the fact that the histological and immunohistochemistry analysis showed that this patient with a mixed infection present *Helicobacter*-like organisms inside the cells (Figure S1C). These findings suggest that this could be a strategy used by the bacteria to evade to the immune system, and even to resist to the eradication treatment. In any case, contact with pets does not necessarily result in an NHPH infection.

The *H. pylori* eradication therapy usually consists of 2-3 antibiotics (i.e. clarithromycin, amoxicillin, metronidazole, tetracycline, and/or levofloxacin) combined with an acid-suppressive drug (proton pump inhibitor-PPI or H₂-receptor antagonists) (Chey WD 2017). However, treatment efficacy has been declining in the past decade, due

to several factors such as side effects, increasing antibiotic resistant strains, and the cost of the antibiotic regimens (De R 2009). In fact, this problem is so urgent that the WHO considered *H. pylori* a high priority group, for which new drugs and alternative therapies are urgently needed (Blaecher C 2013). Acquired resistance to antibiotics has been described not only for *H. pylori*, but also for some NHPH isolates, such as *H. heilmannii*, *H. ailurogastricus* and *H. suis* (Berlamont H 2019; Matos R 2020). Among the 80 individuals included in this study, 32.5% were subjected to the eradication treatment in the last 18 months. Interestingly, in 46.2% of these patients, *Helicobacter* spp. (including *H. pylori* and *H. felis*), were still detected in gastric tissues. Interestingly, van den Bulck and co-workers demonstrated previously that some *H. felis* isolates showed acquired resistance to metronidazole, one of the antibiotics used in the standard triple therapy (Van den Bulck K 2005b). Unfortunately, we have no information regarding the therapeutical scheme administrated on one of the patients presenting a mixed infection. There are several factors that might explain this finding: 1) the treatment was not strictly carried out by the patient (incomplete or unfinished) or was not effective at all; 2) the treatment was carried out too close to the confirmatory test, so there had not yet been an opportunity for the bacteria to be completely eliminated or 3) the patient was reinfected after the treatment. Curiously, among these after treatment *Helicobacter*-positive patients, the triple standard therapy was the most frequent scheme, followed by treatment with a combination of amoxicillin and levofloxacin, and some patients were even submitted to two different eradication schemes. After the eradication treatment, patients should be submitted to a confirmatory test, either by urea breath test or by other techniques that usually require endoscopy to confirm the elimination of the bacteria. Previously performed confirmatory tests indicated a negative *Helicobacter* result in 4 patients, that later were found infected with *H. pylori* (through PCR and confirmed by sequencing) (33.3%), questioning the efficacy of the treatment.

Portugal has been considered one the countries with the highest resistance percentage to clarithromycin (42.35%), one of the main antibiotics that constitute the standard triple therapy (Ghotaslou R 2015). This could partially explain the relative low efficiency of the eradication protocol, since 42.3% of the patients were submitted to a therapeutical scheme based on this antibiotic.

Overall, it can be concluded that NHPH screening should be included in the diagnostic routine due to the proven occurrence of mixed infection by more than one *Helicobacter* species. Additionally, due to the decreased efficiency of the current *Helicobacter* eradication treatment, the development of new therapeutical strategies is necessary to eradicate infection by these agents.

Supplementary Material

Figure S1. Different methods *Helicobacter* spp. identification in human gastric samples.

Table S1. Oligonucleotide primers used for PCR amplification.

Table S2. Different approaches for *Helicobacter* species detection in human gastric samples (Sequencing, modified-Giemsa stain, IHC, PCR).

Author Contributions

Conceptualization, I.A., F.G., F.H.; methodology, R.M., E.T., S.D.B., A.R.; sample collection, H.S.S., J.N., F.C.; validation, I.A., F.G., F.H.; writing—original draft preparation, R.M., I.A.; writing—review and editing, R.M., F.C., A.M., C.A.R., F.H., F.G., I.A.; supervision, I.A., F.H., F.G. All authors have read and agreed to the published version of the manuscript.

Funding

RM is funded by Fundação para a Ciência e Tecnologia/ Ministério da Ciência, Tecnologia e Inovação (FCT) PhD scholarship SFRH/BD/131159/2017.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Ethics Committee from the Centro Hospitalar Universitário de São João/ Faculdade de Medicina da Universidade do Porto (CHSJ/FMUP) (CES 127-15).

Informed Consent Statement

Written informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Material

Figure S1

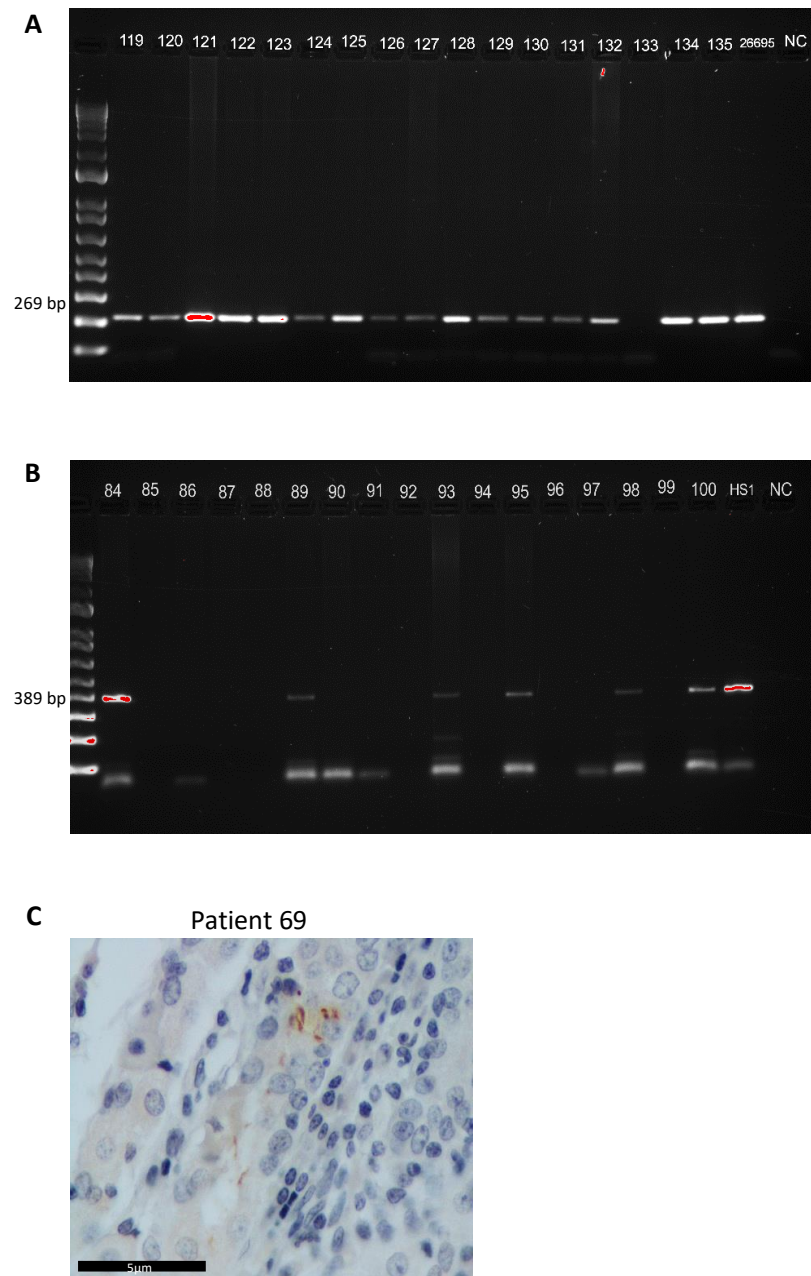


Figure S1. Different methods *Helicobacter* spp. identification in human gastric samples. Agarose gel electrophoresis for identification of *H. pylori* by specific PCR on samples 119 to 135 (A) and for *Helicobacter*-genus PCR on samples 84 to 100 (B). DNA extracted from pure culture of *H. pylori* 26695 Strain (A) and *H. suis* (HS1 strain) (B) were used as a positive control, respectively. Negative control (N consisted solely of mix solution. (C) Immunohistochemistry detection of *Helicobacter* species in patient 69 (intracellular organism). Magnification 600x.

Table S1. Oligonucleotide primers used for PCR amplification.

Target		Primer	Sequence (5'-3')	PCR Conditions
<i>Helicobacter</i>-genus		Hcom1	GTA AAG GCT CAA GGC TAT	5' 94°C
		Hcom2	CCA CCT ACC TCT CCC ACA CTC	40x (1' 94°C-1' 63°C-1' 72°C) 5' 72°C
<i>Helicobacter</i> species- specific	<i>H. pylori</i>	BFHpyl_F1	AAA GAG CGT GGT TTT CAT GGC G	4' 95°C
		BFHpyl_R1	GGG TTT TAC CGC CAC CGA ATT TAA	45x (30" 94°C-30" 59°C-1' 72°C) 10' 72°C
	<i>H. suis</i>	BFHsuis_F1	AAA ACA MAG GCG ATC GCC CTG TA	4' 95°C
		BFHsuis_R1	TTT CTT CGC CAG GTT CAA AGC G	45x (1' 94°C-1' 59°C-1' 72°C) 10' 72°C
	<i>H. heilmannii</i>	IceA_ASB1_Fw	GTG AGC AGC GAA TTT AAA GGG CAG TAT	5' 94°C
		IceA_ASB1_Rv	GTT TGG CTG TAA GTC TAA GTC CAC TTG	30x (30" 94°C-30" 55°C-30" 72°C) 10' 72°C
	<i>H. ailurogastricus</i>	LpsA_ASB7_Fw	CGA TCA AAG ATC GGG TGA AT	5' 94°C
		LpsA_ASB7_Rv	CCA TTA AGG GGT GCT TGA AA	30x (30" 94°C-30" 55°C-30" 72°C) 10' 72°C
	<i>H. felis</i>	BFHfel_F2	GCT GGT GGC ATC GAT ACG CAT	4' 95°C
		BFHfel_R2	TTT TTA GAT TAG CGC GTC CGG GA	45x (30" 94°C-30" 60°C-1' 72°C) 10' 72°C
	<i>H. salomonis</i>	Hsal_FQ_PAR	CTC TTA TGA GGA CTT GGT GCT CAC CAA T	4' 95°C
		Hsal_RQ_PAR	TTT GCC ATC TTT AAT TCC AAT GTC GGC	45x (30" 94°C-30" 61°C-1' 72°C) 10' 72°C
	<i>H. bizzozeronii</i>	BFHbizz_F1	CCA ACA AAT CCC CAC AGC ATT TGC CAG	4' 95°C
		BFHbizz_R1	AGT CCC ATC AGC WGG WCC TGT TCC CCC AC	45x (1' 94°C-1' 58°C-1' 72°C) 10' 72°C

Table S2. Different approaches for *Helicobacter* species detection in human gastric samples (Sequencing, modified-Giemsa stain, IHC, PCR).

ID	Tissue Sample	Histology		IHC	Conventional PCR		Sequencing	Conclusion ³
		Tissue Classification	MG ¹	<i>anti-Helicobacter</i> ²	Genus specific PCR	Species-specific PCR		
1	1	Tumor tissue	-	-	-	-	-	Negative
	2	Adjacent Tissue	-	-	-	-	-	
2	3	Tumor tissue	-	-	-	-	-	Negative
	4	Adjacent Tissue	-	-	-	-	-	
3	5	Tumor tissue	+	+	-	-	-	Negative
	6	Adjacent Tissue	+	+	-	-	-	
4	7	Tumor tissue	+	+	-	-	-	Negative
	8	Adjacent Tissue	+	+	-	-	-	
5	9	Tumor tissue	-	-	-	-	-	Negative
	10	Adjacent Tissue	-	-	-	-	-	
6	11	Tumor tissue	-	-	-	-	-	Negative
	12	Adjacent Tissue	-	-	-	-	-	
7	31	Adjacent Tissue	-	-	-	-	-	Negative
	32	Tumor tissue	-	-	-	-	-	
8	33	Tumor tissue	-	+	-	-	-	Negative
	118	Adjacent Tissue	-	-	-	-	-	
9	34	Adjacent Tissue	+	+	-	-	-	Negative
	35	Tumor tissue	+	+	-	-	-	
10	36	Adjacent Tissue	-	-	-	-	-	Negative
	37	Tumor tissue	-	-	-	-	-	
11	38	Adjacent Tissue	-	-	-	-	-	Negative
	39	Tumor tissue	-	-	-	-	-	
12	40	Adjacent Tissue	-	-	+	Hp +	<i>H. pylori</i>	Positive
	41	Tumor tissue	+	-	-	-	-	
13	42	Adjacent Tissue	+++	-	-	-	-	Negative
	43	Tumor tissue	+	+	-	-	-	
14	44	Adjacent Tissue	+	-	-	-	-	Negative
	45	Tumor tissue	+	+	-	-	-	
15	61	Adjacent Tissue	-	-	-	-	-	Negative
	79	Tumor tissue	+	++	-	-	-	
16	62	Adjacent Tissue	-	+	-	-	-	Negative
	63	Tumor tissue	-	-	-	-	-	
17	89	Adjacent Tissue	-	+	-	-	-	Positive
	90	Tumor tissue	-	-	+	Hp +	<i>H. pylori</i>	

18	13	Non-neoplastic mucosa	-	-	-	-	-	Negative
	14		-	-	-	-	-	
	15		-	-	-	-	-	
19	16	Non-neoplastic mucosa	-	+	-	-	-	Negative
	17		-	+	-	-	-	
	18		-	-	-	-	-	
20	19	Non-neoplastic mucosa	-	-	-	-	-	Negative
	20		-	-	-	-	-	
	21		-	-	-	-	-	
21	22	Non-neoplastic mucosa	+	-	-	-	-	Positive
	23		+	-	+	-	<i>H. pylori</i>	
	24		+	-	-	-	-	
22	25	Non-neoplastic mucosa	-	-	-	-	-	Negative
	26		-	-	-	-	-	
	27		-	-	-	-	-	
23	28	Non-neoplastic mucosa	-	-	-	-	-	Negative
	29		-	-	-	-	-	
	30		-	+	-	-	-	
24	46	Non-neoplastic mucosa	+	+	-	-	-	Negative
	47		+	+	-	-	-	
	48		+	++	-	-	-	
25	49	Non-neoplastic mucosa	+	+	+	-	<i>H. pylori</i>	Positive
	50		+	+	-	-	-	
	51		+	++	+	-	<i>H. pylori</i>	
26	52	Non-neoplastic mucosa	-	+	-	-	-	Negative
	53		-	+	-	-	-	
	54		-	+	-	-	-	
27	55	Non-neoplastic mucosa	+	+	-	-	-	Negative
	56		++	-	-	-	-	
	57		+	-	-	-	-	
28	58	Non-neoplastic mucosa	+	+++	+	-	<i>H. pylori</i>	Positive
	59		+	+	+	Hp +	<i>H. pylori</i>	
	60		+	-	+	Hp +	<i>H. pylori</i>	
29	64	Non-neoplastic mucosa	-	-	-	-	-	Negative
	65		-	-	-	-	-	
	66		-	-	-	-	-	
30	67	Non-neoplastic mucosa	-	-	-	-	-	Negative
	68		-	-	-	-	-	
31	69	Non-neoplastic mucosa	-	-	-	-	-	Negative
	70		-	-	-	-	-	
32	71	Non-neoplastic mucosa	+	+	-	-	-	Negative
	72		+	++	-	-	-	
	133		+	+	-	-	-	
33	73		-	-	-	-	-	Negative
	74		-	-	-	-	-	

	75	Non-neoplastic mucosa	-	-	-	-	-	
34	76	Non-neoplastic mucosa	-	-	-	-	-	Negative
	77		-	-	-	-	-	
	78		-	-	-	-	-	
35	80	Non-neoplastic mucosa	-	-	-	-	-	Negative
	81		-	-	-	-	-	
	82		-	-	-	-	-	
36	83	Non-neoplastic mucosa	+	-	+	Hp +	<i>H. pylori</i>	Positive
	84		+	+	+	Hp +	<i>H. pylori</i>	
	85		+	+	+	Hp +	<i>H. pylori</i>	
37	86	Non-neoplastic mucosa	-	-	+	Hp +	<i>H. pylori</i>	Positive
	87		-	-	+	Hp +	<i>H. pylori</i>	
	88		-	-	+	Hp +	<i>H. pylori</i>	
38	91	Non-neoplastic mucosa	-	-	-	-	-	Negative
	92		-	+	-	-	-	
	93		-	+	-	-	-	
39	94	Non-neoplastic mucosa	-	+	+	Hp +	<i>H. pylori</i>	Positive
	95		+	+	+	Hp +	<i>H. pylori</i>	
	96		-	+	+	-	<i>H. pylori</i>	
40	97	Non-neoplastic mucosa	-	-	-	-	-	Negative
	98		-	-	-	-	-	
	99		-	-	-	-	-	
41	100	Non-neoplastic mucosa	+	+	+	Hp +	<i>H. pylori</i>	Positive
	101		+	++	+	Hp +	<i>H. pylori</i>	
	102		+++	++	+	Hp +	<i>H. pylori</i>	
42	103	Non-neoplastic mucosa	+	+	+	Hp +	<i>H. pylori</i>	Positive
	104		+	++	-	-	-	
	105		-	+	-	-	-	
43	106	Non-neoplastic mucosa	-	-	-	-	-	Negative
	107		-	-	-	-	-	
	108		-	+	-	-	-	
44	109	Non-neoplastic mucosa	+	+	-	-	-	Negative
	110		+	+	-	-	-	
	111		+	+	-	-	-	
45	112	Non-neoplastic mucosa	-	+	-	-	-	Negative
	113		-	+	-	-	-	
	114		-	-	-	-	-	
46	115	Non-neoplastic mucosa	-	-	+	Hp +	<i>H. pylori</i>	Positive
	116		-	-	+	Hp +	<i>H. pylori</i>	
	117		-	-	+	Hp +	<i>H. pylori</i>	
47	119	Non-neoplastic mucosa	+	-	-	-	-	Positive
	120		+	+	-	-	-	
	121		+	+	+	Hp +	<i>H. pylori</i>	
48	122		+	+	+	Hp +	<i>H. pylori</i>	Positive

	123	Non-neoplastic mucosa	+	+	+	Hp +	<i>H. pylori</i>	
	124		+	+	-	-	-	
49	125	Non-neoplastic mucosa	-	-	-	-	-	Negative
	126		-	-	-	-	-	
	127		-	-	-	-	-	
50	128	Non-neoplastic mucosa	-	-	-	-	-	Negative
	129		-	-	-	-	-	
	130		-	-	-	-	-	
51	131	Non-neoplastic mucosa	-	-	-	-	-	Positive
	132		-	-	-	-	-	
	134		+	-	+	Hp +	<i>H. pylori</i>	
52	135	Non-neoplastic mucosa	+	+	+	-	<i>H. pylori</i>	Positive
	136		+	-	+	-	<i>H. pylori</i>	
	137		+	+	-	-	-	
53	138	Non-neoplastic mucosa	-	+	-	-	-	Negative
	139		-	+	-	-	-	
	140		-	+	-	-	-	
54	141	Non-neoplastic mucosa	-	-	-	-	-	Positive
	142		+	+	-	-	-	
	143		+	+	+	-	<i>H. pylori</i>	
55	144	Non-neoplastic mucosa	+	-	+	-	<i>H. pylori</i>	Positive
	145		+	+	+	-	<i>H. pylori</i>	
	146		++	+	-	H. felis +	<i>H. felis</i>	
56	147	Non-neoplastic mucosa	-	+	-	-	-	Positive
	148		-	-	-	-	-	
	149		-	+	+	-	<i>H. pylori</i>	
57	150	Non-neoplastic mucosa	++	+++	+	-	<i>H. pylori</i>	Positive
	151		++	++	+	Hp +	<i>H. pylori</i>	
	152		+	-	-	-	-	
58	153	Non-neoplastic mucosa	+	+	-	-	-	Positive
	154		-	+	-	-	-	
	155		-	+	+	-	<i>H. pylori</i>	
59	156	Non-neoplastic mucosa	+	-	+	-	<i>H. pylori</i>	Positive
	157		+	+	+	-	<i>H. pylori</i>	
	158		-	++	+	-	<i>H. pylori</i>	
60	159	Non-neoplastic mucosa	+	+++	+	-	<i>H. pylori</i>	Positive
	160		-	++	+	Hp +	<i>H. pylori</i>	
	161		+++	+++	-	-	-	
61	162	Non-neoplastic mucosa	+	++	-	-	-	Positive
	163		-	++	-	-	-	
	164		+	++	+	Hp +	<i>H. pylori</i>	
62	165	Non-neoplastic mucosa	+++	+++	-	-	-	Positive
	166		+++	+++	+	Hp +	<i>H. pylori</i>	
	167		+++	+++	+	-	<i>H. pylori</i>	
63	168		+	+++	-	-	-	Negative

	169	Non-neoplastic mucosa	-	+	-	-	-	
	170		-	+	-	-	-	
64	171	Non-neoplastic mucosa	++	+++	+	-	<i>H. pylori</i>	Positive
	172		++	+++	+	-	<i>H. pylori</i>	
	173		++	+++	-	-	-	
65	174	Non-neoplastic mucosa	++	++	+	-	<i>H. pylori</i>	Positive
	175		+++	+++	+	-	<i>H. pylori</i>	
	176		+++	+++	-	-	-	
66	177	Non-neoplastic mucosa	-	-	-	-	-	Negative
	178		-	+	-	-	-	
	179		-	+	-	-	-	
67	180	Non-neoplastic mucosa	++	+++	-	-	-	Negative
	181		++	+	-	-	-	
	182		+	++	-	-	-	
68	183	Non-neoplastic mucosa	+++	+++	-	-	-	Negative
	184		++	+++	-	-	-	
	185		+++	+++	-	-	-	
69	186	Non-neoplastic mucosa	-	-	-	H. felis +	<i>H. felis</i>	Positive
	187		-	-	-	-	-	
	188		-	-	+	Hp +	<i>H. pylori</i>	
70	189	Non-neoplastic mucosa	++	+++	+	-	<i>H. pylori</i>	Positive
	190		+++	+++	+	-	<i>H. pylori</i>	
	191		+++	+++	+	-	<i>H. pylori</i>	
71	192	Non-neoplastic mucosa	+	++	-	-	-	Negative
	193		++	+	-	-	-	
	194		++	++	-	-	-	
72	195	Non-neoplastic mucosa	-	++	+	-	<i>H. pylori</i>	Positive
	196		+++	+++	+	-	<i>H. pylori</i>	
	197		+++	+++	-	-	-	
73	198	Non-neoplastic mucosa	-	-	+	-	<i>H. pylori</i>	Positive
	199		-	-	+	-	<i>H. pylori</i>	
	200		-	-	+	-	<i>H. pylori</i>	
74	201	Non-neoplastic mucosa	+++	+++	+	-	<i>H. pylori</i>	Positive
	202		++	+++	+	-	<i>H. pylori</i>	
	203		++	+++	-	-	-	
75	204	Non-neoplastic mucosa	-	-	+	-	<i>H. pylori</i>	Positive
	205		-	+	+	-	<i>H. pylori</i>	
	206		-	-	+	-	<i>H. pylori</i>	
76	207	Non-neoplastic mucosa	+++	++	-	-	-	Negative
	208		++	++	-	-	-	
	209		+	++	+	-	-	
77	210	Non-neoplastic mucosa	+++	+++	+	-	<i>H. pylori</i>	Positive
	211		+++	+++	+	-	<i>H. pylori</i>	
	212		+++	+++	+	-	<i>H. pylori</i>	
78	213		+++	+++	+	Hp +	<i>H. pylori</i>	Positive

	214	Non-neoplastic mucosa	+++	+++	+	Hp +	<i>H. pylori</i>	
	215		+++	+++	+	-	<i>H. pylori</i>	
79	216	Non-neoplastic mucosa	++	-	+	-	<i>H. pylori</i>	Positive
	217		-	-	+	-	<i>H. pylori</i>	
	218		-	-	+	-	<i>H. pylori</i>	
80	219	Non-neoplastic mucosa	-	-	-	-	-	Positive
	220		-	+	+	-	<i>H. pylori</i>	
	221		-	+	-	-	-	
80 patients								

¹ The amount of bacteria detected on the Giemsa-modified staining was scored as absent (-), low amount (+), moderate amount (++), and high amount of bacteria (+++).

² The immunostaining was scored according to the intensity (-, absent; +, weakly positive; ++, moderately positive; +++, intense stain), as described by Prachasilpchai *et al.*, 2007.

³ Criteriums for final classification: if a sample has a positive qPCR is considered a positive sample regardless the other techniques. If a sample has a positive result on two techniques, then is considered positive.

***Helicobacter* species
binding to the human
gastric mucosa**

4 CHAPTER

Paper IV

Helicobacter species binding to the human gastric
mucosa

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Fátima Carneiro, Irina Amorim, Freddy Haësebrouck, Fátima Gärtner

2021

Submitted to *Helicobacter*

20th September 2021

Article

***Helicobacter* species binding to the human gastric mucosa**

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Conceptualization, I.A., F.G.; methodology, R.M.; sample collection, H.S.S., J.N., F.C.; validation, I.A., F.G., F.H.; writing—original draft preparation, R.M., I.A.; writing—review and editing, R.M., F.C., A.M., C.A.R., F.H., F.G., I.A.; supervision, I.A., F.H., F.G. All authors have read and agreed to the published version of the manuscript.

Abstract

Helicobacter pylori infects half of the world population, being associated with several gastric disorders, such as chronic gastritis and gastric carcinoma. The *Helicobacter* genus also includes other gastric helicobacters, such as *H. heilmannii*, *H. ailurogastricus*, *H. suis*, *H. felis*, *H. bizzozeronii* and *H. salomonis*. These gastric helicobacters colonize both the human and animal stomach. The prevalence of gastric non-*Helicobacter pylori* *Helicobacter* (NHPH) species in humans has been described as low, and the *in vitro* binding to the human gastric mucosa was never assessed. Herein, human gastric tissue sections were used for the evaluation of the tissue glycophenotype and for the binding of gastric NHPH strains belonging to different species. Histopathological evaluation showed that 37.5% of the patients enrolled in our cohort presented chronic gastritis, while the presence of neutrophil or eosinophilic activity (chronic active gastritis) was observed in 62.5% of the patients. The secretor phenotype was observed in 68.8% of the individuals, based on the expression of Lewis B antigen and binding of the UleX lectin. The *in vitro* binding assay showed that all the NHPH strains evaluated were able to bind, albeit in low frequency, to the human gastric mucosa. The *H. heilmannii*, *H. bizzozeronii* and *H. salomonis* strains displayed the highest binding ability both to the gastric superficial epithelium and to the deep glands. Interestingly, we observed binding of NHPH to the gastric mucosa of individuals with

severe chronic inflammation and intestinal metaplasia, suggesting that NHPH binding may not be restricted to the healthy gastric mucosa or slight chronic gastritis. Furthermore, the *in vitro* binding of NHPH strains was observed both in secretor and non-secretor individuals in a similar frequency. In conclusion, this study is the first report of the *in vitro* binding ability of gastric NHPH species to the human gastric mucosa. The results suggest that other glycans, besides the Lewis antigens, could be involved in the bacterial adhesion mechanism, however the molecular intervenients remain unknown.

Keywords: Gastric Helicobacters; NHPH; Binding ability; Gastric mucosa; Lewis Antigens; Secretor status

Introduction

The *Helicobacter* genus is a group of Gram-negative bacteria, composed of at least 45 different species, which are able to colonize the stomach or intestinal tract of humans and animals (Ali B 2018; Ansari S 2017; Huang Y 2016). *Helicobacter pylori* (*H. pylori*) is the most common and best studied species that infects half of worldwide human population (Doulberis M 2020; Hooi JKY 2017; van Amsterdam K 2006). Infection with *H. pylori* usually occurs during childhood, but the majority of the individuals remain asymptomatic (Ali B 2018; Hooi JKY 2017). However, some individuals can develop chronic gastritis, gastric or duodenal ulcers (10-20%), gastric carcinoma (1-2%) or mucosa-associated lymphoid tissue (MALT) lymphoma (1-2%) (Blaser N 2019; Hooi JKY 2017), with *H. pylori* being considered a leading cause of gastric adenocarcinoma onset (Blaecher C 2013; Blaser N 2019; Rodrigues MF 2019).

Besides *H. pylori*, other gastric helicobacters, named non-*Helicobacter pylori* Helicobacter (NHPH), have been found both in human and animal stomach (Bento-Miranda M 2014; Berlamont H 2019; De Witte C 2018; Matos R 2020). The most common gastric NHPH in humans are *H. suis*, *H. heilmannii*, *H. felis*, *H. salomonis* and *H. bizzozeronii* (Boyanova L 2003; Haesebrouck F 2009; Sýkora J 2003; Yakob J 2012). Human NHPH infection is significantly lower than with *H. pylori* (0.2-6.0% of human gastric biopsies) (Haesebrouck F 2009), but these values are probably underestimated due to the lack of proper diagnostic tools (Haesebrouck F 2009). Similar to *H. pylori*, NHPH infection may be associated with gastric disorders, being usually accompanied by gastritis, antral erosions and duodenal ulcers (Ali B 2018; Haesebrouck F 2009; Joosten M 2016). Interestingly, the risk of developing gastric low-grade MALT lymphoma is higher with NHPH infection than with *H. pylori* (Matos et al. 2021; Morgner A 2000).

Glycosylation is a post-translational modification consisting of the attachment of single sugars to other saccharides, proteins and lipids (Eichler 2019; Endo 2019; Mereiter S 2019). This is a highly regulated mechanism, essential for several biological processes, such as cell-cell and cell-extracellular matrix adhesion (Eichler 2019; Endo 2019; Pinho SS 2015). Glycans are also important players in the pathogenesis of several diseases (Etzold S 2014; Formosa-Dague C 2018; Thompson AJ 2019), and the role of glycosylation in infection, inflammation and immune surveillance is very well accepted (Lubbers, Rodriguez, and van Kooyk 2018; Pereira et al. 2018; Rabinovich, van Kooyk, and Cobb 2012). The first step is the interaction between the pathogen and the host cell, which is mediated by glycans, and this step is crucial for a successful colonization (Kreisman and Cobb 2012). The infection of the human gastric mucosa by *H. pylori* is one of the most studied host-pathogen interactions (Aspholm-Hurting M 2004; Benktander J 2012; Borén T 1994). First, the bacteria reach the surface of the gastric mucosa, and then adheres

to the gastric epithelial cells via outer membrane proteins (Thompson AJ) (Aspholm-Hurting M 2004; Benktander J 2012; Borén T 1993; Borén T 1994). The adhesion occurs through the interaction between bacterial adhesins, such as BabA, SabA, LabA, AlpA/B and HopQ/Z, with the respective ligand expressed at the host cell surface (Ansari S 2017; Bauwens E 2018; Benktander J 2012; Benktander J 2018; Borén T 1993; Borén T 1994; Ilver D 1998; Rossez Y 2014).

The BabA adhesin, expressed at the bacterial surface, mediates the first contact between *H. pylori* and the human gastric epithelial cells, binding to the fucosylated blood-group antigens H-type 1 and Lewis B (LeB) (Benktander J 2012; Borén T 1993). The LeB antigen, the main receptor for BabA adhesin, is expressed mainly on MUC5AC mucin, but it can also be present on other mucins, such as MUC1 and MUC2 (Ansari S 2017; Linden SK 2008; Magalhaes A 2010a; Mahdavi MR 2013). The type 2 Lewis antigens are usually co-expressed with MUC6 mucin (Reis CA 2000).

The Lewis antigens are terminal fucosylated glycans and they are key players in the recognition and binding of several pathogens (Amorim I 2014; De Oliveira DM 2017; Le Pendu and Ruvoen-Clouet 2020; Magalhaes A 2015). These structures can be classified as type 1 or type 2 antigens, based on their biosynthesis (Pinho SS 2015). The type 1 Lewis antigens are characterized by a Gal β 1,3GlcNAc linkage, while the type 2 structures present a Gal β 1,4GlcNAc linkage (Pinho SS 2015). The synthesis of the H- type 1 antigen results from the addition of a fucose residue to the terminal galactose on type 1 chains, and it can be further modified with a fucose on the GlcNAc, resulting in the formation of LeB antigen. Alternatively, a fucose can be added to the GlcNAc residue of the type 1 chain, and lead to the synthesis of Lewis A (Oleastro M) antigen, which can be modified by the addition of a sialic acid, leading to the synthesis of sialyl-Lewis A (sLeA). The type 2 Lewis antigens biosynthesis is similar to the type 1, but it occurs in the Gal β 1,4GlcNAc chains, resulting in the production of Lewis X (LeX), Lewis Y (LeY) and sialyl-Lewis X (sLeX) (Pinho SS 2015).

The glycosylation profile of the human gastric mucosa has been widely studied in the last years (De Bolos C 1995; López-Ferrer A 2000; Magalhaes A 2009; Mollicone R 1985; Moran 2008; Teixeira A 2002). Furthermore, Amorim *et al.*, demonstrated that the canine gastric mucosa presents a different glycophenotype in comparison with human gastric mucosa, mainly characterized by the solely expression of type 2 Lewis antigens (Amorim I 2014). Evaluation of the *in vitro* adhesion of *Helicobacter* species to the canine gastric mucosa showed that, amongst the NHPH species tested, *H. heilmannii* presented the highest adhesion score (Amorim I 2014). These results suggest that the *H. pylori* and NHPH adhesion abilities are distinct and involve different glycans which have not been identified for NHPH (Amorim I 2014). The *in vitro* binding of NHPH to the human gastric mucosa has never been addressed before.

In the present study, the adhesion of zoonotically important gastric NHPH species to the human gastric mucosa was studied. Our findings may help to obtain relevant insights in the molecules involved in the adhesion mechanism.

Material and Methods

Patients samples

Gastric samples from 80 human patients were used in this study and included tumor-adjacent tissues collected from patients diagnosed with gastric cancer (GC) ($n=17$) and tissues from patients diagnosed with morbid obesity (Ob) and subjected to laparoscopic sleeve gastrectomy ($n=63$). These were collected between March 2017 and July 2020, during surgical

procedures that occurred at Centro Hospitalar Universitário de São João (CHUSJ), Porto, Portugal. All the patients included in the study were informed about the project and their written consent was requested. None of the participants was considered a vulnerable individual. All the actions were approved by the Ethics Committee from the CHSJ/FMUP (CES 127-15). Each sample was divided in two fragments: one was formalin-fixed for routine histological processing and the other was frozen at -80°C for further molecular studies.

Histopathologic evaluation

Samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Serial 3 µm thick, paraffin embedded sections were made, one of which was subjected to haematoxylin-eosin (H&E) stain for histopathological evaluation.

Then, microscopical examination was performed by a pathologist in order to confirm the gastric anatomical region and to perform a semi-quantitative evaluation of the inflammatory cells and other relevant alterations, according to the Updated Sidney Classification System (Dixon MF 1996a), graded on a scale of absent, mild, moderate and severe (Table 1).

Table 1. Histopathological parameters evaluated, according to criteria proposed in the Updated Sidney System (Dixon MF 1996a).

Histologic Properties	Definition	Mild		Moderate		Severe	
Chronic Inflammation	Presence of lymphocytes and plasma cells	+	1	++	2	+++	3
Chronic Active Inflammation	Presence of eosinophils and neutrophil infiltration in the lamina propria or superficial epithelium	+	1	++	2	+++	3
Glandular Atrophy	Loss of corpus and antral glands	+	1	++	2	+++	3
Intestinal Metaplasia	Intestinal Metaplasia in the mucosal epithelium	Focal		Multifocal			

Immunohistochemistry

Other consecutive gastric paraffin-embedded sections were used for the evaluation of type 1 and type 2 Lewis Antigens expression (Table 2). These were first deparaffinized and hydrated. Then antigen retrieval was performed in 10 mmol/L sodium citrate buffer, pH 6.0, for 10 minutes. Slides were cooled for 20 minutes at room temperature (RT) and rinsed twice in phosphate buffered saline with 0.05% Tween 20 for 5 minutes. After blocking endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 minutes, slides were blocked for 30 minutes with normal goat serum (Dako, California, USA), diluted 1:5 in 10% bovine serum albumin (BSA 10%). Next, slides were incubated overnight at 4°C with a primary monoclonal antibody (Table 6). Sections were rinsed with PBS-T 0.05% and incubated with secondary antibody goat anti-mouse diluted 1:200 in BSA 5%, for 30 minutes (Dako, California, USA). Slides were rinsed once more and incubated with avidin-biotin-peroxidase complex solution for 30 minutes (Vectastain® Elite ABC HRP Kit, PK-6100, Vector Laboratories, USA). Colour was developed with 3,3'-diamino-benzidine (DAB; Sigma, St. Louis, MO, USA) for 5 to 7 minutes at RT. Sections were then counterstained with haematoxylin, dehydrated and mounted. Negative

controls were performed by replacing the primary antibody with an antibody not reacting with Lewis antigens and belonging to the same immunoglobulin isotype.

Table 2. Antibodies used for the immunohistochemistry study of the Lewis Antigens expression.

Antigen	Antibody Clone	Dilution	Reference
Lewis A (Oleastro M)	CA3F4	1:5	(Young, Portoukalian, and Hakomori 1981)
Lewis B (LeB)	BG-6 (T218)	1:50	Signet
Sialyl-Lewis A (sLeA)	CA19-9	1:500	Santa Cruz Biotechnonology
Lewis X (LeX)	SH1	1:5	(Fukushi Y 1984)
Lewis Y (LeY)	AH6	1:10	(Abe K 1993)
Sialyl-Lewis X (sLeX)	CSLEX-1	1:80	BD Bioscience

UleX Lectin staining

For the lectin study, gastric paraffin embedded sections were subjected to deparaffination and rehydration, and then, endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Sections were washed twice with PBS and incubated with 10% BSA in PBS for 30 minutes at RT. After, sections were incubated with biotinylated *Ulex Europaeus* Agglutinin I (EUA-1) lectin solution, diluted 1:100 in PBS, for 1h at RT. Slides were rinsed again and incubated with avidin-biotin-peroxidase complex solution for 30 minutes (Vectastain® Elite ABC HRP Kit, PK-6100, Vector Laboratories, USA). Finally, colour was developed with DAB, counterstained with haematoxylin, dehydrated and mounted. Negative controls were performed by replacing the lectin by PBS.

Evaluation of secretor status

The Lewis Antigens expression was evaluated and the immunostaining was classified according to the distribution (0: 0% positive cells; 1: 1-20% positive cells; 2: 21-50% positive cells; 3: 51-75% positive cells; 4: 76-100% positive cells) and intensity (0: absent; 1: weak; 2: moderate; 3: strong), as previously described by Lee *et al.*, 2006 (Lee HS 2006). The classification obtained regarding both immunoreactivity distribution and intensity was then converted into a final score, where samples were considered positive (score >2) or negative (score ≤2).

The secretor status of each patient was also evaluated and classified as secretor and non-secretor, depending on the expression of both *Ulex* lectin and LeB antigen. Individuals presenting expression of LeB antigen and UleX staining, are considered secretors, with two possible phenotypes: LeA⁺B⁺ or LeA⁻B⁺. Individuals not expressing one of the markers (LeB or *Ulex*) are classified as non-secretors (LeA⁺B⁻ or LeA⁻B⁻ phenotypes).

***Helicobacter* strains growth conditions**

Bacterial species were grown using specific growth conditions in a microaerobic atmosphere, as previously described (Baele M 2004; Berlamont H 2019). Briefly, *H. suis* (HS1 strain), *H. heilmannii* (ASB1.4) and *H. ailurogastricus* (ASB7.1) were grown in biphasic conditions consisting of brucella agar supplemented with fetal calf serum, amphotericin B, Skirrow antibiotics, and Vitox supplements at pH5, with brucella broth (pH 5) on top.

H. pylori (26695 strain), *H. felis* (CS1), *H. bizzozeronii* (R1051) and *H. salomonis* (R1053) were grown in brain heart infusion agar supplemented with horse blood, amphotericin B, Skirrow antibiotics and Vitox supplement.

***In vitro* binding assay of *Helicobacter* species to the human gastric mucosa**

For the *in vitro* binding ability assay, paraffin embedded gastric sections were used. The different *Helicobacter* species (*H. suis*, *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. salomonis* and *H. bizzozeronii*) were labelled with fluorescein isothiocyanate (FITC), as previously described (Falk P 1993). The FITC-labelled bacteria were kept at -80°C until used in the binding assay. Additionally, *H. pylori* 26695 strain was also labelled with FITC, and used as a positive control for the *in vitro* binding assay.

Adhesion of *Helicobacter* to the human gastric mucosa was studied as described before (Amorim I 2014; Magalhaes A 2009; Mollicone R 1985). Briefly, gastric sections were deparaffinized, rehydrated and incubated overnight at 4°C and 1h at RT with blocking buffer (1% BSA in PBS-Tween 0.05%). BSA was previously submitted to periodate oxidation to destroy competitive carbohydrate receptors for *Helicobacter* binding (Aspholm M 2006). Each section was then incubated with the FITC-labelled bacterial suspension (diluted 5-fold in blocking buffer) for 2h at RT and washed five times with PBS-Tween 0.1%. Finally, slides were stained with DAPI (diluted 1:100 in PBS) for 10 minutes at RT.

For binding evaluation, sections were observed with a fluorescence microscope (Zeiss Apotome Z1), under 200x magnification, and at least five different fields per case were analysed. The number of labelled-bacteria was quantified using the ImageJ Software (NIH, Bethesda, MD, USA). In order to avoid quantification of unspecific binding, a minimal quantity of 100 visible adhered bacteria was required to consider a case as positive, i.e as presenting consistent binding.

Statistical Analysis

Statistical analysis was performed using GraphPad PRISM 7.0 software. The One-way ANOVA with Kruskal-Wallis Test was used for the comparison of the histopathological evaluation, as well for the binding ability of each *Helicobacter* species. For each bacterial species, additional comparison of the binding ability to superficial epithelium and deep glands was performed using an ordinary two-way ANOVA analysis. P values <0.05 were considered as statistically significant (95% confidence interval).

Results

Histopathological evaluation of the human gastric samples

Histological evaluation demonstrated that all the patients presented signs of chronic inflammation, with presence of lymphocytes and plasma cells. The presence of neutrophils and eosinophils was also detected in some patients. A total of 30 out of 80 (37.5%) patients were classified as presenting chronic inflammation (presence of lymphocytes and plasma cells exclusively). The presence of chronic active inflammation, with preponderance of lymphocytes and plasma cells, but also presence of neutrophils and/or eosinophils, was observed in 50 out of 80 patients (62.5%). Glandular atrophy was detected in 24 of the overall individuals (30.0%), while focal or multifocal intestinal metaplasia was observed in 15 out of 80 patients (18.8%).

These results are summarized in Table 3 and S1 Table.

Table 3. Histological evaluation of the human gastric samples.

	Chronic Inflammation			Chronic Active Inflammation			Glandular Atrophy			Intestinal Metaplasia	
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe	Focal	Multifocal
Human Gastric samples (n=80)	60.0% (18/30)	30.0% (9/30)	10.0% (3/30)	48.0% (24/50)	32.0% (16/50)	20.0% (10/50)	25.0% (6/24)	41.7% (10/24)	33.3% (8/24)	46.7% (7/15)	53.3% (8/15)
	37.5% (30/80)			62.5% (50/80)			30.0% (24/80)			18.8% (15/80)	

Expression of type 1 and type 2 Lewis Antigens

Type 1 Lewis Antigens immunoexpression was mainly found within the surface mucus and in the superficial gastric epithelium (Fig 1A).

Moderate and generalized immunoexpression of LeA antigen covered the gastric superficial epithelium of 52 out of 80 (65.0%) individuals. Similar LeB expression location and distribution was observed, although LeB immunointensity varied from weak to strong. LeB immunoreactivity was recorded in 55 out of 80 (68.8%) cases. Multifocal to generalized sLeA immunoexpression was detected in 36 out of 80 cases (45.0%), mainly in superficial foveolar epithelium and displaying weak to strong intensity.

The type 2 Lewis Antigens were found in the surface epithelium but also in the neck cells, gastric pits and deep gastric glands (Fig 1A). Almost all the patients (79 out of 80; 98.8%), exhibited generalized and moderate to strong LeX immunoexpression. The LeY antigen was detected with moderate to strong intensity, in 79 out of 80 individuals (98.8%). Only one case did not present immunoexpression of these two Lewis Antigens. Expression of sLeX was detected in 17 out of 80 cases (21.3%), limited to the cytoplasm of the foveolar superficial epithelium and cells of the pyloric glands. In 7 of these, weak immunoexpression restricted to few cells was observed thus, these cases were considered negative. In the remaining 10 cases, the expression was multifocal and varied from weak to strong.

Detailed information of Type 1 and 2 Lewis Antigens immunoexpression results are presented in Fig 1A and S2 Table.

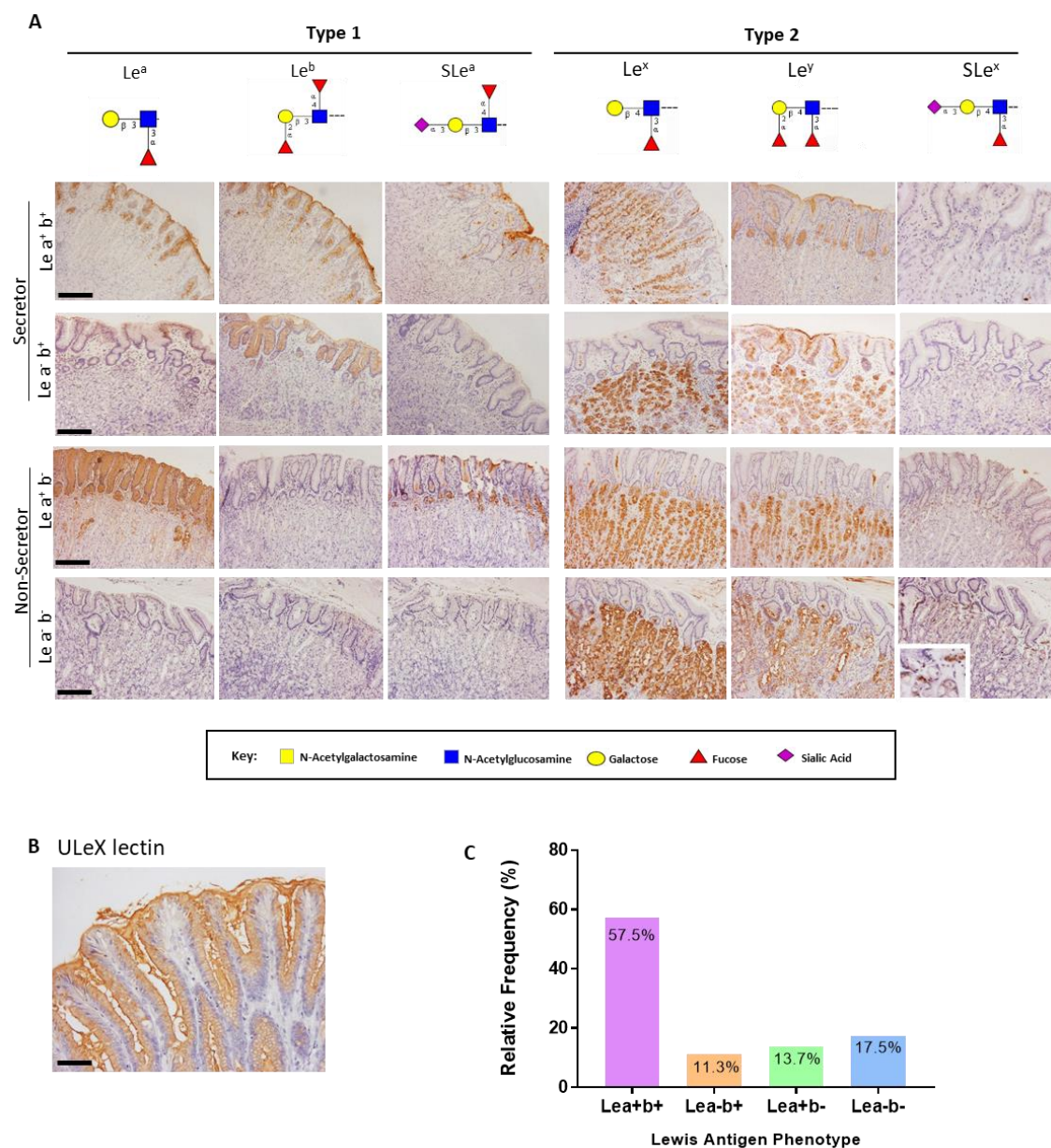


Figure 1. Expression of Lewis Antigens in human gastric mucosa sections. (A) Type 1 (Lewis A, B and sialyl-Lewis A) and Type 2 (Lewis X, Y and sialyl-Lewis X) Lewis Antigens expression in human gastric tissue. Magnification 100x (200x for sLeX insert). (B) Ulex Europaeus Agglutinin I (UEA I) lectin staining in human gastric mucosa, Magnification 200x. (C) Relative frequency of secretor and non-secretor phenotype in human stomach samples.

Secretor and non-secretor phenotype

The secretor status of each patient was evaluated through the simultaneous expression of LeB antigen and binding of the UleX lectin. The detailed results and representative images are presented in Fig 1B and S2 Table. The secretor phenotype was the most predominant, being found in 55 out of 80 cases (68.8%); the phenotype LeA⁺B⁺ was the most common (46 out of 80; 57.5%), while the LeA⁻B⁺ phenotype was found in 9 out of 80 cases (11.3%). Regarding the non-secretor status, it was observed in 25 out of 80 patients (32.1%), with two possible phenotypes: LeA⁺B⁻ (11

out of 80; 13.8%) and LeA⁻ B⁻ (14 out of 80; 17.5%). These results are summarized in Table 4 and Fig 1C.

Table 4. Secretor and Non-secretor phenotype of the human gastric mucosa.

Gastric Samples	Secretor phenotype		Non-Secretor phenotype	
	Le A ⁺ B ⁺	Le A ⁻ B ⁺	Le A ⁺ B ⁻	Le A ⁻ B ⁻
Total (n=80)	57.5% (46/80)	11.3% (9/80)	13.8% (11/80)	17.5% (14/80)
Gastric Cancer (GC) patients (n=17)	58.8% (10/17)	11.8% (2/17)	0% (0/17)	29.4% (5/17)
Obese (Ob) patients (n=63)	57.1% (36/63)	11.1% (7/63)	17.5% (11/63)	14.3% (9/63)

***Helicobacter* species *in vitro* binding to the human gastric mucosa**

The *H. pylori* strain 26695 *in vitro* binding assay was used as a positive control for this experiment. As expected, *H. pylori* presented a high adhesion to the human gastric mucosa, both to gastric superficial epithelium and deep glands (S1 Fig). In addition, to minimize quantification errors associated with possible unspecific binding, a minimal quantity of 100 adhered bacteria was used as a criterion to consider a case as positive for *Helicobacter* spp. binding.

The *in vitro* adhesion ability of almost all the *Helicobacter* species evaluated was higher to the superficial foveolar epithelium, in comparison to the deep glands, being this difference in binding statistically significant (S2 Fig A-F). The only exception was for *H. suis* (HS1), where no significant differences in the adhesion values between these gastric structures were detected (S2A Fig).

Regarding the binding to the superficial epithelium, *H. heilmannii* (ASB1.4) was the strain with the highest adhesion capacity (27 out of 80; 33.8%) followed by *H. salomonis* (R1053) and then *H. felis* (CS1), binding detected in 22 out of 80 (27.5%) and 20 out of 80 (25.0%) patient tissue samples, respectively (Fig 2A-G). *H. suis* (HS1) presented the lowest *in vitro* adhesion potential, being detected on gastric section from only in one patient (1.2%). On the other hand, *H. bizzozeronii* (R1051), *H. heilmannii* (ASB1.4) and *H. salomonis* (R1053) presented the highest *in vitro* binding ability to the deep glands (16; 15 and 13 out of 80, respectively), while *H. suis* (HS1) exhibited the lowest *in vitro* binding (2 out of 80; 2.5%) (Fig 2H).

The results of the *in vitro* binding of the *Helicobacter* spp. are summarized in Table 5 and Fig 2.

Table 5. *Helicobacter* species *in vitro* binding to human gastric mucosa tissue sections, according to the tissue classification and gastric structures evaluated.

	GC patients (n=17)				Ob patients (n=63)		Overall patients (n=80)	
<i>Helicobacter</i> spp.	Antral Mucosa (n=5)		Oxyntic Mucosa (n=12)		Oxyntic Mucosa			
	<i>Epithelium</i>	<i>Deep Glands</i>	<i>Epithelium</i>	<i>Deep Glands</i>	<i>Epithelium</i>	<i>Deep glands</i>	<i>Epithelium</i>	<i>Deep Glands</i>
<i>H. pylori</i>	2/5 (40.0%)	2/5 (40.0%)	9/12 (75.0%)	6/12 (50.0%)	39/63 (61.9%)	27/63 (42.8%)	50/80 (62.5%)	35/80 (43.8%)
<i>H. suis</i>	-	-	-	-	1/63 (1.6%)	2/63 (3.2%)	1/80 (1.3%)	2/80 (2.5%)
<i>H. heilmannii</i>	2/5 (40.0%)	1/5 (20.0%)	7/12 (58.3%)	2/12 (16.7%)	18/63 (28.6%)	12/63 (19.0%)	27/80 (33.8%)	15/80 (18.8%)
<i>H. ailurogastricus</i>	2/5 (40.0%)	-	3/12 (25.0%)	2/12 (16.7%)	6/63 (9.5%)	6/63 (9.5%)	11/80 (13.8%)	8/80 (10.0%)
<i>H. felis</i>	2/5 (40.0%)	1/5 (20.0%)	2/12 (16.7%)	1/12 (8.3%)	16/63 (25.4%)	10/63 (15.9%)	20/80 (25.0%)	12/80 (15.0%)
<i>H. bizzozeronii</i>	2/5 (40.0%)	1/5 (20.0%)	1/12 (8.3%)	2/12 (16.7%)	13/63 (20.6%)	13/63 (20.6%)	16/80 (20.0%)	16/80 (20.0%)
<i>H. salomonis</i>	3/5 (60.0%)	1/5 (20.0%)	6/12 (50.0%)	1/12 (8.3%)	13/63 (20.6%)	11/63 (17.5%)	22/80 (27.5%)	13/80 (16.3%)

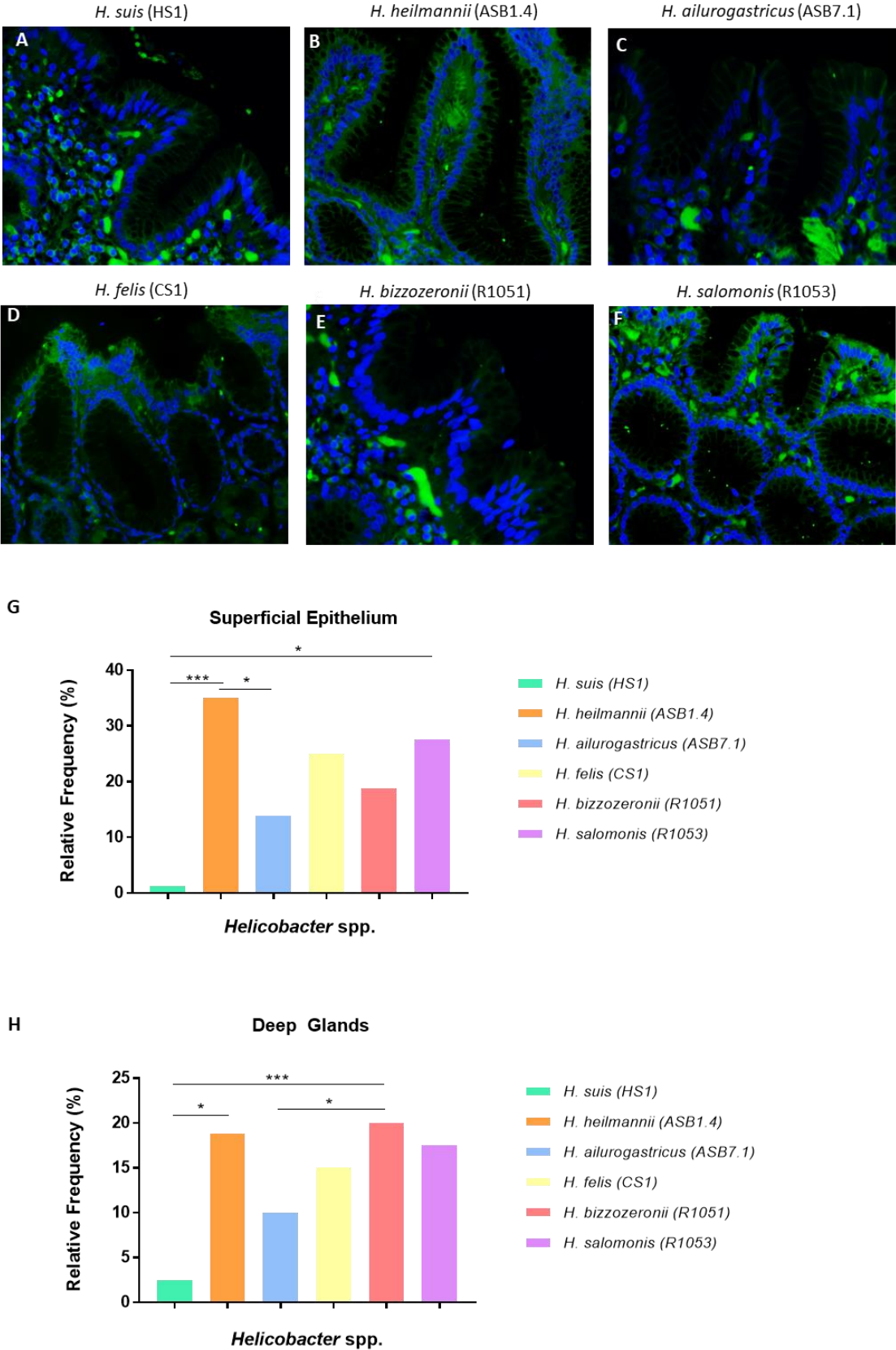


Figure 2. *In vitro* evaluation of *Helicobacter* binding to human gastric mucosa. Adhesion of fluorescein-labelled *Helicobacter* spp. to human gastric mucosa: (A) *H. suis* (HS1 Strain), (B) *H. heilmannii* (ASB1.4 strain), (C) *H. ailurogastricus* (ASB7.1 strain), (D) *H. felis* (CS1 strain), (E) *H. bizzozeronii* (R1051 strain) and (F) *H. salomonis* (R1053 strain). Magnification 200x. (G-H)

Quantification of the *in vitro* adhesion ability of different *Helicobacter* spp. strains. Each symbol represents the mean value obtained by evaluating at least five different fields within the superficial epithelium (G) or deep glands (H), as defined in Materials and Methods. The statistical significance of the differences of the mean values between the groups was evaluated using one-way ANOVA analysis (Kruskal-Wallis test) (*, $p < 0.05$; ***, $p < 0.001$).

Correlation of *Helicobacter* spp. *in vitro* adhesion with histopathological evaluation and gastric tissue glycophenotype

Histopathological evaluation demonstrated that 18 out of 80 (22.5%) patients presented severe chronic inflammation, 15 of them with chronic active gastritis (S1 Table). Amongst these individuals, *H. heilmannii* was the gastric *Helicobacter* species with the highest ability (5 out of 18; 27.8%) to adhere to the gastric superficial epithelium, followed by *H. felis* and *H. bizzozeronii*, both adhering to gastric samples from 3 out of 18 patients (16.7%). Regarding the binding to the deep glands, again *H. felis* (5 out of 18; 27.8%) and *H. bizzozeronii* (4 out of 18; 22.2%) were the species with higher *in vitro* binding ability (Table 6).

Intestinal metaplasia was observed in 15 gastric tissue samples (18.8%): 5 belonged to the gastric cancer (GC) group and 10 to the obese (Ob) group. Gastric *Helicobacter* species were able to bind to tissue specimens presenting intestinal metaplasia. *H. heilmannii* exhibited the highest binding ability both to the gastric epithelium (8 out of 15; 53.3%) and to the deep glands (4 out of 15; 26.7%) of these tissue fragments. *H. bizzozeronii* and *H. salomonis* also presented a high *in vitro* binding capacity to both superficial epithelium and deep glands. The results are summarized in Table 6.

Regarding the secretor status and the possible correlation with histologic features, the secretor phenotype was most frequent among the patients with severe chronic inflammation (13 out of 15; 72.5%). On the other hand, the frequency of the secretor and non-secretor phenotype was very similar for the patients with intestinal metaplasia, with a prevalence of 53.3% (8 out of 15) and 46.7% (7 out of 15), respectively.

Among the 25 non-secretor individuals, adhesion of *H. pylori* was not observed in only 3 cases (3 out of 25; 12.0%), all of them belonging to the double negative phenotype (Le A-B-) (S3 Table). In the remaining 22 non-secretor individuals (88.0%), not only *H. pylori*, but also NHPH, were able to bind to gastric mucosa (S3 Table).

Regarding the secretor individuals, *H. pylori* and NHPH successfully attached to gastric mucosa, being detected in almost all cases. No *H. pylori* adhesion was observed in 3 individuals (3 out of 55; 5.5%), but other NHPH species were detected (S3 Table).

Table 6. Correlation between the *in vitro* binding ability of *Helicobacter* species and the histopathologic characteristics of the gastric tissue.

<i>Helicobacter</i> spp.	Severe Inflammation (n=18)					
	GC patients (n=5)		Ob patients (n=13)		Overall (n=80)	
	Epithelium	Deep glands	Epithelium	Deep glands	Epithelium	Deep Glands
<i>H. pylori</i>	3/5 (60.0%)	1/5 (20.0%)	8/13 (61.5%)	5/13 (38.5%)	11/18 (61.1%)	6/18 (33.3%)
<i>H. suis</i>	-	-	-	1/13 (7.7%)	-	1/18 (5.5%)
<i>H. heilmannii</i>	4/5 (80.0%)	-	1/13 (7.7%)	2/13 (15.4%)	5/18 (27.8%)	2/18 (11.1%)
<i>H. ailurogastricus</i>	1/5 (20.0%)	-	-	-	1/18 (5.5%)	-
<i>H. felis</i>	-	-	3/13 (23.1%)	5/13 (38.5%)	3/18 (16.7%)	5/18 (27.8%)
<i>H. bizzozeronii</i>	1/5 (20.0%)	2/5 (40.0%)	2/13 (15.4%)	2/13 (15.4%)	3/18 (16.7%)	4/18 (22.2%)

<i>H. salomonis</i>	1/5 (20.0%)	-	1/13 (7.7%)	1/13 (7.7%)	2/18 (11.1%)	1/18 (5.5%)

	Intestinal Metaplasia (n=15)					
<i>Helicobacter</i> spp.	GC patients (n =5)		Ob patients (n =10)		Overall (n=80)	
	Epithelium	Deep glands	Epithelium	Deep glands	Epithelium	Deep Glands
<i>H. pylori</i>	4/5 (80.0%)	1/5 (20.0%)	-	-	4/15 (26.7%)	1/15 (6.7%)
<i>H. suis</i>	-	-	-	1/10 (10.0%)	-	1/15 (6.7%)
<i>H. heilmannii</i>	3/5 (60.0%)	-	5/10 (50.0%)	4/10 (40.0%)	8/15 (53.3%)	4/15 (26.7%)
<i>H. ailurogastricus</i>	-	-	1/10 (10.0%)	3/10 (30.0%)	1/15 (6.7%)	3/15 (20.0%)
<i>H. felis</i>	-	-	2/10 (20.0%)	1/10 (10.0%)	2/15 (13.3%)	1/15 (6.7%)
<i>H. bizzozeronii</i>	1/5 (20.0%)	1/5 (20.0%)	3/10 (30.0%)	2/10 (20.0%)	3/15 (20.0%)	3/15 (20.0%)
<i>H. salomonis</i>	-	-	3/10 (30.0%)	3/10 (30.0%)	3/15 (20.0%)	3/15 (20.0%)

Discussion

The *H. pylori* pathogenesis in the human stomach has been widely studied and its role in gastric carcinogenic processes is obvious (Ahn HJ 2015; Ansari S 2017; De Falco M 2015; Huang Y 2016; Jenab et al. 2008; Magalhaes A 2010b; van Amsterdam K 2006). Initially, *H. pylori* infection is associated with chronic gastritis, that may persist and evolve to intestinal metaplasia, dysplasia and, ultimately, to gastric carcinoma (Ahn HJ 2015; Camilo V 2017; Carneiro and Chaves 2006; Chmiela M 2019; Magalhaes A 2015; P 1992, 2004). Chronic inflammation is one the major clinical outcomes of human *H. pylori* infection. In our cohort, 37.5% of the patients presented a chronic inflammation, varying from mild (60.0%), to moderate (30.0%) or severe (10.0%). The presence of neutrophils and eosinophils, in combination with lymphocytes and plasma cells, a phenotype called chronic active inflammation, was observed in 62.5% of the patients, mostly with mild activity (48.0%).

Glycosylation is a post-translational modification essential for several biological processes such as the interactions of a pathogen with its host (Eichler 2019; Endo 2019; Magalhaes A 2015). Glycans are responsible for the modulation of the interplay between several microorganisms and the host microenvironment (Etzold S 2014; Formosa-Dague C 2018; Thompson AJ 2019). The infection of the human gastric mucosa by *H. pylori* is one of the most well-studied interactions (Aspholm-Hurting M 2004; Benktander J 2012; Borén T 1994; Magalhaes A 2010b; Magalhaes A 2016) which firstly relies on the contact of the pathogen with the glycan receptors at the cell surface, namely the Lewis antigens. This step is crucial for colonization and subsequent infection success (Ansari S 2017; Huang Y 2016; Kreisman and Cobb 2012). The expression of type 1 Lewis antigens (LeA and LeB) within our samples was observed in the gastric superficial foveolar epithelium and mucous cells, as previously described (López-Ferrer A 2000; Magalhaes A 2009; Mollicone R 1985; Teixeira A 2002). On the other hand, the type 2 antigens (LeX and LeY) are present mainly on the gastric pits and deep glands, but they can also be expressed in the superficial epithelium (Magalhaes A 2009; Moran 2008).

Human infection by *H. pylori* is considered a major health issue, affecting half of the worldwide population (Doulberis M 2020; Hooi JKY 2017; van Amsterdam K 2006). Gastric NHPH species are mainly found in the stomach of several animals, such as dogs, cats and pigs (Haesebrouck F 2009), but several of these bacteria can also colonize the human stomach (Bento-Miranda M 2014; Berlamont H 2019; De Witte C 2018; Matos R 2020). Compared to *H. pylori*, NHPH infection rate is lower, being found in 0.2-6.0% of gastric biopsies from human patients with gastric disease (Haesebrouck F 2009). In our experiments, we were able to observe, for the first time, *in vitro* binding of several NHPH strains to the human gastric mucosa. All the

Helicobacter species analysed were able to bind, both to the superficial foveolar epithelium and to the deep glands. With exception of the *H. suis* HS1 strain, the strains belonging to all other *Helicobacter* species assessed had a higher binding ability to the superficial foveolar epithelium than to the deeper glands. *H. heilmannii* (ASB1.4 strain) presented the highest *in vitro* binding ability to the superficial foveolar epithelium, with statistical significance compared to *H. suis* HS1 (** $p < 0.001$) and *H. ailurogastricus* ASB7.1 (* $p < 0.05$). Interestingly, the patient with the highest percentage of *H. heilmannii* adhered bacteria belongs to the GC group (patient 16) and presented a non-secretor phenotype with no expression of type 1 nor type 2 Lewis antigens and a moderate chronic active inflammation. In this patient, a high *H. felis* and *H. salomonis* adhesion value was also detected. Amongst the other NHPH, *H. salomonis* (R1053 strain) and *H. felis* (CS1 strain) also presented a good capacity to adhere to human gastric epithelial cells. The patient 31 (Ob group) exhibited a moderate chronic inflammation, and the highest number of *H. suis* and *H. felis* adhered bacteria. The highest number of *H. ailurogastricus* adhered to the gastric epithelium was observed for patient 1 (GC group), who presented a severe sub-acute inflammation. Additionally, this patient also presented multifocal intestinal metaplasia. Patient 9 (GC group), presented the highest binding ability for *H. salomonis* R1053, concomitantly with a moderate chronic active inflammation. For the *H. bizzozeronii* R1051, patient 50, showing a severe sub-acute gastritis with a high amount of neutrophils and presence of intestinal metaplasia, presented the highest percentage of adhered bacteria. Amongst the 3 NHPH species with the highest *in vitro* binding, there are 7 common patients, all with signs of inflammation and moderate to severe gastritis.

H. bizzozeronii (R1051 strain) exhibited the highest *in vitro* binding to the deep glands. The gastric tissue with the highest number of adhered bacteria was from patient 53 (Ob group), who presented a chronic moderate inflammation. The binding ability was significantly higher when compared to *H. suis* HS1 (** $p < 0.001$) and to *H. ailurogastricus* ASB7.1 (* $p < 0.05$). Besides this, *H. heilmannii* (ASB1.4 strain) and *H. salomonis* (R1053 strain) also exhibited high capacity to bind to the gastric deep glands. Samples from patient 32 (Ob group; moderate sub-acute inflammation) presented the highest number of *H. heilmannii* ASB1.4 adhered bacteria, while gastric tissue from patient 10 (GC group; moderate active inflammation) exhibited the highest binding of *H. salomonis* R1053 and *H. felis* CS1. Amongst the 3 NHPH species with the highest *in vitro* binding ability, there are 2 common patients (patient 38 and patient 45), both secretors and with moderate active gastritis. Interestingly, patient 28 (non-secretor phenotype and with a severe chronic inflammation) exhibited the highest binding ability to the deep glands for *H. suis* HS1, although this patient also exhibits a high number of adhered bacteria for *H. heilmannii* ASB1.4 and *H. ailurogastricus* ASB7.1. Furthermore, the *H. heilmannii*, *H. felis*, *H. bizzozeronii* and *H. salomonis* strains tested here, were also able to bind to gastric tissues with severe chronic inflammation, and even to tissues presenting intestinal metaplasia, suggesting that these bacteria do not bind exclusively to a healthy gastric microenvironment.

The *in vitro* binding of the *H. heilmannii*, *H. felis*, and *H. ailurogastricus* strains was higher in the patients presenting a chronic active inflammation when compared to the chronic gastritis phenotype. On the other hand, the adhesion of the *H. bizzozeronii* and *H. salomonis* strains was highest for individuals with chronic, mostly mild, gastritis (data not shown). The differences might suggest that the NHPH strains tested here can survive and persist in different conditions. The *H. heilmannii*, *H. ailurogastricus* and *H. felis* strains seem to bind to gastric epithelial cells in a more initial phase of the inflammatory process, since the presence of acute inflammation with neutrophilic activity was observed in the majority of the individuals. In contrast, the *H. bizzozeronii* and *H. salomonis* strains tested here preferentially adhered to the tissues presenting chronic inflammation (mainly mild). Some studies reported the presence of genes encoding the neutrophil-activating protein (NapA) in the genome of *H. felis*, *H. bizzozeronii*, *H. suis* and *H. heilmannii* (Arnold IC 2011). In *H. pylori*, NapA has been described as a modulator of the oxidative burst, that contributes for the establishment of a balanced and chronic inflammation of the gastric mucosa (Olczak, Wang, and Maier 2005; Petersson et al. 2006). The binding of *H. felis* and *H. heilmannii* to individuals with chronic active inflammation, characterized by periods of massive amounts of neutrophils, and the described occurrence of mixed infections by *H. pylori* and these

NHPH, may suggest that these bacteria could support the establishment of a chronic inflammation, through NapA. Additionally, the *H. heilmannii* adhesins HofE and HofF have been associated with the activation of IL-1 β production by gastric epithelial cells (Liu C 2016; De Witte C 2016). Tissues presenting signs of active immune response are usually characterized by the expression of several proinflammatory molecules, such as IL-1 β , and this could be another possible explanation for the high *in vitro* binding of *H. heilmannii* ASB1.4 strain to inflamed gastric tissues.

The biosynthesis of the ABO(H) blood-group antigens in the human gastric epithelium is mediated by the expression of the *FUT2* gene (Mollicone R 1985), which encodes the fucosyltransferase 2 (FUT2) enzyme (De Oliveira DM 2017; Heneghan M 1998). The presence of an active FUT2 enzyme results in the production of the corresponding blood-group antigens, such as LeB and LeY, and individuals harbouring a functional FUT2 enzyme are called secretors (Ikehara Y 1998; Magalhaes A 2009; Serpa J 2004). It has been described that 70.0% of the world's population belongs to the secretor phenotype (De Oliveira DM 2017). The infection by *H. pylori* is highly associated with the secretor status, with individuals being more prone to colonization and persistent infection (Azevedo M 2008; Ikehara Y 1998; Magalhaes A 2009; Magalhaes A 2016; Serpa J 2004). Additionally, the expression of the *UleX Europaeus* Agglutinin I (EAU I) lectin, which binds to α -linked fucose residues, such as ABO blood group, has also been used as a marker of the secretor status (Kulhlmann WD 1983). In this study, 68.8% of the individuals (55 out of 80) were classified as secretors, which is similar to the values previously described (De Oliveira DM 2017). Curiously, in 3 of these individuals absence of *in vitro* adhesion of *H. pylori* was observed (3 out of 55; 5.5%), however several NHPH strains were able to bind to the gastric mucosa of these patients.

On the other hand, non-secretor individuals are characterized by the absence of ABO(H) antigens on the gastric epithelial cells (Heggelund JE 2017). This phenotype has been correlated to decreased susceptibility to infection by several pathogens, including *H. pylori* (Ikehara Y 1998; Magalhaes A 2009). These individuals do not express the LeB antigen, but albeit in smaller frequency can also be infected with *H. pylori*, which means that the BabA-LeB interaction is important but not essential for a successful colonization (Benktander J 2018). In the non-secretor individuals, the infection process seems to occur through the interaction between the SabA adhesin and sialylated glycans, such as sialyl-Lewis X and sialyl-Lewis A (Benktander J 2018; Linden SK 2008; Magalhaes A 2015; Mahdavi J 2002). In our cohort, the non-secretor phenotype was observed in 25 out of 80 patients (31.2%). For the majority of these patients (88.0%), *in vitro* binding of *H. pylori*, as well as of several NHPH strains, was observed. Indeed, 3 of them (12.0%) presented NHPH *in vitro* binding to the human gastric mucosa, but no *H. pylori* binding. These results suggest that the binding of the NHPH species to the human gastric mucosa is not correlated to the secretor status, highlighting the possibility that other unknown molecules can be essential players in the NHPH adhesion and colonization process. Unlike for *H. pylori*, the NHPH infection mechanism of the human stomach is practically unknown, but the absence of the major *H. pylori* adhesins, such as BabA, SabA and AlpA/B, can be a possible explanation for the low infection rate of NHPH (Amorim I 2014; De Witte C 2016; Padra M 2018). However, it has been hypothesized that NHPH species express other OMPs, which may be essential for the adhesion mechanism (Arnold IC 2011; Kondadi PK 2012; Schott T 2011b; Smet A 2013). Recently, some studies described the presence of possible virulence factors for *H. heilmannii* and *H. suis* (Liu C 2016; Padra M 2018). Furthermore, Kondadi *et al.*, described that *H. bizzozeronii* binds preferentially to α 2,3-sialylated structures (Kondadi PK 2012). Interestingly, in our cohort, 80% of the individuals with *H. bizzozeronii* R1051 *in vitro* binding expressed sialylated antigens, mostly sLeA, suggesting that these glycan structures could be a potential recognition domain for these bacteria.

Conclusion

In conclusion, the *in vitro* screening of the *Helicobacter* binding ability showed that all the NHPH species evaluated were able to bind to the human gastric mucosa, albeit in lower frequency when compared with *H. pylori*. In descendent order, the *H. heilmannii*, *H. salomonis* and *H. bizzozeronii* strains testes here, presented the highest *in vitro* binding ability, both to the superficial epithelium and to the deep glands. Interestingly, no correlation was found between the individuals secretor status and the binding of NHPH, reinforcing that other glycans, besides Lewis antigens, are involved in the NHPH adhesion mechanisms to the human gastric mucosa.

Supplementary Material

S1 Table. Detailed information of the histopathological evaluation based on the Updated Sidney System (Dixon 1996).

S2 Table. Evaluation of type 1 and 2 Lewis Antigens expression and characterization of secretor status of human gastric mucosa.

S3 Table. Quantification of the *in vitro* binding ability of *Helicobacter* species.

Figure S1. Quantification of the *in vitro* adhesion ability of fluorescein-labelled *Helicobacter* species to the superficial epithelium and deep glands.

Figure S2. *In vitro* adhesion ability of *Helicobacter pylori* to the human gastric mucosa.

Funding

RM is funded by Fundação para a Ciência e Tecnologia/ Ministério da Ciência, Tecnologia e Inovação (FCT) PhD scholarship SFRH/BD/131159/2017.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Ethics Committee from the Centro Hospitalar Universitário de São João/ Faculdade de Medicina da Universidade do Porto (CHSJ/FMUP) (CES 127-15).

Informed Consent Statement

Written informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Material

Figure S1

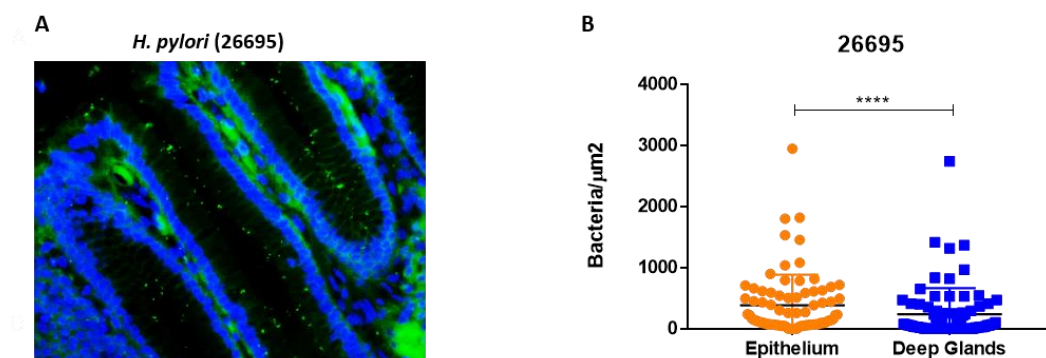


Figure S1. *In vitro* adhesion ability of *Helicobacter pylori* to the human gastric mucosa.

Adhesion of *Helicobacter pylori* (26695 strain) fluorescein-labelled to the human gastric mucosa. (A) Representative image of the *in vitro* binding of the *H. pylori* to the human gastric tissue. (B) Quantification of the *in vitro* adhesion ability of *H. pylori* to the superficial epithelium and to the deep glands. Each symbol represents the mean value obtained by evaluating at least five different fields, as defined in Materials and Methods. The differences of the mean values between the groups were statistically significant using ordinary two-way ANOVA analysis (****, $p < 0.0001$).

Figure S2

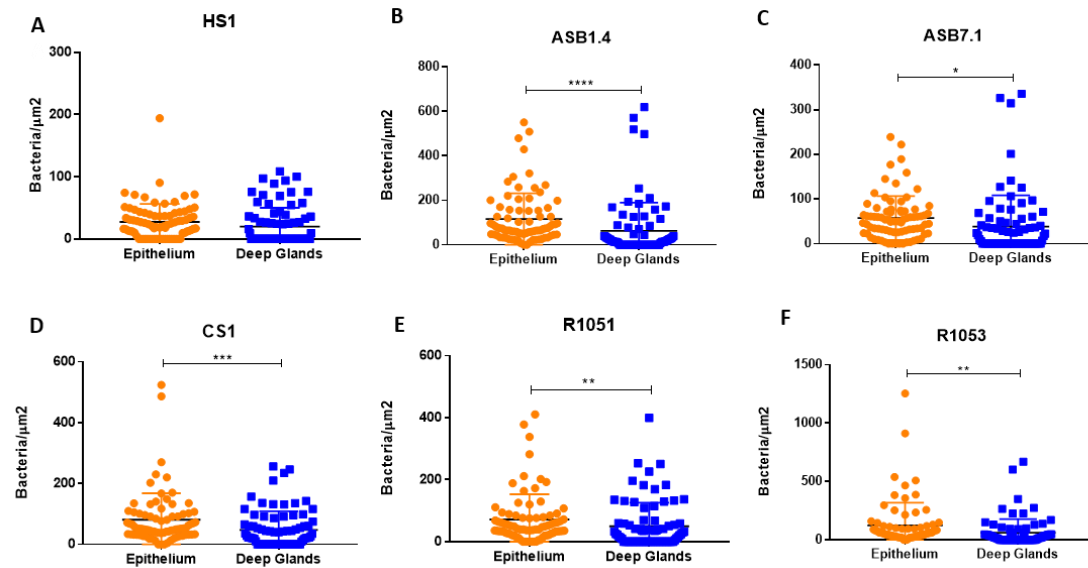


Figure S2. Quantification of the *in vitro* adhesion ability of fluorescein-labelled *Helicobacter* species to the superficial epithelium and deep glands.

Comparisons of the adhesion ability of fluorescein-labelled *Helicobacter* spp. to superficial epithelium and deep glands of the human gastric sample. (A) *H. suis* (HS1 strain), (B) *H. heilmannii* (ASB1.4 strain), (C) *H. ailurogastricus* (ASB7.1 strain), (D) *H. felis* (CS1 strain), (E) *H. bizzozeronii* (R1051 strain) and (F) *H. salomonis* (R1053 strain). Each symbol represents the mean value obtained by evaluating at least five different fields, as defined in Materials and Methods. The differences of the mean values between the groups were statistically significant using ordinary two-way ANOVA analysis (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Table S1

S1 Table. Detailed information of the histopathological evaluation based on the Updated Sidney System (Dixon 1996).

Patient	Clinical Indication	Tissue region	Lymphocytes and plasma cells	Score	Neutrophils and/or eosinophils	Score	Glandular Atrophy	Score	Intestinal Metaplasia	Score	Final Score	Observations
1	GC	Corpus/Fundus	Severe	3	Severe	3	Severe	3	Multifocal	2	11	Hyperplasia of the foveolar superficial epithelium
2	GC	Corpus/Fundus	Moderate	2	Moderate	2	Severe	3	Multifocal	2	9	Lymphoid follicles
3	GC	Corpus/Fundus	Severe	3	Moderate	2	Severe	3	Multifocal	3	11	Dysplasia
4	GC	Corpus/Fundus	Severe	3	Mild	1	Severe	3	Multifocal	1	8	Dysplasia
5	GC	Corpus/Fundus	Moderate	2	Severe	3	-	-	-	-	5	Lymphoid follicles
6	GC	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	
7	GC	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Hyperplasia of the foveolar superficial epithelium and dysplasia
8	GC	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Hyperplasia of the foveolar superficial epithelium
9	GC	Corpus/Fundus	Moderate	2	Moderate	2	Moderate	2	-	-	6	Edema
10	GC	Corpus/Fundus	Moderate	2	Moderate	2	Mild	1	-	-	5	Hyperplasia of the foveolar superficial epithelium and dysplasia
11	GC	Corpus/Fundus	Moderate	2	-	-	-	-	-	-	2	
12	GC	Corpus/Fundus	Moderate	2	-	-	-	-	-	-	2	
13	GC	Corpus/Fundus	Severe	3	Severe	3	Mild	1	-	-	7	
14	GC	Antrum	Moderate	2	Moderate	2	-	-	-	-	4	
15	GC	Corpus/Fundus	Moderate	2	-	-	Mild	1	-	-	3	Severe dysplasia
16	GC	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	
17	GC	Antrum	Severe	3	Severe	3	Severe	3	Multifocal	2	11	Lymphoid follicles, dysplasia, erosion, ulceration; hyperplasia of the superficial epithelium
18	Ob	Corpus/Fundus	Moderate	2	-	-	Moderate	2	Focal	1	5	Lymphoid follicles
19	Ob	Corpus/Fundus	Moderate	2	-	-	Moderate	2	-	-	4	Severe dysplasia

20	Ob	Corpus/Fundus	Mild	1	Mild	1	-	-	-	-	2	
21	Ob	Corpus/Fundus	Moderate	2	Severe	3	Moderate	2	Focal	2	9	
22	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	
23	Ob	Corpus/Fundus	Mild	1	Mild	1	Moderate	2	Focal	2	6	Hiperplasia of the foveolar superficial epithelium
24	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles and mild hyperplasia of the foveolar superficial epithelium
25	Ob	Corpus/Fundus	Moderate	2	-	-	-	-	-	-	2	Erosion
26	Ob	Corpus/Fundus	Moderate	2	Moderate	2	Moderate	2	Focal	1	7	
27	Ob	Corpus/Fundus	Moderate	2	Mild	1	Severe	3	Multifocal	2	8	Hiperplasia of the foveolar superficial epithelium and dysplasia
28	Ob	Corpus/Fundus	Severe	3	-	-	-	-	-	-	3	
29	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	
30	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	
31	Ob	Corpus/Fundus	Moderate	2	-	-	-	-	-	-	2	
32	Ob	Corpus/Fundus	Moderate	2	Severe	3	-	-	-	-	5	Lymphoid follicles, dysplasia and hiperplasia of the foveolar superficial epithelium
33	Ob	Corpus/Fundus	Moderate	1	Mild	1	-	-	-	-	2	Dysplasia
34	Ob	Corpus/Fundus	Severe	3	Severe	3	Severe	3	Multifocal	3	12	
35	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	
36	Ob	Corpus/Fundus	Moderate	2	Moderate	2	-	-	-	-	4	
37	Ob	Corpus/Fundus	Severe	3	Mild	1	-	-	-	-	4	
38	Ob	Antrum	Moderate	2	Moderate	2	-	-	-	-	4	Dysplasia
39	Ob	Corpus/Fundus	Severe	3	Moderate	2	-	-	-	-	5	Dysplasia
40	Ob	Corpus/Fundus	Mild	1	Mild	1	-	-	-	-	2	Lymphoid follicles
41	Ob	Corpus/Fundus	Moderate	2	-	-	-	-	-	-	2	
42	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Dysplasia

43	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	Hiperplasia of the foveolar superficial epithelium
44	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	
45	Ob	Corpus/Fundus	Moderate	2	Moderate	2	-	-	-	-	4	Lymphoid follicles
46	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
47	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
48	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	Lymphoid follicles
49	Ob	Corpus/Fundus	Moderate	2	Mild	1	Moderate	2	Focal	1	6	Lymphoid follicles
50	Ob	Corpus/Fundus	Severe	3	Severe	3	Moderate	2	Focal	1	9	Lymphoid follicles and dysplasia
51	Ob	Corpus/Fundus	Severe	3	Severe	3	-	-	-	-	6	Hiperplasia of the foveolar superficial epithelium, lymphoid follicles and erosion
52	Ob	Corpus/Fundus	Severe	3	Severe	3	-	-	-	-	6	Lymphoid follicles, severe dysplasia and cystic dilation
53	Ob	Antrum	Moderate	2	-	-	-	-	-	-	2	
54	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
55	Ob	Corpus/Fundus	Mild	1	Mild	1	-	-	-	-	2	
56	Ob	Corpus/Fundus	Severe	3	Moderate	2	-	-	-	-	5	Lymphoid follicles
57	Ob	Corpus/Fundus	Mild	1	Mild	1	-	-	-	-	2	Lymphoid follicles
58	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	Lymphoid follicles and cystic dilation
59	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	
60	Ob	Corpus/Fundus	Severe	3	-	-	Severe	3	Multifocal	2	8	Dysplasia
61	Ob	Corpus/Fundus	Severe	3	Mild	1	-	-	-	-	4	Lymphoid follicles, dysplasia and erosion
62	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
63	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
64	Ob	Corpus/Fundus	Severe	3	-	-	-	-	-	-	3	Lymphoid follicles
65	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	
66	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles, edema and cystic dilation

67	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	
68	Ob	Corpus/Fundus	Severe	3	Moderate	2	Moderate	2	Focal	1	8	Lymphoid follicles, erosion, dysplasia and ulceration
69	Ob	Corpus/Fundus	Severe	3	Moderate	2	-	-	-	-	5	Lymphoid follicles, erosion and dysplasia
70	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
71	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	Lymphoid follicles
72	Ob	Corpus/Fundus	Moderate	2	Moderate	2	-	-	-	-	4	Lymphoid follicles
73	Ob	Corpus/Fundus	Moderate	2	Mild	1	Mild	1	-	-	4	Lymphoid follicles
74	Ob	Corpus/Fundus	Moderate	2	Mild	1	Mild	1	-	-	4	Lymphoid follicles
75	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	Lymphoid follicles
76	Ob	Corpus/Fundus	Moderate	2	Moderate	2	Moderate	2	-	-	6	Lymphoid follicles
77	Ob	Corpus/Fundus	Moderate	2	Mild	1	-	-	-	-	3	Lymphoid follicles
78	Ob	Corpus/Fundus	Moderate	2	Moderate	2	Mild	1	-	-	5	Lymphoid follicles and dysplasia
79	Ob	Corpus/Fundus	Mild	1	Mild	1	-	-	-	-	2	
80	Ob	Corpus/Fundus	Mild	1	-	-	-	-	-	-	1	

Table S2

S2 Table. Evaluation of type 1 and 2 Lewis Antigens expression and characterization of secretor status of human gastric mucosa.

Patient	Tissue Type	Secretor status ⁴			Type I Lewis Antigens									Type II Lewis Antigens								
					Le ^a CA3F4 (1:5)			Le ^b BG6 (1:50)			Sle ^a CA19-9 (1:500)			Le ^x SH1 (1:5)			Le ^y AH6 (1:10)			Sle ^x CSLEX-1 (1:80)		
		ULeX			D. ¹	I. ²	Score ³	D. ¹	I. ²	Score ³	D. ¹	I. ²	Score ³	D. ¹	I. ²	Score ³	D. ¹	I. ²	Score ³	D. ¹	I. ²	Score ³
1	Adjacent Tissue	Le (a+ b+)	S	+	2	2	4	3	1	3	0	0	0	4	1	4	4	1	4	1	2	2
2	Adjacent Tissue	Le (a- b+)	S	+	0	0	0	3	1	3	0	0	0	4	2	8	4	1	4	0	0	0
3	Adjacent Tissue	Le (a+ b+)	S	+	4	3	12	3	2	6	1	2	2	4	2	8	4	1	4	2	1	2
4	Adjacent Tissue	Le (a- b-)	NS	-	2	1	2	1	2	2	1	3	3	4	3	12	4	2	8	2	1	2
5	Adjacent Tissue	Le (a+ b+)	S	+	3	1	3	3	2	6	3	2	6	4	3	12	4	2	8	1	1	1
6	Adjacent Tissue	Le (a- b-)	NS	-	0	0	0	2	1	2	0	0	0	4	3	12	4	3	12	1	2	2
7	Adjacent Tissue	Le (a+ b+)	S	+	1	2	2	4	2	8	1	2	2	3	2	6	3	3	9	0	0	0
8	Adjacent Tissue	Le (a+ b+)	S	+	1	1	1	3	2	6	2	3	6	3	3	9	3	1	3	2	1	2
9	Adjacent Tissue	Le (a- b-)	NS	-	0	0	0	0	0	0	0	0	0	3	3	9	4	1	4	1	3	3
10	Adjacent Tissue	Le (a+ b+)	S	+	1	2	2	3	1	3	1	3	3	4	2	8	4	2	8	3	1	3
11	Adjacent Tissue	Le (a- b+)	S	+	1	1	1	4	1	4	1	3	3	3	3	9	3	2	6	0	0	0
12	Adjacent Tissue	Le (a+ b+)	S	+	4	1	4	3	1	3	0	0	0	4	3	12	3	1	3	0	0	0
	Adjacent Tissue				2	1	2	3	1	3	1	2	2	3	3	9	4	1	4	0	0	0
13	Adjacent Tissue	Le (a+ b+)	S	+	3	1	3	3	2	6	2	2	4	3	1	3	3	1	3	0	0	0
14	Adjacent Tissue	Le (a+ b+)	S	+	3	1	3	2	1	2	1	3	3	3	1	3	3	1	3	0	0	0
	Adjacent Tissue				3	1	3	2	2	4	2	1	2	3	1	3	3	1	3	0	0	0
15	Adjacent Tissue	Le (a- b-)	NS	-	0	0	0	0	0	0	0	0	0	3	1	3	2	1	2	0	0	0
	Adjacent Tissue				0	0	0	0	0	0	0	0	0	3	1	3	2	1	2	0	0	0
16	Adjacent Tissue	Le (a- b-)	NS	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Adjacent Tissue				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1

17	Adjacent Tissue	Le (a+ b+)	S	+	3	2	6	3	2	6	3	2	6	4	2	8	4	1	4	0	0	0
	Adjacent Tissue				3	2	6	3	1	3	2	1	2	3	2	6	4	2	8	1	1	1
18	Non-neoplastic Tissue	Le (a+ b+)	S	+	1	3	3	4	2	8	0	0	0	4	2	8	4	2	8	0	0	0
					1	3	3	4	1	4	1	2	2	4	3	12	4	1	4	0	0	0
					0	0	0	4	2	8	0	0	0	4	3	12	4	2	8	0	0	0
19	Non-neoplastic Tissue	Le (a+ b+)	S	+	1	2	2	4	2	8	1	2	2	4	3	12	4	3	12	0	0	0
					1	3	3	4	2	8	1	2	2	4	3	12	4	2	8	0	0	0
					3	3	9	3	3	9	2	2	4	4	2	8	4	2	8	0	0	0
20	Non-neoplastic Tissue	Le (a+ b-)	NS	-	3	1	3	0	0	0	0	0	0	4	1	4	4	1	4	0	0	0
					3	1	3	0	0	0	1	1	1	4	2	8	4	1	4	0	0	0
					3	3	9	0	0	0	0	0	0	4	2	8	4	2	8	0	0	0
21	Non-neoplastic Tissue	Le (a+ b-)	NS	-	3	2	6	0	0	0	2	1	2	3	2	6	3	1	3	1	1	1
					3	2	6	1	1	1	3	2	6	3	2	6	3	1	3	0	0	0
					3	1	3	0	0	0	3	2	6	3	3	9	0	0	0	0	0	0
22	Non-neoplastic Tissue	Le (a- b+)	S	+	1	1	1	3	1	3	0	0	0	4	3	12	4	1	4	0	0	0
					0	0	0	3	2	6	0	0	0	4	3	12	4	2	8	0	0	0
					0	0	0	3	2	6	0	0	0	4	3	12	4	3	12	0	0	0
23	Non-neoplastic Tissue	Le (a+ b-)	NS	-	3	2	6	0	0	0	3	1	3	4	3	12	4	1	4	1	1	1
					3	2	6	0	0	0	3	1	3	4	3	12	4	1	4	1	1	1
					3	2	6	0	0	0	3	2	6	4	3	12	4	1	4	1	1	1
24	Non-neoplastic Tissue	Le (a+ b+)	S	+	2	1	2	3	1	3	0	0	0	3	1	3	3	1	3	0	0	0
					3	1	3	3	2	6	2	1	2	3	2	6	3	1	3	0	0	0
					3	1	3	3	2	6	2	1	2	3	2	6	3	1	3	0	0	0
25	Non-neoplastic Tissue	Le (a+ b+)	S	+	3	3	9	3	2	6	3	2	6	4	3	12	4	1	4	0	0	0
					3	3	9	3	1	3	3	3	9	4	2	8	4	3	12	0	0	0
					3	3	9	4	1	4	3	2	6	3	2	6	4	3	12	0	0	0
26		Le (a- b+)	S	+	1	1	1	2	1	2	0	0	0	4	3	12	4	1	4	0	0	0

	Non-neoplastic Tissue				0	0	0	3	1	3	0	0	0	4	3	12	4	1	4	0	0	0
					1	1	1	3	2	6	0	0	0	4	3	12	4	1	4	0	0	0
27	Non-neoplastic Tissue	Le (a-b)	NS	-	0	0	0	0	0	0	0	0	0	4	2	8	4	3	12	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	4	3	12	0	0	0
					0	0	0	0	0	0	0	0	0	4	3	12	4	1	4	0	0	0
28	Non-neoplastic Tissue	Le (a-b)	NS	-	0	0	0	0	0	0	0	0	0	4	2	8	4	1	4	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	4	1	4	0	0	0
29	Non-neoplastic Tissue	Le (a+b)	S	+	4	2	8	3	1	3	0	0	0	4	2	8	3	1	3	0	0	0
					4	1	4	3	2	6	0	0	0	4	2	8	2	2	4	0	0	0
30	Non-neoplastic Tissue	Le (a+b)	S	+	3	1	3	3	1	3	1	1	1	4	2	8	2	1	2	0	0	0
					3	1	3	3	1	3	1	1	1	4	2	8	3	1	3	0	0	0
31	Non-neoplastic Tissue	Le (a+b)	S	+	4	3	12	4	3	12	3	3	9	4	2	8	4	3	12	0	0	0
					4	3	12	4	3	12	3	1	3	3	3	9	4	3	12	0	0	0
					4	3	12	4	1	4	3	2	6	4	3	12	4	1	4	0	0	0
32	Non-neoplastic Tissue	Le (a+b)	S	+	3	2	6	3	2	6	2	2	4	4	2	8	4	3	12	0	0	0
					3	2	6	3	1	3	3	2	6	3	3	9	4	3	12	0	0	0
					3	2	6	3	1	3	3	2	6	4	3	12	4	1	4	0	0	0
33	Non-neoplastic Tissue	Le (a+b)	S	+	1	1	1	3	2	6	0	0	0	3	3	9	4	3	12	0	0	0
					1	1	1	3	1	3	0	0	0	4	3	12	4	2	8	0	0	0
					2	2	4	3	1	3	1	1	1	4	3	12	4	3	12	0	0	0
34	Non-neoplastic Tissue	Le (a+b)	S	+	4	3	12	4	3	12	3	3	9	4	2	8	4	1	4	0	0	0
					4	3	12	4	2	8	3	2	6	4	2	8	3	1	3	1	3	3
					4	3	12	4	2	8	3	2	6	3	2	6	4	1	4	0	0	0
35	Non-neoplastic Tissue	Le (a+b)	S	+	4	1	4	4	2	8	1	3	3	4	2	8	4	1	4	0	0	0
					4	2	8	4	1	4	2	3	6	4	2	8	4	1	4	0	0	0
					4	3	12	3	2	6	1	2	2	4	1	4	4	1	4	0	0	0
36		Le (a+b)	S	+	4	3	12	3	2	6	0	0	0	4	2	8	4	1	4	0	0	0

	Non-neoplastic Tissue				3	2	6	3	2	6	0	0	0	4	2	8	4	1	4	0	0	0
					4	3	12	3	2	6	0	0	0	4	2	8	4	1	4	0	0	0
37	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	4	3	12	3	3	9	2	2	4	4	1	4	3	2	6	0	0	0
					4	2	8	3	2	6	2	2	4	4	2	8	3	2	6	0	0	0
					4	2	8	4	1	4	1	1	1	4	2	8	4	1	4	0	0	0
38	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	3	1	3	4	2	8	0	0	0	4	2	8	4	1	4	0	0	0
					3	1	3	4	2	8	0	0	0	4	1	4	4	1	4	0	0	0
					3	2	6	4	2	8	0	0	0	4	1	4	4	1	4	0	0	0
39	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	4	3	12	4	2	8	2	3	6	4	2	8	4	2	8	1	1	1
					4	3	12	4	2	8	2	3	6	4	3	12	4	2	8	1	1	1
					4	3	12	4	3	12	3	3	9	4	2	8	4	2	8	2	3	6
40	Non-neoplastic Tissue	Le (a+b ⁻)	NS	-	4	2	8	0	0	0	3	2	6	4	2	8	4	1	4	0	0	0
					4	2	8	0	0	0	2	1	2	4	2	8	4	1	4	0	0	0
					4	2	8	1	1	1	3	2	6	4	1	4	4	2	8	0	0	0
41	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	2	1	2	3	2	6	0	0	0	4	1	4	4	1	4	0	0	0
					3	1	3	3	1	3	2	1	2	4	2	8	4	1	4	0	0	0
					3	1	3	3	1	3	0	0	0	4	2	8	4	1	4	0	0	0
42	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	3	1	3	3	1	3	0	0	0	4	2	8	4	2	8	0	0	0
					4	1	4	3	1	3	0	0	0	4	1	4	4	1	4	0	0	0
					2	1	2	3	1	3	1	2	2	4	2	8	4	1	4	0	0	0
43	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	3	1	3	4	1	4	1	2	2	3	2	6	4	1	4	0	0	0
					2	1	2	4	1	4	1	2	2	4	2	8	4	1	4	0	0	0
					3	1	3	4	1	4	2	1	2	4	1	4	3	1	3	0	0	0
44	Non-neoplastic Tissue	Le (a+b ⁻)	NS	-	0	0	0	0	0	0	0	0	0	4	2	8	4	1	4	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	4	1	4	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	4	1	4	0	0	0
45		Le (a+b ⁺)	S	+	4	3	12	4	1	4	4	1	4	3	1	3	3	1	3	0	0	0

	Non-neoplastic Tissue				4	2	8	4	1	4	4	2	8	3	1	3	3	1	3	0	0	0
					4	3	12	4	2	8	4	1	4	3	1	3	3	1	3	0	0	0
46	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	3	1	3	4	1	4	0	0	0	3	1	3	3	1	3	0	0	0
					3	2	6	1	1	1	0	0	0	3	1	3	3	1	3	0	0	0
					3	1	3	4	1	4	0	0	0	4	2	8	3	1	3	0	0	0
47	Non-neoplastic Tissue	Le (a-b)	NS	-	1	1	1	1	1	1	0	0	0	4	2	8	4	1	4	0	0	0
					1	1	1	0	0	0	0	0	0	4	1	4	4	2	8	0	0	0
					2	1	2	2	1	2	0	0	0	4	1	4	4	1	4	0	0	0
48	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	3	1	3	3	1	3	0	0	0	4	2	8	4	1	4	0	0	0
					0	0	0	4	1	4	0	0	0	4	1	4	4	1	4	0	0	0
					0	0	0	4	1	4	0	0	0	4	2	8	4	1	4	0	0	0
49	Non-neoplastic Tissue	Le (a+b ⁻)	NS	-	4	1	4	0	0	0	3	2	6	4	1	4	4	1	4	0	0	0
					4	1	4	0	0	0	3	2	6	4	2	8	4	2	8	0	0	0
					4	1	4	0	0	0	3	2	6	4	2	8	4	2	8	1	1	1
50	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	2	2	4	4	1	4	1	1	1	4	2	8	4	2	8	0	0	0
					3	2	6	4	1	4	1	1	1	4	2	8	4	1	4	0	0	0
					2	2	4	4	1	4	1	1	1	4	1	4	4	1	4	0	0	0
51	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	4	1	4	4	2	8	0	0	0	4	1	4	4	2	8	0	0	0
					4	1	4	4	1	4	0	0	0	4	1	4	4	2	8	0	0	0
					4	2	8	4	1	4	0	0	0	3	2	6	4	2	8	0	0	0
52	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	4	2	8	4	1	4	2	1	2	4	2	8	4	2	8	0	0	0
					4	2	8	3	1	3	2	1	2	4	1	4	4	1	4	0	0	0
					3	2	6	3	1	3	1	1	1	4	1	4	4	1	4	0	0	0
53	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	1	1	1	4	2	8	0	0	0	4	2	8	4	1	4	0	0	0
					2	1	2	4	1	4	0	0	0	4	1	4	4	2	8	0	0	0
					3	1	3	3	1	3	2	1	2	4	2	8	4	2	8	0	0	0
54		Le (a+b ⁻)	NS	-	4	1	4	0	0	0	3	1	3	4	2	8	4	2	8	0	0	0

	Non-neoplastic Tissue				4	1	4	0	0	0	2	1	2	4	2	8	4	1	4	0	0	0
					4	3	12	0	0	0	4	2	8	3	1	3	4	1	4	0	0	0
55	Non-neoplastic Tissue	Le (a ⁺ b ⁺)	S	+	2	1	2	4	1	4	0	0	0	4	1	4	4	1	4	0	0	0
					2	1	2	3	1	3	0	0	0	4	1	4	4	1	4	0	0	0
					0	0	0	4	1	4	0	0	0	3	1	3	4	1	4	0	0	0
56	Non-neoplastic Tissue	Le (a ⁺ b ⁺)	S	+	2	1	2	4	2	8	2	2	4	4	3	12	4	2	8	0	0	0
					2	2	4	4	2	8	4	2	8	4	3	12	4	2	8	0	0	0
					0	0	0	4	1	4	2	3	6	4	2	8	4	3	12	0	0	0
57	Non-neoplastic Tissue	Le (a ⁺ b ⁺)	S	+	0	0	0	4	1	4	0	0	0	4	3	12	4	3	12	0	0	0
					2	2	4	3	2	6	2	3	6	4	3	12	4	2	8	0	0	0
					0	0	0	0	0	0	0	0	0	4	3	12	4	3	12	0	0	0
58	Non-neoplastic Tissue	Le (a ⁺ b ⁻)	NS	-	4	3	12	0	0	0	2	3	6	4	3	12	4	2	8	0	0	0
					4	3	12	0	0	0	2	3	6	4	3	12	4	2	8	0	0	0
					4	3	12	0	0	0	4	3	12	4	2	8	4	2	8	0	0	0
59	Non-neoplastic Tissue	Le (a ⁺ b ⁺)	S	+	2	2	4	3	2	6	3	2	6	4	3	12	3	2	6	0	0	0
					2	2	4	3	3	9	3	3	9	4	3	12	3	2	6	0	0	0
					2	2	4	3	2	6	3	2	6	4	3	12	4	2	8	0	0	0
60	Non-neoplastic Tissue	Le (a ⁺ b ⁻)	NS	-	2	2	4	0	0	0	0	0	0	4	3	12	4	2	8	0	0	0
					3	2	6	0	0	0	0	0	0	4	3	12	4	3	12	0	0	0
					3	3	9	0	0	0	0	0	0	4	2	8	4	2	8	0	0	0
61	Non-neoplastic Tissue	Le (a ⁻ b ⁻)	NS	-	1	2	2	0	0	0	0	0	0	4	2	8	3	2	6	0	0	0
					0	0	0	0	0	0	0	0	0	3	2	6	3	2	6	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	4	2	8	0	0	0
62	Non-neoplastic Tissue	Le (a ⁻ b ⁺)	S	+	1	1	1	2	1	2	0	0	0	4	2	8	4	2	8	0	0	0
					0	0	0	3	1	3	0	0	0	3	2	6	4	2	8	0	0	0
					0	0	0	2	2	4	0	0	0	4	3	12	4	2	8	0	0	0
63		Le (a ⁺ b ⁺)	S	+	2	2	4	4	3	12	0	0	0	3	2	6	4	2	8	0	0	0

	Non-neoplastic Tissue				2	3	6	4	2	8	2	1	2	4	2	8	4	2	8	0	0	0
					1	2	2	0	0	0	0	0	0	4	2	8	4	3	12	0	0	0
64	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	2	3	6	4	3	12	1	3	3	4	3	12	4	2	8	0	0	0
					1	2	2	4	2	8	0	0	0	4	3	12	4	3	12	0	0	0
					2	3	6	4	3	12	0	0	0	4	3	12	4	2	8	0	0	0
65	Non-neoplastic Tissue	Le (a+ b-)	NS	-	4	2	8	0	0	0	0	0	0	2	2	4	2	1	2	0	0	0
					4	2	8	0	0	0	0	0	0	4	3	12	4	2	8	0	0	0
					0	0	0	0	0	0	3	2	6	4	2	8	3	2	6	0	0	0
66	Non-neoplastic Tissue	Le (a- b-)	NS	-	0	0	0	0	0	0	0	0	0	4	2	8	4	1	4	0	0	0
					0	0	0	0	0	0	0	0	0	3	2	6	4	2	8	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	3	3	9	0	0	0
67	Non-neoplastic Tissue	Le (a- b-)	NS	-	0	0	0	1	1	1	0	0	0	3	2	6	4	2	8	0	0	0
					0	0	0	2	1	2	0	0	0	4	2	8	4	2	8	0	0	0
					0	0	0	0	0	0	0	0	0	4	3	12	4	3	12	0	0	0
68	Non-neoplastic Tissue	Le (a- b-)	NS	-	1	2	2	0	0	0	0	0	0	3	3	9	4	3	12	0	0	0
					0	0	0	0	0	0	0	0	0	3	3	9	2	2	4	0	0	0
					0	0	0	0	0	0	0	0	0	4	3	12	4	2	8	0	0	0
69	Non-neoplastic Tissue	Le (a- b ⁺)	S	+	0	0	0	4	2	8	0	0	0	4	3	12	4	3	12	0	0	0
					1	2	2	0	0	0	0	0	0	4	3	12	4	3	12	0	0	0
					0	0	0	2	1	2	0	0	0	4	3	12	4	3	12	0	0	0
70	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	0	0	0	2	1	2	1	1	1	4	2	8	4	1	4	0	0	0
					0	0	0	0	0	0	1	1	1	4	2	8	4	2	8	0	0	0
					2	2	4	3	1	3	1	1	1	4	3	12	4	2	8	0	0	0
71	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	3	2	6	0	0	0	1	1	1	2	2	4	4	2	8	0	0	0
					0	0	0	4	1	4	1	1	1	3	3	9	3	1	3	0	0	0
					1	2	2	2	1	2	1	1	1	4	2	8	2	1	2	0	0	0
72		Le (a+b ⁺)	S	+	4	2	8	4	1	4	4	3	12	4	2	8	4	3	12	0	0	0

	Non-neoplastic Tissue				4	3	12	4	2	8	4	3	12	4	2	8	4	3	12	0	0	0
					0	0	0	4	2	8	4	3	12	3	1	3	4	3	12	0	0	0
73	Non-neoplastic Tissue	Le (a+b ⁺)	S	+	4	3	12	4	2	8	4	3	12	4	3	12	3	1	3	0	0	0
					4	3	12	2	3	6	4	3	12	4	2	8	4	3	12	0	0	0
					4	3	12	4	3	12	4	3	12	4	3	12	4	3	12	0	0	0
74	Non-neoplastic Tissue	Le (a+ b-)	NS	-	4	1	4	0	0	0	4	3	12	4	2	8	4	3	12	0	0	0
					0	0	0	0	0	0	4	3	12	4	3	12	4	1	4	0	0	0
					0	0	0	0	0	0	4	3	12	4	3	12	2	1	2	0	0	0
75	Non-neoplastic Tissue	Le (a+ b-)	NS	-	0	0	0	0	0	0	4	2	8	4	2	8	4	2	8	0	0	0
					4	2	8	0	0	0	4	2	8	4	2	8	4	2	8	0	0	0
					0	0	0	0	0	0	4	2	8	4	2	8	4	3	12	0	0	0
76	Non-neoplastic Tissue	Le (a- b ⁺)	S	+	0	0	0	3	2	6	3	2	6	4	2	8	4	2	8	0	0	0
					0	0	0	4	2	8	3	2	6	4	2	8	4	2	8	0	0	0
					0	0	0	3	2	6	3	2	6	3	2	6	3	2	6	0	0	0
77	Non-neoplastic Tissue	Le (a ⁺ b ⁺)	S	+	2	1	2	4	3	12	1	1	1	4	3	12	4	2	8	0	0	0
					2	2	4	4	2	8	1	1	1	4	3	12	4	2	8	0	0	0
					0	0	0	3	2	6	1	1	1	4	2	8	4	2	8	0	0	0
78	Non-neoplastic Tissue	Le (a- b ⁺)	S	+	1	1	1	4	2	8	1	1	1	4	2	8	4	2	8	0	0	0
					0	0	0	4	2	8	1	1	1	4	2	8	3	1	3	0	0	0
					0	0	0	4	1	4	1	1	1	4	3	12	4	2	8	0	0	0
79	Non-neoplastic Tissue	Le (a- b)	NS	-	0	0	0	0	0	0	0	0	0	4	2	8	4	2	8	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	0	0	0	0	0	0
					0	0	0	0	0	0	0	0	0	4	2	8	4	2	8	0	0	0
80	Non-neoplastic Tissue	Le (a ⁺ b ⁺)	S	+	2	2	4	4	2	8	0	0	0	4	2	8	4	2	8	0	0	0
					2	2	4	3	1	3	0	0	0	4	2	8	3	2	6	0	0	0
					0	0	0	3	1	3	0	0	0	4	2	8	4	2	8	0	0	0

Distribution and intensity of Lewis Antigens immunoreactivity on superficial epithelium and deep glands was classified according with Lee et al., 2006.

1 Distribution: Immunostaining distribution was classified as 0 (0% positive cells); 1 (0-20% positive cells); 2 (21-50% positive cells); 3 (51-75% positive cells); 4 (76-100% positive cells).

2 Intensity: Immunostaining intensity was evaluated as 0 (absent); 1 (weak); 2 (moderate); 3 (Goodwin CS).

3 The score was calculated based on the product of distribution x intensity. If the value was ≤ 2 , the tissue was considered negative, and if the score was >2 the tissue was considered positive.

4 The individual was classified as Secretor when both LeB and UleX lectin were positive. If the individual had no expression of LeB or UleX staining, or only had one of them, then it was classified as Non-Secretor.

Table S3

S3 Table. Quantification of the *in vitro* binding ability of *Helicobacter* species.

Patient	Gastric localization	<i>H. suis</i> (HS1)	<i>H. heilmannii</i> (ASB1.4)	<i>H. ailurogastricus</i> (ASB7.1)	<i>H. felis</i> (CS1)	<i>H. bizzozeronii</i> (R1051)	<i>H. salomonis</i> (R1053)	<i>H. pylori</i> (26695)
1	Superficial Epithelium	17,2	154,2	239,2	58,0	94,2	509,1	260,7
	Deep Glands	0,0	0,0	0,0	59,7	183,1	0,0	0,0
2	Superficial Epithelium	36,8	158,2	53,8	84,9	91,3	14,1	689,0
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
3	Superficial Epithelium	31,0	152,9	61,8	4,9	39,9	45,1	174,1
	Deep Glands	0,0	0,0	0,0	22,5	32,5	0,0	0,0
4	Superficial Epithelium	0,0	127,3	10,0	68,4	79,2	80,8	305,2
	Deep Glands	0,0	0,0	0,0	0,0	135,2	47,8	241,9
5	Superficial Epithelium	30,5	97,2	30,5	32,6	34,0	5,3	711,0
	Deep Glands	0,0	24,9	0,0	0,0	21,0	0,0	357,3
6	Superficial Epithelium	17,3	0,0	145,2	66,1	49,7	297,9	51,3
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
7	Superficial Epithelium	18,2	60,1	6,9	0,0	85,0	466,3	5,7
	Deep Glands	0,0	0,0	0,0	0,0	69,8	0,0	0,0
8	Superficial Epithelium	10,1	259,3	80,2	61,7	378,1	38,5	1083,7
	Deep Glands	0,0	0,0	0,0	65,5	169,0	32,1	1322,9
9	Superficial Epithelium	10,0	126,7	58,9	0,0	0,0	1254,1	0,0
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
10	Superficial Epithelium	0,0	96,7	177,2	132,2	29,4	912,4	85,0
	Deep Glands	0,0	0,0	0,0	256,2	0,0	602,7	57,1
11	Superficial Epithelium	12,1	256,4	67,7	31,8	36,3	149,3	1457,0
	Deep Glands	0,0	21,7	125,6	0,0	0,0	0,0	535,1
12	Superficial Epithelium	42,0	21,6	104,0	51,8	0,0	384,2	819,5
	Deep Glands	75,9	0,0	60,9	54,3	0,0	265,2	406,3
13	Superficial Epithelium	0,0	429,5	0,0	0,0	0,0	0,0	0,0
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
14	Superficial Epithelium	16,5	479,8	60,8	167,5	19,8	147,4	443,7
	Deep Glands	59,4	168,8	0,0	0,0	0,0	0,0	297,2
15	Superficial Epithelium	0,0	83,8	103,9	149,2	0,0	0,0	1041,1
	Deep Glands	43,7	128,1	335,9	116,1	0,0	0,0	473,7
16	Superficial Epithelium	0,0	551,0	59,8	270,3	33,5	215,8	666,1

	Deep Glands	0,0	519,3	90,7	75,4	0,0	170,9	837,2
17	Superficial Epithelium	0,0	0,0	0,0	29,0	410,9	37,2	0,0
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
18	Superficial Epithelium	0,0	126,7	25,7	66,5	5,6	358,6	0,0
	Deep Glands	0,0	254,0	127,6	35,2	0,0	669,8	0,0
19	Superficial Epithelium	16,3	32,9	3,3	92,3	15,0	120,7	143,6
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
20	Superficial Epithelium	0,0	103,2	32,1	52,6	39,6	95,7	618,5
	Deep Glands	0,0	0,0	0,0	91,9	58,8	38,3	268,8
21	Superficial Epithelium	4,7	51,9	3,3	27,9	5,0	56,6	595,8
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
22	Superficial Epithelium	19,4	269,2	34,4	31,6	22,5	21,9	236,7
	Deep Glands	32,7	135,7	0,0	43,0	0,0	28,8	235,7
23	Superficial Epithelium	23,8	154,5	3,8	37,4	172,6	38,8	789,5
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0
24	Superficial Epithelium	13,7	73,7	57,5	12,9	107,7	539,9	496,8
	Deep Glands	9,3	16,5	55,9	0,0	132,7	226,8	534,6
25	Superficial Epithelium	25,8	220,1	70,9	32,4	164,0	258,7	162,3
	Deep Glands	27,1	210,4	71,6	0,0	59,6	152,5	177,9
26	Superficial Epithelium	49,7	306,0	77,0	50,5	34,1	254,5	456,5
	Deep Glands	100,2	571,9	76,5	53,3	22,2	350,7	134,6
27	Superficial Epithelium	74,4	199,0	74,5	230,2	76,6	388,7	902,3
	Deep Glands	88,8	185,1	326,5	234,6	129,9	274,0	973,0
28	Superficial Epithelium	24,9	59,1	95,0	140,6	28,5	235,4	242,7
	Deep Glands	108,7	117,3	0,0	135,9	0,0	63,2	174,4
29	Superficial Epithelium	47,2	321,6	0,0	107,3	125,7	0,0	720,5
	Deep Glands	75,7	0,0	0,0	91,0	0,0	0,0	254,4
30	Superficial Epithelium	0,0	170,2	9,1	220,4	72,2	101,6	499,6
	Deep Glands	0,0	88,5	47,0	98,7	15,2	0,0	220,2
31	Superficial Epithelium	194,0	236,7	0,0	524,0	25,2	81,4	797,4
	Deep Glands	0,0	71,2	0,0	157,2	114,8	35,5	298,0
32	Superficial Epithelium	12,6	508,9	122,6	170,3	0,0	22,6	1805,9
	Deep Glands	0,0	619,6	23,7	28,8	0,0	0,0	1370,5
33	Superficial Epithelium	0,0	284,5	0,0	91,7	0,0	58,4	631,9
	Deep Glands	0,0	194,3	0,0	246,2	0,0	16,9	412,1
34	Superficial Epithelium	0,0	201,0	222,3	486,5	0,0	82,0	429,4
	Deep Glands	0,0	159,1	201,1	44,9	0,0	0,0	0,0

35	Superficial Epithelium	0,0	33,0	47,6	202,6	52,5	84,6	262,7
	Deep Glands	0,0	18,1	0,0	97,7	41,1	9,8	179,1
36	Superficial Epithelium	0,0	84,1	63,7	102,7	13,7	34,5	613,6
	Deep Glands	0,0	85,5	31,9	36,2	0,0	0,0	259,3
37	Superficial Epithelium	0,0	11,6	43,3	117,7	12,3	31,2	1535,4
	Deep Glands	0,0	13,8	17,5	142,7	0,0	19,6	1418,6
38	Superficial Epithelium	0,0	205,5	159,2	77,2	20,6	29,3	2952,3
	Deep Glands	0,0	172,8	141,5	61,3	127,8	108,9	2743,9
39	Superficial Epithelium	0,0	20,8	89,5	135,4	5,5	68,8	508,2
	Deep Glands	0,0	12,8	5,9	131,6	0,0	13,0	549,4
40	Superficial Epithelium	0,0	75,7	61,4	101,4	66,9	53,6	1820,0
	Deep Glands	0,0	11,7	35,1	44,3	50,6	63,2	823,1
41	Superficial Epithelium	0,0	49,3	10,9	105,6	34,6	21,1	433,7
	Deep Glands	0,0	0,0	0,0	44,8	0,0	0,0	154,6
42	Superficial Epithelium	0,0	53,1	43,7	52,7	84,9	160,5	546,6
	Deep Glands	0,0	39,8	0,0	23,0	182,4	33,7	372,3
43	Superficial Epithelium	20,6	166,1	135,1	110,5	188,7	105,0	587,1
	Deep Glands	26,1	78,6	106,5	49,2	131,7	227,2	474,6
44	Superficial Epithelium	27,1	65,3	85,0	80,9	39,5	62,4	224,1
	Deep Glands	35,9	0,0	97,1	43,2	0,0	94,6	75,6
45	Superficial Epithelium	69,4	209,1	189,6	132,9	212,0	131,6	514,5
	Deep Glands	75,3	498,2	314,6	209,9	226,3	131,7	656,3
46	Superficial Epithelium	35,9	46,7	72,2	73,2	282,0	33,2	388,6
	Deep Glands	54,6	0,0	24,6	86,6	196,6	48,6	415,1
47	Superficial Epithelium	33,9	21,5	23,8	69,6	202,5	63,2	275,1
	Deep Glands	56,4	0,0	0,0	49,6	253,5	90,1	536,3
48	Superficial Epithelium	14,7	23,0	22,7	33,8	130,0	45,8	105,9
	Deep Glands	24,7	0,0	0,0	0,0	40,4	15,6	63,3
49	Superficial Epithelium	15,7	32,5	9,0	34,4	75,8	27,7	396,2
	Deep Glands	41,0	8,5	22,4	10,5	35,1	0,0	148,8
50	Superficial Epithelium	33,9	60,8	34,2	91,7	338,3	54,7	126,5
	Deep Glands	57,9	33,4	57,2	40,5	250,7	42,9	129,9
51	Superficial Epithelium	9,6	23,4	23,2	19,1	34,2	20,1	225,4
	Deep Glands	0,0	6,3	0,0	0,0	41,5	15,8	0,0
52	Superficial Epithelium	43,7	92,6	50,0	46,7	77,4	84,3	70,8
	Deep Glands	93,8	16,9	26,1	132,2	136,6	138,1	81,4
53	Superficial Epithelium	90,4	77,5	95,4	135,8	192,6	82,7	142,8

	Deep Glands	97,0	125,9	68,9	136,7	399,8	60,2	105,8
54	Superficial Epithelium	28,9	119,9	35,3	33,2	111,3	95,3	101,7
	Deep Glands	36,3	44,2	10,0	95,6	53,4	51,0	70,2
55	Superficial Epithelium	43,2	92,1	59,9	38,6	36,8	61,5	97,0
	Deep Glands	69,2	0,0	44,0	0,0	39,5	44,8	17,5
56	Superficial Epithelium	18,3	65,3	49,1	43,6	51,1	38,2	29,1
	Deep Glands	25,4	13,6	78,9	60,4	25,4	0,0	9,0
57	Superficial Epithelium	13,2	7,0	21,3	46,1	35,5	22,9	74,4
	Deep Glands	0,0	0,0	0,0	21,3	58,0	40,0	0,0
58	Superficial Epithelium	37,1	41,3	25,5	30,8	49,8	12,5	86,3
	Deep Glands	15,8	0,0	0,0	22,6	5,1	47,2	27,9
59	Superficial Epithelium	67,1	92,1	64,3	51,4	56,9	61,3	71,2
	Deep Glands	0,0	37,0	35,2	0,0	54,4	19,9	40,7
60	Superficial Epithelium	23,0	53,3	32,1	48,5	33,6	41,5	73,8
	Deep Glands	33,6	0,0	95,9	18,8	24,7	0,0	7,6
61	Superficial Epithelium	44,4	42,3	77,2	46,5	33,4	61,7	148,2
	Deep Glands	0,0	0,0	0,0	40,4	13,4	45,9	32,4
62	Superficial Epithelium	41,6	45,7	13,9	42,8	57,5	27,9	56,0
	Deep Glands	9,2	47,9	0,0	0,0	0,0	97,0	74,7
63	Superficial Epithelium	71,6	58,9	44,9	38,4	61,9	43,6	114,6
	Deep Glands	23,9	0,0	18,8	0,0	32,6	0,0	0,0
64	Superficial Epithelium	51,3	48,3	69,9	19,2	120,3	49,6	122,4
	Deep Glands	0,0	0,0	0,0	0,0	37,2	33,8	0,0
65	Superficial Epithelium	36,8	24,1	27,2	19,3	42,4	20,5	98,6
	Deep Glands	39,0	39,9	26,2	0,0	0,0	0,0	32,6
66	Superficial Epithelium	58,6	64,3	60,6	99,6	64,2	96,2	97,3
	Deep Glands	27,2	13,3	0,0	0,0	0,0	100,8	0,0
67	Superficial Epithelium	50,6	61,0	33,0	12,8	37,3	112,7	66,0
	Deep Glands	70,7	13,8	41,1	60,1	0,0	130,0	52,5
68	Superficial Epithelium	29,4	33,5	6,8	24,0	26,7	26,8	54,4
	Deep Glands	0,0	17,3	0,0	20,4	35,8	48,8	0,0
69	Superficial Epithelium	47,7	47,2	33,8	27,2	21,0	46,2	76,5
	Deep Glands	24,4	30,0	50,3	0,0	0,0	0,0	0,0
70	Superficial Epithelium	26,9	65,4	54,0	26,8	37,8	24,9	60,7
	Deep Glands	0,0	0,0	33,9	22,2	36,2	0,0	57,9
71	Superficial Epithelium	59,7	71,3	61,1	49,5	35,9	64,1	69,4
	Deep Glands	0,0	0,0	36,4	19,2	67,8	23,5	35,9

72	Superficial Epithelium	28,8	58,0	29,7	49,7	39,6	90,1	51,0
	Deep Glands	0,0	0,0	0,0	10,0	16,4	44,9	32,3
73	Superficial Epithelium	39,7	65,0	59,5	53,5	68,9	36,7	35,4
	Deep Glands	26,6	26,8	0,0	116,4	29,7	22,4	38,2
74	Superficial Epithelium	30,2	74,3	54,3	71,6	55,4	58,5	62,6
	Deep Glands	0,0	24,9	40,0	21,4	40,8	35,8	20,0
75	Superficial Epithelium	20,4	104,2	28,0	42,7	27,4	22,6	50,2
	Deep Glands	0,0	29,9	0,0	0,0	0,0	4,9	0,0
76	Superficial Epithelium	0,0	25,6	37,0	32,2	20,0	44,1	46,0
	Deep Glands	0,0	0,0	7,1	14,8	34,8	6,7	0,0
77	Superficial Epithelium	71,1	65,2	50,0	39,8	47,0	45,8	71,0
	Deep Glands	0,0	13,7	36,8	27,0	9,4	0,0	22,2
78	Superficial Epithelium	12,0	75,2	73,0	52,0	62,6	50,8	81,2
	Deep Glands	0,0	32,2	28,4	0,0	10,5	0,0	59,7
79	Superficial Epithelium	57,0	31,9	113,4	96,6	60,8	73,3	139,4
	Deep Glands	0,0	23,7	0,0	0,0	109,2	63,6	21,8
80	Superficial Epithelium	36,6	26,1	46,6	42,5	89,1	120,2	65,7
	Deep Glands	0,0	0,0	0,0	0,0	0,0	0,0	0,0

The impact of
Helicobacter species
infection in human
gastric cell lines

5 CHAPTER

Paper V

The impact of *Helicobacter* species infection in human
gastric cell lines

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Manuscript in preparation

2021

H. pylori has been the focus of several studies during the last decades, due to its high prevalence in humans. Other gastric *Helicobacter* species are commonly found in several animal hosts, and they can cross the species barrier and infect the human gastric mucosa. However, there are a lot of questions that remain to answer regarding human infection with NHPH. How many time are these bacteria able to persist *in vitro*? Which are the effects of these bacteria at cellular level? Are they able to induce cellular alterations or to produce glycophenotype modifications? In order to answer some of these questions, we decided to pursue an *in vitro* approach using human gastric cell lines to evaluate the effects of gastric NHPH species infection.

These are preliminary results, and the manuscript is currently in preparation.

Material and Methods

Bacterial growth conditions

H. pylori and six gastric *Helicobacter* species (*H. suis*, *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. salomonis* and *H. bizzozeronii*) were used on this study. The bacteria were grown under microaerophilic conditions, as previously described (Vermoote 2011, Berlamont 2019, Baele 2004). Briefly, *H. suis* (HS1 strain), *H. heilmannii* (ASB1.4) and *H. ailurogastricus* (ASB7.1) were grown in biphasic conditions consisting of brucella agar supplemented with fetal calf serum, amphotericin B, Skirrow antibiotics, and Vitox supplements at pH 5, with brucella broth (pH 5) on top.

H. pylori (26695 strain), *H. felis* (CS1), *H. bizzozeronii* (R1051) and *H. salomonis* (R1053) were grown in brain heart infusion agar supplemented with horse blood, amphotericin B, Skirrow antibiotics and Vitox supplement.

Helicobacter infection of human gastric cell lines

To evaluate the capacity of *Helicobacter* species to infect human cells, three different cell lines were used: a normal gastric epithelial cell line (GES-1, gently provided by Prof. Dawit Kidane-Mulat, University of Texas, USA) and two gastric neoplastic epithelial cell lines (AGS and MKN74). Cells were plated in 24-well plates at a concentration 2×10^5 cells/mL, in RPMI medium supplemented with 10% fetal bovine serum, and incubated at 37°C 5% CO₂ for 24h. After 24h, the cell viability was evaluated using the PrestoBlue Reagent method (fluorescence 560/590 nm; ThermoFisher Scientific, USA). Bacterial suspensions of each *Helicobacter* species were collected and added to the cells (multiplicity of infection (moi) 100), in triplicates to each condition.

Plates were incubated at 37°C for different time-points according to the experiment. Negative controls without bacterial suspension (only cells) were included. Additionally, the same conditions were repeated in 24-well plates containing slides that will be used for detection of *Helicobacter* species by immunofluorescence.

Helicobacter species detection by immunofluorescence

Cells were grown in 24-well plates containing slides and incubated with bacterial suspensions for different time-points (from 30 min to 96h post-infection). Slides were fixed with PFA 4% at each time-point and kept at -20°C until use. Slides were washed 3 times with PBS and once with PBS-T 0.05%. After, slides were blocked for 30 min with normal goat serum (Dako, California, USA), diluted 1:5 in 10% bovine serum albumin (BSA 10%). Next, slides were incubated overnight at 4°C with a primary polyclonal antiserum against *H. pylori* (RBK012; Zytomed, Berlin, Germany), diluted 1:100 in BSA 5%, known to present immunoreactivity with a wide range of bacteria belonging to the *Helicobacter* genus (Amorim 2015). Sections were rinsed with PBS and incubated with a fluorescent secondary antibody (Alexa 488 goat anti-rabbit or Alexa 594 goat anti-rabbit) diluted 1:500 in BSA 5%, for 45 min (Dako, California, USA). Slides were rinsed again and incubated with DAPI (1:100) for 10 min for nucleus staining. Then, washed with PBS and mounted with Vectashield (Vector Laboratories, USA). Slides were observed at fluorescence microscope Zeiss Z1 Apotome.

Immunofluorescence

Slides containing the cells infected with the different gastric *Helicobacter* species were screened for some immunomarkers, such as the indicator of apoptosis caspase 3. Furthermore, the glycophenotype of these cell lines was also characterized, regarding the expression of Lewis antigens. The protocol is similar to the described in the section above and the antibodies used are listed on Table 1.

Table 1. Antibodies used in the immunofluorescence analysis of the gastric cell lines infected with *Helicobacter* species.

Antigen	Antibody Clone	Dilution	Reference
Lewis A (Oleastro M)	CA3F4	1:5	Young 1991
Lewis B (LeB)	BG-6 (T218)	1:50	Signet
Sialyl-Lewis A (sLeA)	CA19-9	1:500	Santa Cruz Biotechnology
Lewis X (LeX)	SH1	1:5	Fukushi 1984
Lewis Y (LeY)	AH6	1:10	Abe 1993

Sialyl-Lewis X (sLeX)	CSLEX-1	1:80	BD Bioscience
Anti-cleaved caspase 3	9661	1:200	Cell Signaling Technology

1. Effects on cell viability

The effect of *Helicobacter* species infection in the cellular viability was evaluated using the PrestoBlue Reagent after 24h, 48h and 72h of infection. The PrestoBlue Reagent was used accordingly to the manufacturer's instruction and the fluorescence (560/590 nm) was measured before the infection and at each time-point. The results are presented as fold changes to the expected growth under normal conditions (growth of non-infected cells was considered as expected growth and the differences observed for each *Helicobacter* were normalized to that value).

GES-1 cell line (normal gastric epithelial cell line)

The infection of this cell line with all the gastric *Helicobacter* species tested led to a decrease in cell viability. With exception of *H. suis* (HS1 strain) and *H. pylori* (26695 strain), this effect was observed after only 24h post-infection (p.i.) (Figure 1). Remarkably, for *H. pylori*, and in lesser extent for *H. suis*, at 24h p.i. it was observed an increase in the cell viability, suggesting that the cells can profit from the presence of these bacteria. However, although these experiments were already performed in triplicates, this should be repeated one more time to confirm.

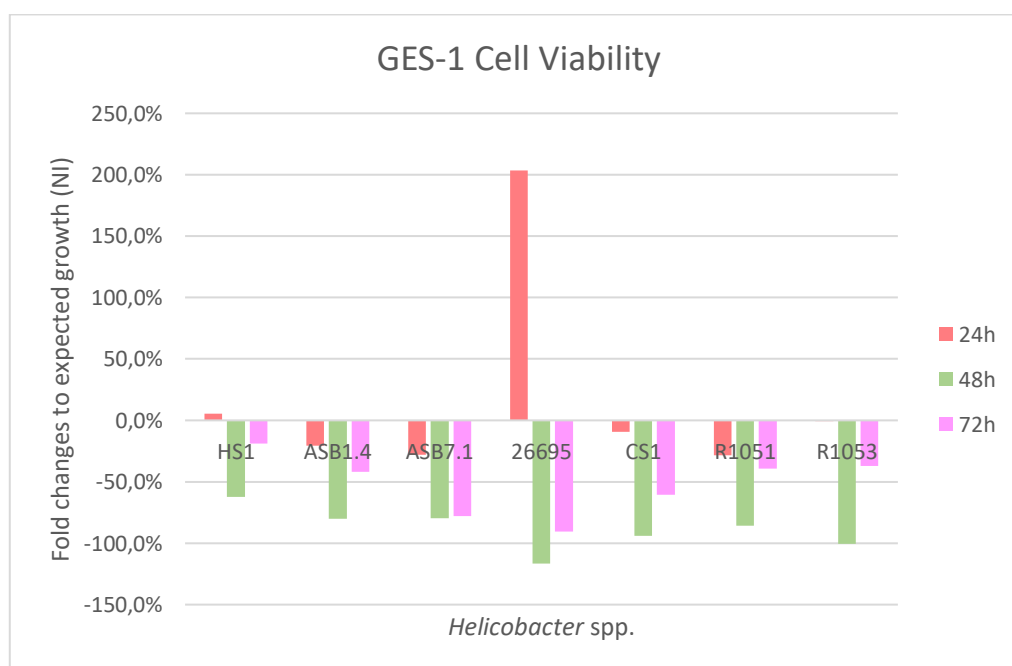


Figure 1. Impact of gastric *Helicobacter* species infection in GES-1 cell line viability. HS1: *H. suis*; ASB1.4: *H. heilmannii*; ASB7.1: *H. ailurogastricus*; 26695: *H. pylori*; CS1: *H. felis*; R1051: *H. bizzozeronii*; R1053: *H. salomonis*. The results were obtained through the PrestoBlue Reagent method and measure of the fluorescence emitted at 560/590nm.

MKN74 cell line (neoplastic gastric epithelial cell line)

Regarding MKN74 cell line, almost all the gastric *Helicobacter* species tested, resulted in a decrease on cell viability for all the time-points evaluated (Figure 2). Interestingly, *H. bizzozeronii* (R1051 strain) result in an increase in cell viability at 24h, 48h and 72h p.i..

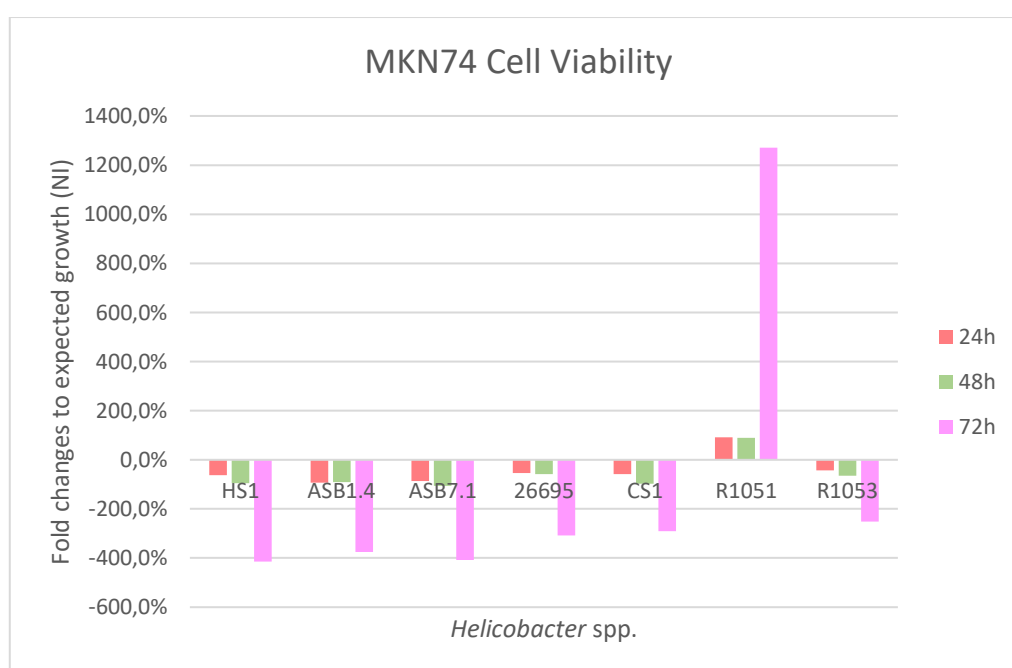


Figure 2. Impact of gastric *Helicobacter* species infection in MKN74 cell line viability. HS1: *H. suis*; ASB1.4: *H. heilmannii*; ASB7.1: *H. ailurogastricus*; 26695: *H. pylori*; CS1: *H. felis*; R1051: *H. bizzozeronii*; R1053: *H. salomonis*. The results were obtained through the PrestoBlue Reagent method and measure of the fluorescence emitted at 560/590nm.

AGS cell line (neoplastic gastric epithelial cell line)

The impact of the gastric *Helicobacter* species infection on AGS cell line seems to be dependent of the bacterial species involved, and of the time of infection (Figure 3).

For *H. suis* (HS1 strain) and *H. felis* (CS1 strain), the infection resulted in a decrease in the cell viability at 24h and 48h p.i.. However, at 72h post-infection, the cell viability increased. For *H. heilmannii* (ASB1.4 strain) and *H. ailurogastricus* (ASB7.1 strain), a decrease on cell viability was observed in all the time-points evaluated. *H. pylori* (26695 strain) infection of AGS cell line led to decrease on cell viability at 24h p.i., but this effect was reversed at 48h and 72h p.i.. For *H. salomonis* (R1053 strain), a slight increase on cell viability was observed at 24h and 48h p.i., but at 72h p.i. a huge decrease on cell viability was obtained. Finally, similarly to the results obtained for MKN74 cell line, *H. bizzozeronii* (R1051 strain) *in vitro* infection was the only bacterial species that lead to an increase on AGS viability for all the time-points tested.

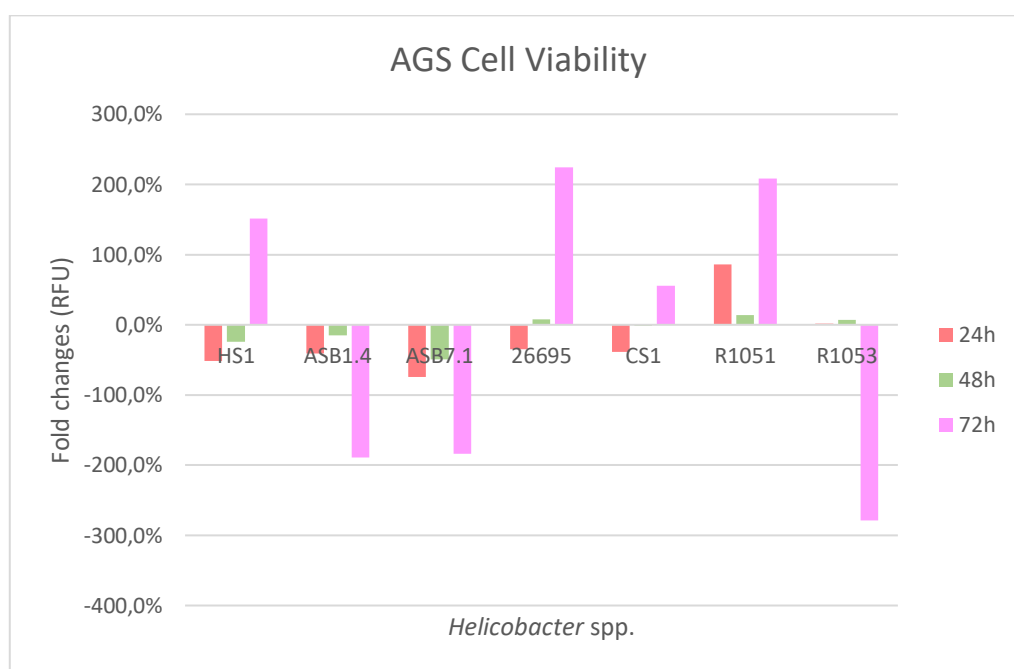


Figure 3. Impact of gastric *Helicobacter* species infection in AGS cell line viability.

HS1: *H. suis*; ASB1.4: *H. heilmannii*; ASB7.1: *H. ailurogastricus*; 26695: *H. pylori*; CS1: *H. felis*; R1051: *H. bizzozeronii*; R1053: *H. salomonis*. The results were obtained through the PrestoBlue Reagent method and measure of the fluorescence emitted at 560/590nm.

These results were somewhat unexpected and difficult to interpret. First, we were not expecting that infection by NHPH would result in such huge decrease on cell viability, both in normal and neoplastic gastric cell lines. Interestingly, with exception of *H. bizzozeronii*, the pattern was very similar for GES-1 and MKN74 cell lines. GES-1 is a normal epithelial cell line and AGS and MKN74 cell lines are both classified as Lauren

intestinal-type gastric adenocarcinoma cell lines. However, the results of the *in vitro* infection with gastric *Helicobacter* species were very different. It would be interesting to assess cell viability in the presence of the different *Helicobacter* species using another cell line with a different histogenesis, like diffuse-type gastric adenocarcinoma cell line, such as MKN45.

Taking these results, the next question was: is this cell viability reduction due to an apoptosis activation related with the bacterial presence, or is this the result of cell proliferation inhibition? To answer this question, relevant approach would be the analysis of the expression of the cleaved-caspase 3 (indicator of apoptosis) and ki67 (indicator of proliferation). Unfortunately, we only conducted preliminary experiments assessing the expression of cleaved-caspase 3 in MKN74-infected cells (Figure 4).

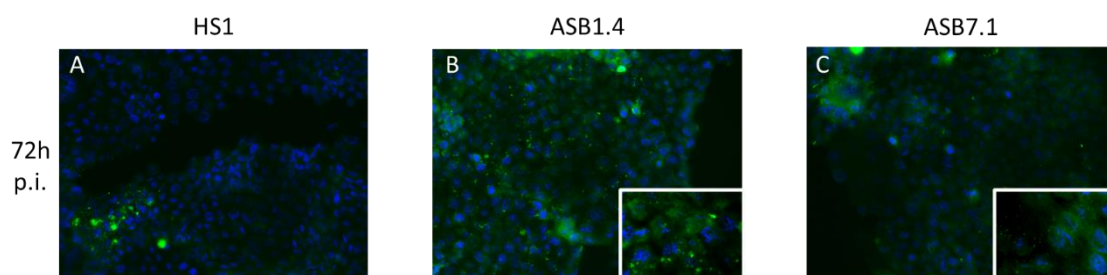


Figure 4. Expression of cleaved-caspase 3 (Vinall LE) in MKN74 cell line infected with *H. suis* (A), *H. heilmannii* (B) or *H. ailurogastricus* (C), after 72h of infection. Magnification 200x and 400x (insert panel B and C).

As demonstrated in Figure 4, MKN74 cells infected with *H. suis* (HS1), *H. heilmannii* (ASB1.4) or *H. ailurogastricus* (ASB7.1) strains showed an expression of cleaved-caspase 3 (active form of caspase 3) at 72h p.i.. Regarding this, we can speculate that the decrease in cell viability, can be related with cell apoptosis activation under the presence of bacteria. Further studies in the other cell lines available and using the remaining NHPH species are needed to confirm this hypothesis.

2. Detection of gastric *Helicobacter* species

One of the goals was to determine the time that the bacteria need to start binding to human gastric cell lines *in vitro*. For that, the presence of *Helicobacter* was evaluated from early incubation times to long-time infections: from 30min p.i. to 72h p.i.. The

presence of *Helicobacter* was detected by immunofluorescence using a polyclonal antibody for *Helicobacter pylori* (Table 1).

GES-1 cell line

The presence of *Helicobacter* species was detected after 24h, 48h and 72h after infection of GES-1 cells. All the bacterial strains tested were visible at all the time-points checked (Figure 5). The number of detected bacteria increase in line with the time of infection, for instance, at 72h p.i. the number of bacteria was usually higher in comparison to 24h p.i..

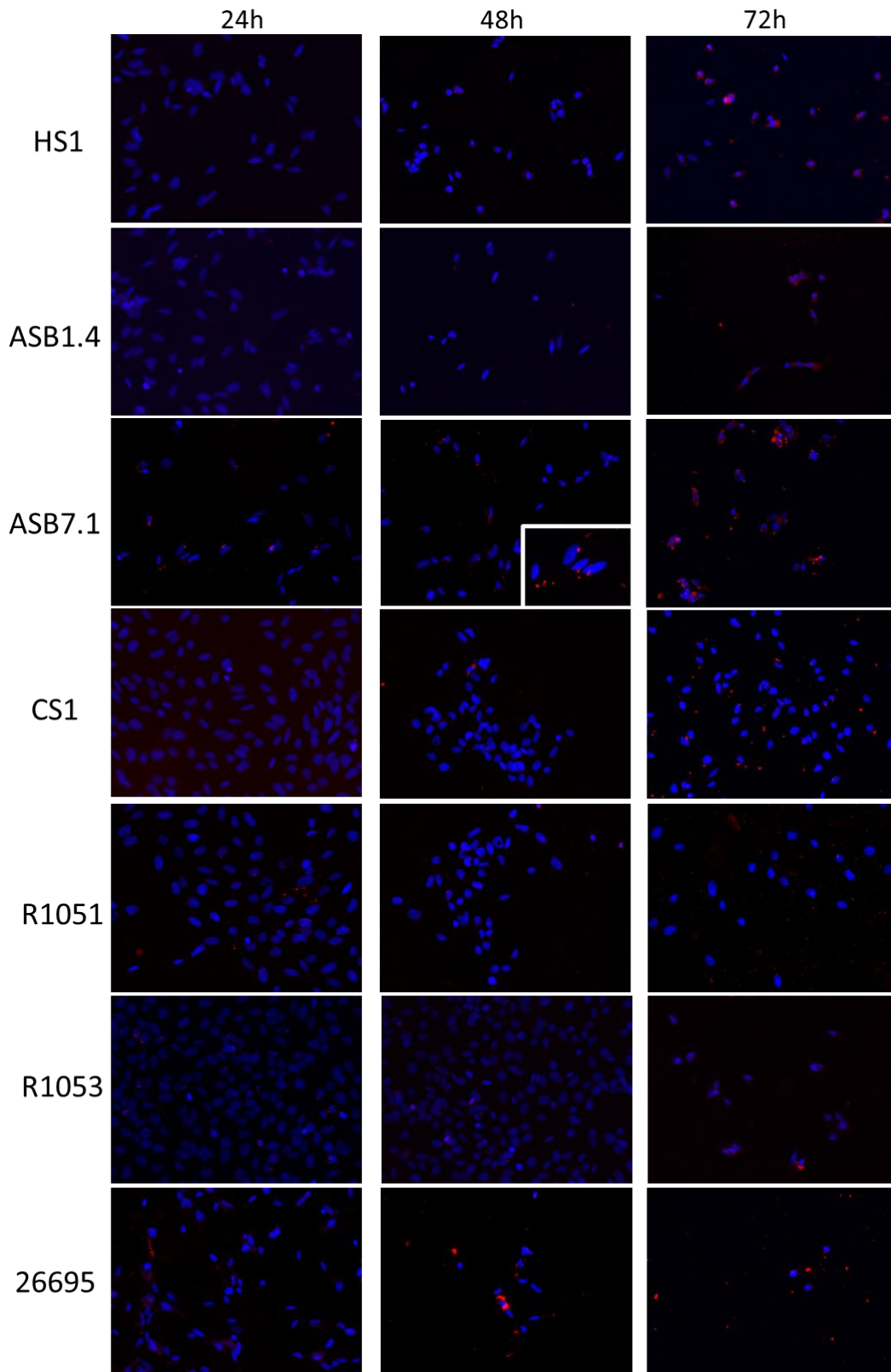


Figure 5. Detection of *Helicobacter* spp. presence in GES-1 cell line infected with different gastric *Helicobacter* species. The bacteria were detected using a polyclonal antibody (red). HS1: *H. suis*; ASB1.4: *H. heilmannii*; ASB7.1: *H. ailurogastricus*; 26695: *H. pylori*; CS1: *H. felis*; R1051: *H. bizzozeronii*; R1053: *H. salomonis*. Magnification 200x and 400x (insert).

MKN74 cell line

The presence of *Helicobacter* species was detected after 24h, 48h and 72h after infection. For MKN74 cell line, only *H. heilmannii*, *H. ailurogastricus*, *H. felis*, *H. bizzozeronii* and *H. salomonis* were analysed by immunofluorescence. The presence of *H. heilmannii* was really difficult to detect, with only visible bacteria at 48h p.i., and some possible bacteria at 72h p.i.. For the remaining bacterial strains tested, bacteria were visible at all the time-points tested (Figure 6). Similarly to observed for GES-1 cell line, the number of detected bacteria increase in line with the time of infection, for instance, at 72h p.i. the number of bacteria was usually higher in comparison to 24h p.i..

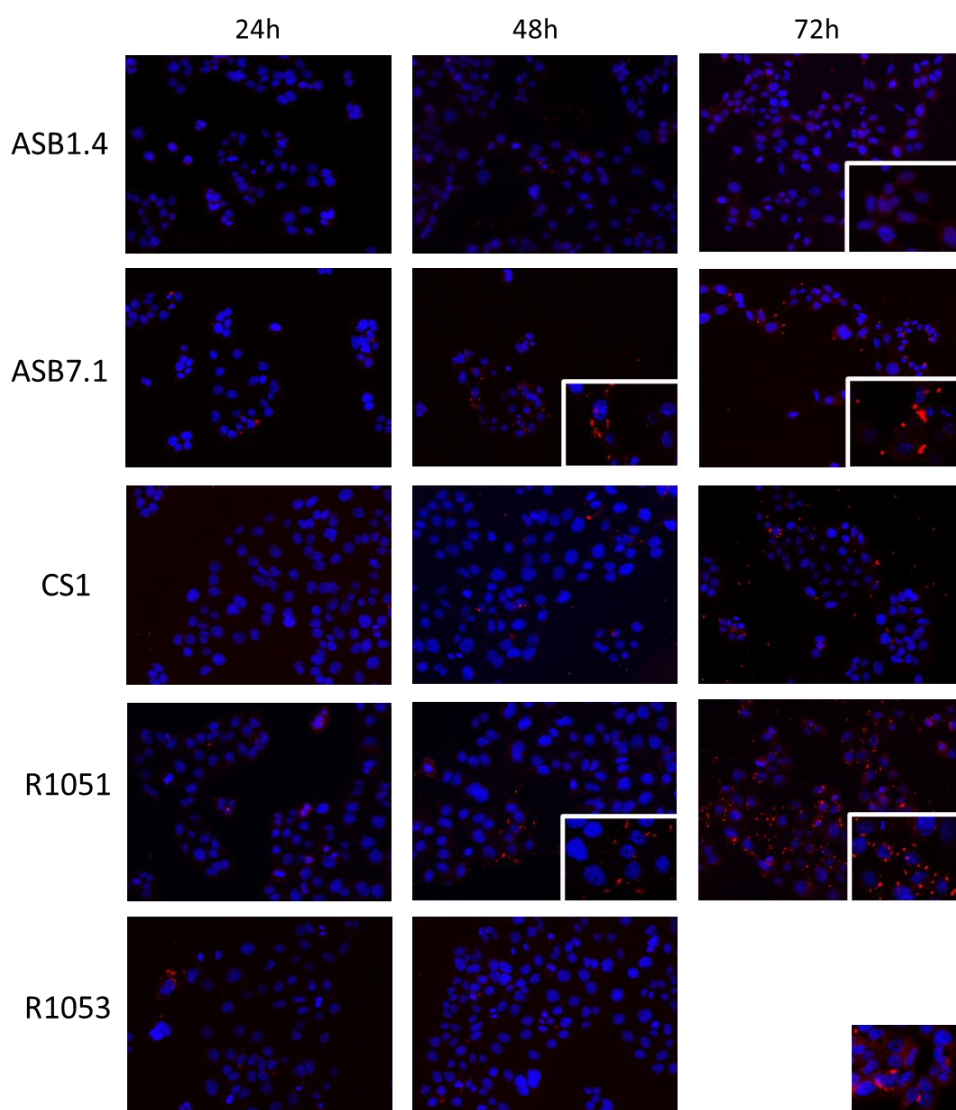


Figure 6. Detection of *Helicobacter* spp. presence in MKN74 cell line infected with different gastric *Helicobacter* species. The bacteria were detected using a polyclonal antibody (red). ASB1.4: *H. heilmannii*; ASB7.1: *H. ailurogastricus*; CS1: *H. felis*; R1051: *H. bizzozeronii*; R1053: *H. salomonis*. Magnification 200x and 400x (insert).

For AGS cell line, the detection of *Helicobacter* species through immunofluorescence was not performed yet.

3. Gastric glycophenotype

It has been very well established that some pathologic conditions, such as cancer, can lead to modifications on the cell glycophenotype, changing the glycans usually expressed in homeostasis (Pinho 2015).

As described on Section 2 and 3, and on Paper I, of Chapter 1, the Lewis Antigens are strictly involved in the binding of *H. pylori* to the human gastric mucosa. The major intervenients are the LeB and the sialylated antigens, SleA and SleX. In this study, we decide to look to these gastric cell lines and evaluate if infection by NHPH results in changes of the glycans expressed at the cell surface, modifying the gastric glycophenotype profile.

GES-1 cell line

In normal conditions (non-infected cells), a slight expression of type 1 Lewis Antigens (LeA and LeB) was observed on GES-1 cells, with almost no expression of SleA. On the other hand, a more pronounced expression of type 2 antigens (LeX, LeY and SleX) was recorded (Figure 7A). Unfortunately, at 72h p.i. the number of viable cells were very low, making difficult/impossible to detect the expression of these antigens.

Infection with *H. suis* (HS1 strain), result in the expression of SleX, mostly at 24h and 48h p.i.. Unfortunately, at 72h p.i. the number of viable cells were very low, not allowing the detection of this antigen. On the other hand, infection with *H. heilmannii* (ASB1.4 strain), *H. ailurogastricus* (ASB7.1 strain), *H. felis* (CS1 strain) and *H. salomonis* (R1053 strain) seems to not have an impact on the *de novo* synthesis of sialylated antigens, with almost no expression of these glycans after infection of GES-1 cells. *H. bizzozeronii* (R1051 strain) infection leads to a sparse expression of SleA at 24h p.i., and a clear increased expression of SleX upon infection (at least at 24h p.i.).

Infection with *H. pylori* (26695 strain) was also performed, and it results in expression of SleA and SleX antigens, especially at 24h p.i. (other time-points were not possible to evaluate).

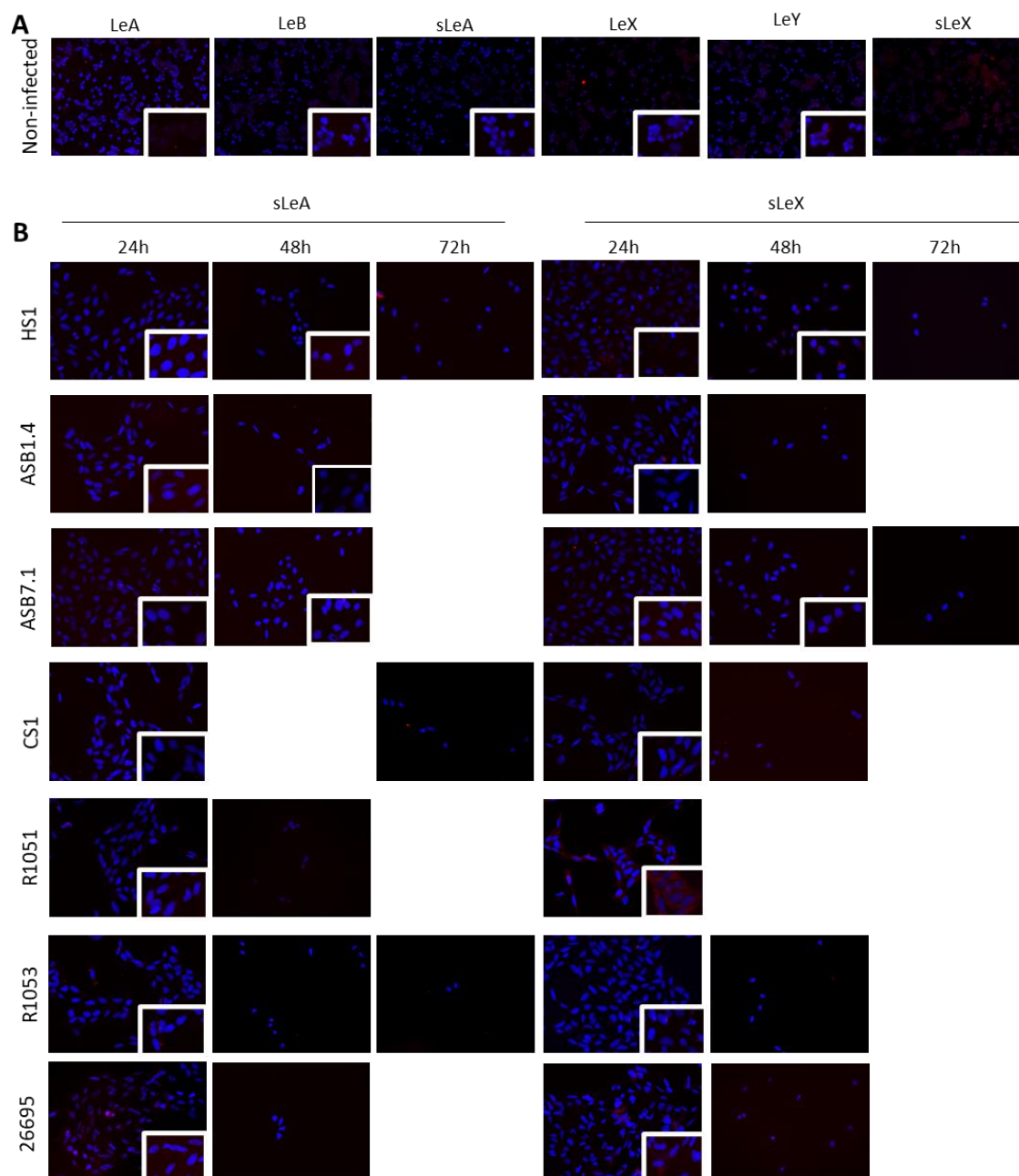


Figure 7. Expression of Lewis Antigens on GES-1 cell line infected with different gastric *Helicobacter* species. (A) Type 1 and type 2 Lewis Antigens expression (red) in non-infected GES-1 cells. (B) Expression of sialylated antigens (SleA and SleX) (red) on GES-1 cells infected with gastric *Helicobacter* species. HS1: *H. suis*; ASB1.4: *H. heilmannii*; ASB7.1: *H. ailurogastricus*; CS1: *H. felis*; R1051: *H. bizzozeronii*; R1053: *H. salomonis*; 26695: *H. pylori*. Magnification 200x and 400x (insert).

Analysis of the expression of Lewis Antigens and stimulation of *de novo* synthesis of sialylated glycans upon *Helicobacter* infection is still being performed for other gastric cell lines.

4. Inflammatory response

H. pylori infection of the human gastric mucosa is usually accompanied by gastritis and the induction of an inflammatory response. We would like to evaluate if infection with NHPH can also result in the stimulation of the inflammatory response. For that, gastric cell lines were infected with different gastric *Helicobacter* species and the expression of some cytokines, such as IL-1 β , IL-8, TNF- α , IFN- γ and others, are being assessed through qPCR. These experiments are still being performed.

6 CHAPTER

General Discussion

Summary and conclusions

Future perspectives

General Discussion

The decreased efficacy of eradication of *H. pylori* with the standard triple therapy has become a huge health problem. Indeed, the WHO considered *H. pylori* a high priority group, for which new drugs and alternative therapies are urgently needed (Blaecher C 2013; Jang S 2017). Regarding the resistance mechanisms, the most common strategies are the production of enzymes that inactivate the antibiotics, the increase in active drug efflux, and point mutations in genes encoding the targets of the antibiotics (Blaecher C 2013).

As described in the General Introduction (Chapter 1), human infection by NHPH species has been associated with gastritis, gastric and duodenal ulcers, and MALT lymphoma (Haesebrouck F 2009). *H. suis* has been described as the most prevalent NHPH species in humans, but *H. felis*, *H. bizzozeronii*, *H. salomonis* and *H. heilmannii* can also be found in the human gastric mucosa (Bento-Miranda M 2014; De Groote D 2005; Van den Bulck K 2005a). Interestingly, some studies reported an *H. suis* infection in association with gastric diseases, and the remission of these lesions, including MALT lymphoma, after the elimination of the bacteria (Joosten M 2013; Morgner A 2000; Rimbara E 2021). Additionally, *H. suis* has been the focus of antimicrobial susceptibility tests and acquired resistance to some antimicrobial compounds have been described (Berlamont H 2019; Van den Bulck K 2005b; Vermoote M 2011). Similar studies have also been performed for canine- and feline- *H. felis*, *H. bizzozeronii* and *H. salomonis* isolates, with acquired resistance to compounds tested being also observed (Rimbara E 2021; Schott T 2012; Van den Bulck K 2005b). The susceptibility of others gastric *Helicobacter* spp., such as *H. heilmannii* and the recently discovered *H. ailurogastricus*, to the most common antibiotics used in the clinic was never evaluated before, and it was one of the aims of this thesis.

According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, antimicrobial susceptibility tests for *H. pylori* should be performed using the agar dilution method. However, the growth conditions of *H. heilmannii* and *H. ailurogastricus* (biphasic medium with acidic pH) require a different approach for the determination of the antimicrobial susceptibility pattern. Since the growth conditions of these bacteria are very similar to *H. suis*, a combined agar and broth dilution method followed by qPCR was used to determine the susceptibility of *H. heilmannii* and *H. ailurogastricus* (Baele M 2008; Berlamont H 2019; Vermoote M 2011). However, this technique is very laborious and time-consuming, which makes it difficult to apply in the clinic. In this study, the activity

of 15 antimicrobial compounds against 7 feline isolates of *H. heilmannii* and 6 feline isolates of *H. ailurogastricus* was evaluated (Chapter 2). The main criterium used for the interpretation of MIC (minimal inhibitory concentration) values and determine potential acquired resistance was the epidemiological criterion ((ESCMID) 2003; Berlamont H 2019). A monomodal distribution was observed for all the *H. ailurogastricus* isolates, indicating that all the isolates are within the wild type range. The majority of *H. heilmannii* isolates also exhibited a monomodal distribution. However, 5 *H. heilmannii* isolates presented a bimodal distribution, suggesting that isolates with higher MIC endpoints are potentially less susceptible than the WT population to that specific antimicrobial agent.

Feline *H. heilmannii* isolates not belonging to the WT population were detected for azithromycin (one isolate), enrofloxacin (two isolates), lincomycin (one isolate) and spectinomycin (one isolate). Furthermore, *H. heilmannii* isolates showed higher MIC values for neomycin compared to *H. ailurogastricus* isolates. This suggests that *H. heilmannii* is intrinsically less susceptible to neomycin than *H. ailurogastricus*. As pets may constitute a source of infection for humans, this should be kept in mind when dealing with a human patient infected with *H. heilmannii* (Chapter 2). The identification of the mechanisms underlying this decreased susceptibility/resistance is difficult due to the lack of an antimicrobial gene resistance database for the *Helicobacter* genus. Still, manual screening for the presence of SNPs (single nucleotide polymorphisms) in these feline *H. heilmannii* isolates revealed the occurrence of SNPs in several genes of the 30S and 50S ribosomal protein family, with impact on protein stability and activity (Chapter 2). However, interpretation of MIC values obtained *in vitro* needs to be done with caution since it may not always reflect their activity *in vivo* (Gisbert 2011). In addition, the complexity of the gastric microenvironment, further complicates prediction of activity of these antimicrobials. The occurrence of acquired resistance and/or decreased susceptibility to amoxicillin and metronidazole in NHPH species (Rimbara E 2021; Schott T 2012; Van den Bulck K 2005b), suggests that the standard triple therapy used for *H. pylori* eradication, might not always be effective to treat NHPH infection in human patients.

The prevalence of NHPH in human gastric biopsies has been described as very low, with values ranging from 0.2% to 6.0% (Haesebrouck F 2009). Interestingly, co-infection with different gastric *Helicobacter* species may occur (11-16% of the infected individuals) (Bento-Miranda M 2014; De Groote D 2005; Liu J 2015; Van den Bulck K 2005a). In Portugal, the prevalence of *H. pylori* infection has been described as very high, with some reports of 86.4% of incidence (Hooi JKY 2017). In Chapter 3, a study

using human gastric samples for the determination of *Helicobacter* occurrence in the North region of Portugal, as well the efficacy of the eradication treatment is presented. Overall, 46.3% of the individuals were positive for the presence of *H. pylori*. Furthermore, 2.5% of the patients exhibited a mixed infection by *H. pylori* and the canine- and feline-associated *H. felis* (Chapter 3). More interestingly, 32.5% of these individuals had been subjected to *Helicobacter* eradication treatment in the last 18 months prior to the gastric surgery, but in 46.2% bacteria were still detected. The majority of these patients were treated with the standard triple therapy, reinforcing the decreasing efficacy of this therapeutical approach. However, these values are not at all surprising, since Portugal presents one of the highest percentages of clarithromycin resistance in *H. pylori* isolates (Ghotaslou R 2015). Due to this high probability of failure in the elimination of the bacteria, a confirmatory test is usually requested by the clinicians. This test should be performed at least 4 weeks after the treatment, and UBT or endoscopy-based methods are the recommended techniques (Chey WD 2017). However, these techniques do not discriminate *H. pylori* from the other gastric *Helicobacter* species and infections with NHPH are often not detected by UBT. Therefore, molecular techniques (e.g., PCR or qPCR) are needed to confirm at species level (Haesebrouck F 2009). Amongst the patients that were submitted to the eradication of *H. pylori*, it was effective in 30.8% of the population. The percentage of false-negatives (negative in the confirmatory test but with *Helicobacter* detected by PCR analysis/ sequencing) was 33.3%, which can be explained for instance by the low amount of bacteria (below the sensitivity of the technique).

Alternative therapies to the antibiotics, based on antioxidants, vitamins and probiotics, have been proposed to improve the currently available treatment options (Palacios-Espinosa JF 2014). For instance, it was demonstrated that the combined administration of vitamin C with the triple therapy led to an increased rate of bacterial elimination, and possible reduction of *H. pylori*-induced gastric carcinoma risk (Everett SM 2002; Jang S 2017; Yang-Ou YB 2018). Studies in mice models demonstrated that treatment with N-acetylcysteine (NAC) reduced bacterial colonization and prevented the development of severe inflammation, when administrated in the feed, during the initial phase of infection (Jang S 2017). Additionally, administration of certain probiotics seems to improve the bacteria elimination and reduce the side effects associated with the standard treatment (Eslami M 2019; Papastergiou V 2014; Sakamoto I 2001). Besides this, probiotics, when administrated in adequate amounts, contribute for an improvement of the microbial balance and overall health (Eslami M 2019; Homan M 2015; Papastergiou V 2014). In a more recent study, a new promising target has been

explored: the *H. pylori* flavodoxin, which is a bacterial electron carrier, essential for the bacteria and not present in humans (Salillas S 2021). Previous studies described already that treatment with flavodoxin inhibitors decreased *H. pylori* gastric colonization rates in mice model of infection (Salillas S 2019). Interestingly, antimicrobial susceptibility tests showed that a group of compounds were effective against different bacteria, including *H. pylori*. The results suggests that they might help in the reduction of bacterial damage, and they can be used to replace some currently used antimicrobials and improve the currently available eradication therapy (Salillas S 2021). The normal gastric mucosa is characterized by the expression of MUC1, MUC5AC and MUC6 mucins, the major components of the mucous layer (Babu SD 2006; Linden SK 2008; Teixeira A 2002). The MUC5AC mucin is usually co-expressed with LeB and LeA antigens in the superficial epithelium, while MUC6 mucin is expressed in the deeper gastric glands simultaneously with type 2 Lewis antigens, LeX and LeY (Ansari S 2017; De Bolos C 1995; López-Ferrer A 2000; Magalhaes A 2010a; Mollicone R 1985; Moran 2008; Reis CA 2000). Under pathological conditions, such as inflammation and/or cancer, alterations in the glycans pattern (glycophenotype) are usually observed. For instance, upon *H. pylori* infection, an inflammatory response is activated, which may lead to the *de novo* expression of sialylated structures, changing the gastric glycophenotype (Mahdavi J 2003; Mahdavi J 2002). These glycans are recognized by the SabA adhesin and used to reinforce the attachment of the bacteria to the gastric epithelial cells (Mahdavi J 2003; Mahdavi J 2002). In 2014, Amorim *et al.*, evaluated the expression of the Lewis antigens in the canine gastric mucosa, since dogs are one of the major hosts of NHPH bacteria (Amorim I 2014). On the contrary to the human gastric mucosa, dogs presented a different glycosylation profile, characterized by the expression of type 2 Lewis antigens and absence of type 1 structures. Additionally, the *in vitro* binding ability of some NHPH to the canine gastric mucosa was evaluated, and *H. heilmannii* showed the highest adhesion rate, followed by *H. felis* (Amorim I 2014). However, the binding ability of these gastric NHPH to the human gastric mucosa is not fully understood and characterized.

In Chapter 4, histological evaluation of human gastric tissues demonstrated that infection by *H. pylori* was accompanied by chronic active inflammation in the majority of the patients, mostly moderate to severe. Severe conditions, such as dysplasia, presence of lymphoid follicles and IM were also observed. The glycosylation profile of these patients was assessed, and the expression of type 1 and type 2 Lewis antigens was observed as previously described (Ansari S 2017; De Bolos C 1995; López-Ferrer A 2000; Magalhaes A 2010a; Moran 2008; Reis CA 2000). The secretor phenotype (expression of LeB antigen) occurs in 70.0% of the worldwide population and has been

associated with a higher risk of *H. pylori* colonization and persistent infection (Azevedo M 2008; Ikehara Y 1998; Lee YC 2016; Magalhaes A 2009; Magalhaes A 2016; Serpa J 2004). A total percentage of 68.8% of secretor individuals was observed in the patients included in the study, which goes towards to the values described in the literature (De Oliveira DM 2017). The infection by *H. pylori* in the non-secretor individuals suggests that the other molecules, such as SabA, are also involved in the adhesion mechanism and the BabA-LeB interaction is important but not essential for a successful colonization (Benktander J 2018; Linden SK 2008). Additionally, the *in vitro* binding ability of *H. heilmannii*, *H. suis*, *H. ailurogastricus*, *H. felis*, *H. salomonis* and *H. bizzozeronii* to human gastric tissue was also evaluated. All the bacterial species tested were able to bind to the human gastric mucosa. The highest binding was observed for *H. heilmannii*, followed by *H. felis* and *H. salomonis*. This pattern is very similar to that observed for the canine gastric mucosa (Amorim I 2014), suggesting a potential role of the type 2 Lewis antigens in the adhesion of the NHPH both to humans and animals. Furthermore, *H. heilmannii*, *H. felis*, *H. bizzozeronii* and *H. salomonis* were also able to bind to gastric tissues with severe chronic inflammation, and even with intestinal metaplasia, suggesting that these bacteria do not bind exclusively to a normal gastric microenvironment. The binding of gastric NHPH was not correlated to the secretor status. Unfortunately, the mechanism and the key molecules involved in the adhesion remain to be elucidated, but the lack of the major known adhesins, such as BabA, SabA and AlpA/B, could partially explain the low infection rate of NHPH (Amorim I 2014; De Witte C 2016; Padra M 2018). Furthermore, it has also been demonstrated that the expression of some OMPs on NHPH species is essential for the ability of these species to colonize the gastric mucosa (Arnold IC 2011; Kondadi PK 2012; Schott T 2011a; Schott T 2011b; Smet A 2013).

In Chapter 5, the aim was to evaluate the *in vitro* effects of infection of gastric *Helicobacter* species. For that, some human gastric cell lines were used, namely the neoplastic cells AGS and MKN74, and a normal gastric epithelial cell line GES-1. Preliminary results suggests that infection with gastric NHPH leads to a decrease on the cell viability. This could be due to the stimulation of the apoptosis, as indicated by the expression of the active form of caspase 3 (cleaved-caspase 3) in some gastric cells. Besides this, the analysis of the gastric glycophenotype of these cell lines demonstrated that some bacterial species could induce the expression of sialylated antigens. These structures are usually synthesized in response to some pathogens, such as *H. pylori* (Mahdavi J 2002), being an indicator of inflammation. These results suggests that infection by NHPH could have a negative impact on the cell, resulting in activation of

apoptosis, induction of an inflammatory response, and changing the gastric glycan pattern. However, this task is not finished yet and some extra experiments are needed to have more conclusions on the effect of NHPH infection in human gastric cells.

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Summary and Conclusions

H. pylori has been the aim of several studies in the last decades due to its high prevalence in human infection and its crucial role in the development of gastric diseases, ultimately gastric carcinoma. The search for this bacterium allowed the discovery of other gastric *Helicobacter* species, referred to as non-*Helicobacter pylori Helicobacter* (NHPH), in the human gastric mucosa. Additionally, these organisms colonize the stomach of several animal hosts, such as dogs, cats and pigs, and they can cross the species barrier and be transmitted from animals to humans (zoonosis). Infection by NHPH is also associated with gastric disorders (in humans and animals), namely to gastritis, peptic ulcer and MALT lymphoma.

In this study we evaluated the zoonotic potential of gastric NHPH, looking for the occurrence of these bacteria in humans, and trying to determine the binding ability to the human gastric mucosa and possible effects at cellular level (*in vitro*). The acquisition of resistance mechanisms by these NHPH and the efficacy of the currently available *Helicobacter* eradication therapy were also investigated in this project.

In **Chapter 1**, a general description of the current knowledge on gastric *Helicobacter* was performed, focusing on the prevalence and clinical outcomes of infection by *H. pylori* and NHPH (in humans and animals). A closer look into the diagnostic techniques and the therapeutical approaches to treat infection by *Helicobacter* spp. was also included. Additionally, the pathogenesis and the mechanisms used by these organisms to adhere to the gastric epithelial cells, with involvement of adhesins and other virulence factors, were described in detail. In this chapter, the role of glycans and glycosylation in the adhesion of *Helicobacter* species to the human gastric mucosa was highlighted in Paper I.

In **Chapter 2**, we determined for the first time the antimicrobial susceptibility pattern of feline isolates of *H. heilmannii* and *H. ailurogastricus*. These two gastric pathogens colonize preferentially the feline stomach, although human infection by *H. heilmannii* were already described. Some *H. heilmannii* isolates presented acquired resistance and/or decreased susceptibility to azithromycin, enrofloxacin, lincomycin, spectinomycin and neomycin. The discovery of acquired resistance in this *Helicobacter* spp. is very interesting, and it can be useful to the veterinary clinic to improve the current therapy used to treat animal infection. Furthermore, due to the occurrence of human

infection by this bacterial species, it can also be translated into the human medicine and be useful for treatment of human infection by NHPH.

In **Chapter 3**, the prevalence of *H. pylori* and NHPH in the Portuguese population (from North region) was determined. *H. pylori* was found in 46.3% of the patients, and a mixed infection by *H. pylori* and the canine- and feline-associated *H. felis* was detected in 2.5% of the individuals. These values are in agreement with the values described in the literature for the occurrence of these bacteria in humans. Furthermore, evaluation of the efficacy of the *Helicobacter* eradication treatment was also performed. Interestingly, almost half of the patients subjected to the eradication therapy were still infected with *Helicobacter*. This can be partially explained by the increased percentage of acquired resistance to antibiotics, namely to clarithromycin and metronidazole (used in these therapies), highlighting the importance of development of new therapeutical approaches and strategies to treat human infection by *Helicobacter*.

In **Chapter 4**, the study focused on the evaluation of the human gastric glycophenotype and the secretor status of the human patients. Patients presented expression of both type 1 and type 2 Lewis antigens, and the secretor phenotype was observed in almost 70% of the patients. The histopathological evaluation of the gastric tissue demonstrated that the majority of these individuals presented signs of chronic inflammation (moderate to severe), possible correlated to the presence of *Helicobacter*-like organisms (by modified-Giemsa stain). Additionally, the *in vitro* binding ability of gastric NHPH species to the human gastric mucosa was evaluated. Interestingly, *H. heilmannii* presented the highest binding ability, followed by *H. felis* and *H. salomonis*. A clear correlation between the expression of some Lewis antigens and the binding of NHPHs was not established, but the similar binding pattern to the canine gastric mucosa suggests a potential role of the type 2 Lewis antigens in the adhesion of the NHPH both to humans and animals.

Finally, in **Chapter 5**, the ability of these gastric NHPH species to infect human gastric cell lines *in vitro*, and the effects on cell viability were assessed. All the bacterial species evaluated were able to infect the cells and persist for long periods of time (until 96h post-infection). More interestingly, *in vitro* infection lead to a decrease in the cell viability, possibly due to the stimulation of apoptosis, as suggested by the expression of anti-cleaved caspase 3 by these cells. Infection by some NHPHs also seems to induce an inflammatory response, with expression of cytokines being detected. However, further studies are needed to clarify the cellular and molecular effects of NHPH infection *in vitro*.

In conclusion, these results demonstrate that even if the prevalence of NHPH infection in humans is low, these bacteria should not be underestimated. The detection of acquired resistance in these organisms, together with the decreased efficacy of the eradication treatment for the elimination of *H. pylori*, indicates that new strategies are urgently needed. All the gastric NHPH species tested presented *in vitro* binding ability both to the human gastric tissues and to human gastric cell lines, with impact on cell viability. Unfortunately, the full mechanism underlying the adhesion of NHPH, and the main molecules involved remains unknown.

Future Perspectives

H. pylori is well established as a human pathogen and its role in the development of gastric diseases is undeniable. However, other gastric *Helicobacter* spp. can colonize the human stomach, such as *H. felis*, *H. suis*, *H. heilmannii*, *H. salomonis* and *H. bizzozeronii*, as demonstrated here. The results obtained in this study highlight the zoonotic potential of gastric NHPH species, however there is almost no evidence about the mechanisms used by these pathogens to bind. Regarding this, it is important to **identify the glycan receptors involved in the adhesion of NHPH to the human gastric mucosa**. To achieve this, the use of glycan microarray analysis, a powerful high-throughput tool, will be an essential technique. This would provide information on the host-pathogen interaction, more specifically on the glycans involved in the adhesion (host receptors recognized by the bacteria), and on the biological activity of the gastric NHPH species.

The WHO announced recently that *H. pylori* is a high priority group for which new drugs and alternative therapies are urgently needed, due to the decreased efficacy of the eradication treatments currently available. The low efficacy of these therapies in the elimination of the bacteria, and the acquired resistance by *Helicobacter* spp. were demonstrated in the studies included in this thesis. Some vitamins and antioxidants have been suggested as having a potential anti-*H. pylori* effect, when administrated together with the eradication treatment. However, the potential effects of these compounds on NHPH species are unknown. Another interesting goal would be the **evaluation of the potential anti-NHPH effect of these alternative molecules on the elimination of these bacteria**. For instance, it would be very helpful in the swine industry, since infection by *H. suis* is very high, with impact on animal health and well-being, and it has a clear economic impact. So, the discovery of new therapies that would improve the efficacy rate of *Helicobacter* elimination is important.

The development of 3D *in vitro* models (organoids) has been a helpful laboratory tool for a better representation of the human microenvironment. Indeed, the gastric organoids, simple or formed by conjugation of different types of cells, can be a good approach and, in certain ways, a substitute of the *in vivo* animal experiments. It would be very interesting to **apply the 3D models to the study of infectious gastric disorders, such as *Helicobacter***. The 3D structure and the possible formation of organoids from epithelial and parietal cells, would give important insights in the mechanisms used by NHPHs, and which host cells are mainly involved.

Appendix

CHAPTER

Appendix I

Mycobacterium tuberculosis infection up-regulates sialyl Lewis X expression in the lung epithelium

Rita Matos *, Kaori L. Fonseca *, Stefan Mereiter, Ana R. Maceiras, Joana Gomes, Cristina Vilaplana, Fátima Gärtner, Pedro NS Rodrigues, Celso A. Reis, Margarida Saraiva, Ana Magalhães

2021

Microorganisms

<https://doi.org/10.3390/microorganisms9010099>



Article

Mycobacterium tuberculosis Infection Up-Regulates Sialyl Lewis X Expression in the Lung Epithelium

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Citation: Matos, R.; Fonseca, K.L.; Mereiter, S.; Maceiras, A.R.; Gomes, J.; Vilaplana, C.; Gartner, F.; Rodrigues, P.N.S.; Reis, C.A.; Saraiva, M.; et al. *Mycobacterium tuberculosis* Infection Up-Regulates Sialyl Lewis X Expression in the Lung Epithelium. *Microorganisms* **2021**, *9*, 99. <https://doi.org/10.3390/microorganisms9010099>

Received: 8 December 2020

Accepted: 29 December 2020

Published: 4 January 2021

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Abstract: Glycans display increasingly recognized roles in pathological contexts, however, their impact in the host-pathogen interplay in many infectious diseases remains largely unknown. This is the case for tuberculosis (TB), one of the ten most fatal diseases worldwide, caused by infection of the bacteria *Mycobacterium tuberculosis*. We have recently reported that perturbing the core-2 O-glycans biosynthetic pathway increases the host susceptibility to *M. tuberculosis* infection, by disrupting the neutrophil homeostasis and enhancing lung pathology. In the present study, we show an increased expression of the sialylated glycan structure Sialyl-Lewis X (SLeX) in the lung epithelium upon *M. tuberculosis* infection. This increase in SLeX glycan epitope is accompanied by an altered lung tissue transcriptomic signature, with up-regulation of genes coding enzymes that are involved in the SLeX core-2 O-glycans biosynthetic pathway. This study provides novel insights into previously unappreciated molecular mechanisms involving glycosylation, which modulate the host response to *M. tuberculosis* infection, possibly contributing to shape TB disease outcome.

Keywords: *Mycobacterium tuberculosis*; lung glycophenotype; Lewis antigens; Sialyl-Lewis X

1. Introduction

Glycosylation is a common post-translation modification, recognized to regulate several key biological processes, either on homeostasis or pathological conditions [1–3]. This highly regulated enzyme-mediated modification occurs mainly on the Golgi apparatus and results in the production of glycosidic linkages of saccharides to other saccharides, proteins or lipids [4]. Glycans and glycoconjugates can be classified into several classes, including the protein linked N-glycans and O-glycans. The biosynthesis and modification

of glycan structures are tissue- and cell-specific and are dictated by the cellular glycosylation machinery [5].

The crosstalk between glycosylation, infection, inflammation and immune surveillance is well established [6–9]. Glycans are important mediators in host pathogen interactions, constituting ligands that are explored by the pathogen for the colonization of host tissues, as well as important effector molecules for cellular signaling in response to infection [10,11]. Moreover, glycans are key players in immune cell adhesion and recruitment to the site of infection. We have previously described a critical role of glycan structures in the context of two infections with high relevance to human health: *Helicobacter pylori* and *Mycobacterium tuberculosis*. In the case of *H. pylori*, we showed that infection results in increased expression of sialylated Lewis antigens in the gastric epithelium, which are recognized by the bacterial sialic acid binding adhesin (SabA), contributing to a close contact with the gastric mucosal cells and a tighter fit of the infection load with proficient transfer of virulence factors [12,13]. As for *M. tuberculosis*, a pathogen that causes over 1.4 million deaths and over 10 million new cases of tuberculosis (TB) per year [14], we have recently described a link between O-glycans and susceptibility to infection [15]. Briefly, the deficiency of *Gcnt1*, a key enzyme acting on the initial biosynthetic steps of the core-2 O-glycans, was associated with increased susceptibility to *M. tuberculosis* infection, characterized by augmented lung immune pathology, with both hematopoietic and non-hematopoietic compartments being crucial for this process [15]. Previous studies had shown that a combined deficiency in fucosyltransferases 4 and 7 in the mouse model led to accelerated death following *M. tuberculosis* infection, which was not caused by increased bacterial proliferation, nor by exacerbated tissue pathology [16]. This combined deficiency was subsequently shown to be associated with reduced numbers of T cells and antigen-specific effector responses in lymph nodes, but normal T cell responses in the lung [17]. Interestingly, all these glycosyltransferases (*Gcnt1*, *Fut4* and *Fut7*) are involved in the biosynthesis of Lewis antigens [18]. Moreover, it has been recently shown that *Mycobacterium bovis* infection led to increased expression of specific Lewis epitopes on N-glycans [19].

Lewis antigens are terminal fucosylated structures that decorate glycan chains, and their expression has also been implicated in the recognition and binding of several pathogens [11,12]. These antigens are classified based on their biosynthesis: Type 1 chains are characterized by the Gal β 1,3GlcNAc linkage, while type 2 chains display a Gal β 1,4GlcNAc linkage [3,20]. The addition of a fucose to terminal galactose (Gal) on type 1 Gal β 1-3GlcNAc chain leads to H-type 1 structure, that can be further modified with a fucose on the N-acetylglucosamine (GlcNAc) residue resulting on the di-fucosylated Lewis B (LeB) antigen. Alternatively, an α 1,4-fucosyltransferase can act towards the type 1 chain and add a fucose to the GlcNAc residue and lead to the formation of Lewis A (LeA) antigen. The modification of a type 1 chain by an α 2,3-sialyltransferase followed by addition of fucose results on the biosynthesis of sialyl-Lewis A (SLeA). The biosynthesis of the type 2 Lewis antigens is very similar, with addition of the same glycan units, but it occurs in the type 2 Gal β 1-4GlcNAc chains, originating the isomers Lewis X (LeX), Lewis Y (LeY) and sialyl-Lewis X (SLeX) [20,21]. Among the Lewis antigens, SLeX is well studied in inflammation and in infection. Indeed, SLeX mediates the interactions between host cells and some pathogens like *H. pylori* [12,22].

Despite the growing body of evidence attesting a role of terminal sialylated Lewis antigens in infection, the crosstalk between *M. tuberculosis* and the lung sialylation profile has never been assessed. Here, we investigated how *M. tuberculosis* infection impacted the expression of different glycan structures in the lung, using a mouse model of infection. We demonstrate that *M. tuberculosis* infection promotes the expression of the sialylated glycan structure SLeX, but not of the other Lewis antigens. This increase in the SLeX glycan epitope was observed in infection by two different *M. tuberculosis* strains, H37Rv and HN878, and in two distinct genetic mouse backgrounds with different infection susceptibility, C57BL/6 and C3HeB/FeJ. Furthermore, we showed that the up-regulation of SLeX tissue expression is concomitant with the higher expression levels of enzymes involved in

the biosynthetic pathway of SLeX on core-2 O-glycans. Noteworthy, this increase in the lung epithelium sialylation was also observed in mice lacking Gcnt1 glycosyltransferase, suggesting that the overall increased lung sialylation in response to *M. tuberculosis* infection results from up-regulation of SLeX terminal decoration on different glycoconjugate carriers. This work further illustrates the existing cross-talk between glycans and infection, highlighting the importance of glycosylation in infectious diseases.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were performed in accordance with recommendations of the European Union Directive 2010/63/EU, and were approved by the i3S Animal Ethics Committee and the Portuguese National Authority for Animal Health (# 014811/2016-07-13) or by the Animal Experimentation Ethics Committee of the Hospital Universitari Germans Trias i Pujol (#B9900005) and the Dept d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Government.

2.2. Animals

C57BL/6 wild-type and *Gcnt1*^{−/−} mouse strains were bred and housed at the i3S animal house and infected under ABSL3 conditions. The *Gcnt1*^{−/−} mouse [23] was obtained from the Consortium for Functional Glycomics (CFG). C3HeB/FeJ specific-pathogen-free mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were supervised daily following a strict monitoring protocol in order to ensure animal welfare and euthanized with isoflurane (inhalation excess) or by CO₂ inhalation. Food and water were ad libitum.

2.3. Bacteria and Bacterial Growth

M. tuberculosis H37Rv and HN878 were grown in Middlebrook 7H9 liquid media (Becton-Dickinson, Sparks, MD, USA) supplemented with 0.05% Tween 80, 0.2% glycerol and 10% oleic acid/albumin/dextrose/catalase (OADC) enrichment for 7–10 days and then sub cultured in Proskauer Beck (PB) medium, supplemented with 0.05% Tween 80 and 2% glycerol, to the mid-log phase. To determine the concentration of *M. tuberculosis*, 6 frozen aliquots were serially diluted and plated in Middlebrook 7H11 (Becton-Dickinson, Sparks, MD, USA) agar plates supplemented with 10% OADC and 0.5% glycerol. Viable bacteria were determined by CFU enumeration after 21–28 days of incubation at 37 °C.

2.4. *M. tuberculosis* Infection

Mice were infected with *M. tuberculosis* H37Rv or HN878 strains via aerosol route using an inhalation exposure system (Glas-Col), following previously published protocols [15]. A murine model of active TB mimicking human TB was used, as described in Kroesen et al. [24]. C3HeB/FeJ mice were challenged with a single i.v. infection (4×10^4 – 2×10^5 CFU/mL per mouse) with *M. tuberculosis* H37Rv Pasteur strain. At the experimental time points (15, 30, 60 and 90 days post-infection), the lungs of infected animals were harvested and the bacterial load determined by CFU enumeration, as previously described [15,25,26]. The data is presented in Supplementary Figure S1.

2.5. Tissue Samples and Immunohistochemistry

At the indicated time-points, the lungs of control or infected animals were aseptically excised, fixed in 10% buffered-formalin and paraffin-embedded. Serial consecutive sections of 3 µm-thickness were made and used for immunohistochemical analysis. Lung sections were deparaffinized, rehydrated and the endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 30 min. Then, sections were incubated with normal rabbit serum diluted 1:5 in BSA 10%-PBS for 30 min followed by incubation with primary antibody (see Table 1) diluted in BSA 5%-PBS overnight at 4 °C. Sections were incubated with secondary antibody rabbit anti-mouse (DAKO, Denmark) diluted 1:200 in BSA 5%-PBS

at room temperature followed by avidin/biotin complex detection (Vector laboratories, Vectastain, CA, USA). Color reaction was developed for 10 min with 3,3-diamino-benzidine chromogen (DAB, Sigma, St. Louis, MO, USA) activated with 0.02% of H₂O₂ and counter staining of the nucleus was performed using Mayer's Haematoxylin, followed by samples dehydration and mounting, as previously described [27].

The intensity of the immunohistochemistry staining was measured using ImageJ image analysis software with the Fiji image-processing package [28]. First, the area of interest was selected for each image and all brown tones were extracted by defining following cut-offs: Hue 1 to 45 and 205 to 255; brightness 0 to 122. Next, the brown saturation was divided in 11 categories, from lowest to highest saturation, and the number of pixels within each category was quantified. This gave rise to an intensity histogram, which was used to calculate the mean brown intensity value for the area of interest of each image. Three images of each animal per time-point were used, and at least five animals of each group were analyzed.

Table 1. Monoclonal antibodies used for Lewis antigens analysis by immunohistochemistry.

Antigen	Antibody Clone	Dilution (IHC)	Company/Reference
Lewis B (LeB)	BG-6 (T218)	1:50	Signet
Sialyl-Lewis A (SLeA)	CA19-9	1:500	Santa Cruz Biotechnology
Lewis X (LeX)	SH1	1:5	[29]
Lewis Y (LeY)	AH6	1:10	[30]
Sialyl-Lewis X (SLeX)	CSLEX-1	1:80	BD Biosciences

2.6. RNA Extraction, cDNA Synthesis and Real-Time PCR Analysis

At the indicated time-points, the lungs were aseptically excised and cell suspensions prepared as before [25,26]. Total RNA was extracted from lung of C57BL/6 and *Gcnt1*^{−/−} mouse models, using TRI Reagent (Sigma, St. Louis, MO, USA) and converted to cDNA using the ProtoScript First Strand cDNA Synthesis Kit (E6300S, New England Biolabs, Ipswich, MA, USA). For real-time PCR analysis cDNA samples were amplified with PowerUp SYBRGreen Master Mix (Applied Biosystems, Foster City, CA, USA), using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA, USA). Oligonucleotides were selected based on Nairn et al. [18], and their sequences are listed in Table 2. Specificity of amplification was confirmed by melting curve analysis. Standard curves and RQ value were determined for each gene. Ubiquitin was used as a reference gene for normalization of target gene abundance.

Table 2. Oligonucleotide sequences used for mice glycosyltransferases quantitative real-time PCR analysis.

Gene	Forward Primer	Reverse Primer
<i>Gcnt1</i>	5'- CGAAGGCCATGTTTCCAACGG -3'	5'- TCCGAAGACGCACACAGAGC -3'
<i>Fut4</i>	5'- ACCAGAGGGAGCAGTGACG -3'	5'- TCCACACCCACCTCTGCCC -3'
<i>Fut7</i>	5'- GGACGACTTCAGCTCTGCCC -3'	5'- CGCCAAGCAAAGAAGCCACG -3'
<i>Fut9</i>	5'- TCCCATGCGGTCTGATTAC -3'	5'- TTCTGAAAGGGTGCCCTGGC -3'
<i>Fut10</i>	5'- GGGTGTGCAGGACATTAACC -3'	5'- AGCCTACTGTTTGCCACAC -3'
<i>Fut11</i>	5'- GGGTGCTCAGTGTCTGTTCCG -3'	5'- CCCACGGCTCCTCCTCTC -3'
<i>St3gal1</i>	5'- TCCTACAAGTGCACAGCGTCG -3'	5'- TGTTTCGCCTGGTGCCTGG -3'
<i>St3gal2</i>	5'- GCTCTCTCGGGTGTGGTTCC -3'	5'- ATGCTGTGGTGCGAGTAGTG -3'
<i>St3gal3</i>	5'- CAGCAAGAAACCCAGACCAT -3'	5'- ATGAATGGCTCCGTCCATAG -3'
<i>St3gal4</i>	5'- GCTCCTGTGGCTGGCTACG -3'	5'- GGGTCAAAGTGGGCCGACTC -3'
<i>St3gal5</i>	5'- AGCCTCTTGGATATGCTGCC -3'	5'- CGTTCCCAACAACACACAGC -3'
<i>St3gal6</i>	5'- ATGGTGGCATTCCCGTAGTA -3'	5'- AAGTGACCTCGCTGGTTT -3'
<i>Ubiquitin</i>	5'- TGGCTATTAATTATTCGGTCTGCA -3'	5'- GCAAGTGGCTAGAGTGCAGAGTAA -3'

2.7. Statistical Analysis

Data were analyzed using GraphPad Prism 7. Differences between groups were analyzed with One-way ANOVA using Mann-Whitney test for multiple comparisons. Differences were considered significant for $p \leq 0.05$ and represented as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ and **** $p \leq 0.0001$.

3. Results

3.1. *M. tuberculosis* Infected Lungs Display Increased Sialylation

Lewis antigens are terminally fucosylated carbohydrate structures that decorate glycan chains on lipids or proteins. Although Lewis antigens have been associated with the protective roles of epithelial layers and secreted mucus, they are also recognized as important mediators of pathogen binding and the initiation of infection [31]. Although the expression of these glycan structures was shown to be affected in the context of disease, as in cancer and some infections [3,12], whether terminal Lewis antigens are modulated in TB remains largely unknown. To start unveiling this issue, we assessed using immunohistochemistry the presence of Lewis antigens in the lungs of C57BL/6 mice uninfected or infected via aerosol with two different strains of *M. tuberculosis* (H37Rv and HN878).

Type 1 Lewis antigens (Lewis B and Sialyl-Lewis A) were not detected in the lungs of non-infected mice, whereas the type 2 Lewis antigens (Lewis X, Lewis Y and SLeX) were present in the apical side of epithelial cells of bronchi and bronchiole (Figure 1A). In particular, SLeX was the most expressed Lewis antigen, with strong apical labelling of most surface epithelial cells and secreted mucus (Figure 1A). To investigate whether *M. tuberculosis* infection impacted the expression of these terminal structures, we evaluated their expression in lung sections of infected mice. As depicted in Figure 1A, 90 days post-infection there were no visible differences on the expression of the various Lewis antigens, except for SLeX which intensity on positive cells was augmented, particularly evident upon infection with *M. tuberculosis* HN878 strain.

Thus, we further addressed the dynamics of SLeX expression during the course of infection. This evaluation was performed using the CSLEX-1 antibody. Although we and others have shown that SLeX is expressed in human neutrophils [15], using the CSLEX-1 antibody we could not assess such an expression in the mouse model. This is explained by the fact that the majority of SLeX in mice neutrophils displays an O-acetylated form of sialic acid, which is not recognized by the CSLEX-1 antibody [32–34]. Thus, our analysis was focused on the lung epithelium. We observed an increased expression of SLeX antigen over time in the lung epithelia of C57BL/6 mice infected with *M. tuberculosis* (Figure 1B). Interestingly, this increase was much more evident and significant in the case of infection with the *M. tuberculosis* strain HN878. In particular, automatic quantification of SLeX staining, showed a significantly increased expression of this glycan at days 30 and 90 post-infection, when compared to non-infected or even the initial time-points of infection (day 15 post-infection) (Figure 1B).

As compared to the *M. tuberculosis* strain H37Rv, the strain HN878 is known to be hypervirulent [35] and as we have recently described, C57BL/6 mice infected with low doses of H37Rv do not recapitulate features of active TB disease, showing instead a resistance phenotype [36]. Thus, we raised the hypothesis that the upregulation of epithelial SLeX might be associated with disease progression. In support of this hypothesis, we measured the expression of SLeX during infection in a different mouse strain, the susceptible C3HeB/FeJ mice [36,37]. Infection of these animals with the *M. tuberculosis* H37Rv strain, resulted in an increased expression of SLeX antigens in the lung, which was readily seen at day 30 post-infection (Figure 1C). Overall, we provide evidence for the up-regulation of SLeX antigens in the lung during experimental *M. tuberculosis* infection, in conditions associated with susceptibility.

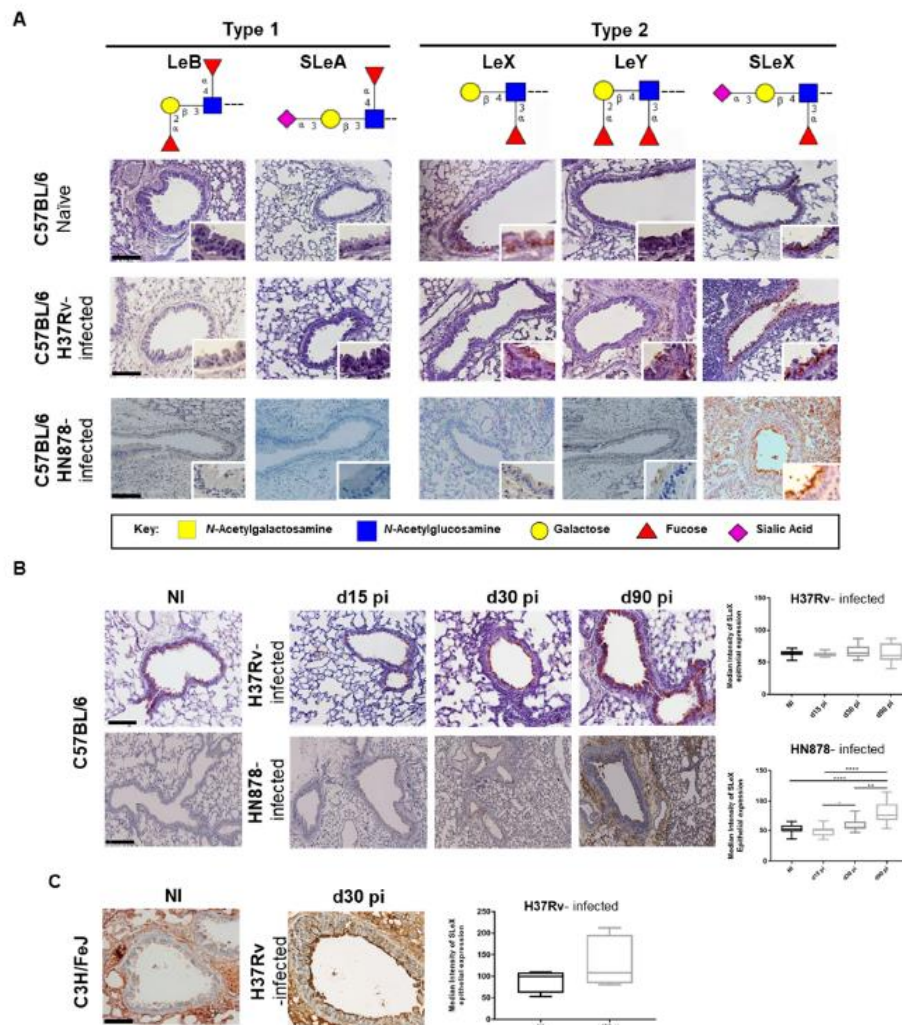


Figure 1. *M. tuberculosis* infection upregulates Sialyl-Lewis X (SLeX) expression in lung tissue. **(A)** Lungs of C57BL/6 mice non-infected or infected after 90 days with two different *M. tuberculosis* strains (H37Rv or HN878) were stained by immunohistochemistry, using a panel of mAbs recognizing type 1 (Lewis B (LeB) and Sialyl-Lewis A (SLeA)) and type 2 (Lewis X (LeX), Lewis Y (LeY) and Sialyl-Lewis X (SLeX)) Lewis antigens. Magnification 200× and inserts at 400×. **(B)** SLeX expression on lung epithelium of non-infected or infected C57BL/6 and C3HeB/FeJ mice. C57BL/6 animals were infected via aerosol with *M. tuberculosis* H37Rv or HN878 strains. Lungs were recovered at the indicated time-points, fixed and lung sections stained for SLeX. Magnification 200×. The right panel represents the quantification of epithelial expression of SLeX. **(C)** C3HeB/FeJ mice were infected intravenously with *M. tuberculosis* H37Rv strain. Lungs were recovered at the indicated time-points, fixed and lung sections stained for SLeX. Magnification 200×. The panel on the right represents the quantification of epithelial expression of SLeX. Each dot represents the median intensity obtained per photograph. At least 5 mice were analyzed. Statistical analysis was performed using one-way ANOVA with Mann-Whitney test for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. Monosaccharide symbols follow the Symbol Nomenclature for Glycans [38].

3.2. *M. tuberculosis* Infection Up-Regulates the Transcription of Enzymes Controlling the Biosynthesis of SLeX on Core-2 O-Glycans

Taking into consideration that core-2 O-glycans are major carriers of terminal SLeX antigens and that their biosynthesis is regulated by the coordinated action of several glycosyltransferases [3,4,39], we measured, using real-time PCR, the transcript levels of key enzymes involved in the core-2 O-glycans SLeX antigens biosynthetic pathway (Figure 2A). Given our previous data (Figure 1), we did so in C57BL/6 mice infected with the *M. tuberculosis* strain HN878. Over the course of infection, we observed a significant upregulation on the transcription of several genes encoding enzymes participating in different steps of SLeX biosynthesis (Figure 2B). This was the case for the core 2 GlcNAcT Gcnt1, the α 1,3-fucosyltransferases Fut4, 7, 9 and 11, and the α 2,3-sialyltransferases St3Gal1, 2, 4 and 5 (Figure 2B). The transcriptomic analysis of glycosyltransferases on the H37Rv-infected C57BL/6 animals showed that, similar to what was observed on mice infected with HN878 strain, there was an increase on the expression of the α 1,3-fucosyltransferases (Fut4, 9, 10 and 11), but no alterations were observed for the other enzymes evaluated (Figure S2). This increased expression in a more restricted number of enzymes could explain the lower amount of SLeX detected on the lung epithelium on these mice, when compared to the mice infected with a more virulent strain (*M. tuberculosis* HN878) (Figure 1). Our data reveals that the increased SLeX expression observed upon *M. tuberculosis* infection is accompanied by alterations at transcriptomic level, with up-regulation of enzymes that regulate the terminal SLeX decoration with fucose and sialic acid residues, constituting a specific glycosylation signature. Moreover, we demonstrate that the lung epithelium increase in SLeX is more evident for a hypervirulent strain of *M. tuberculosis*.

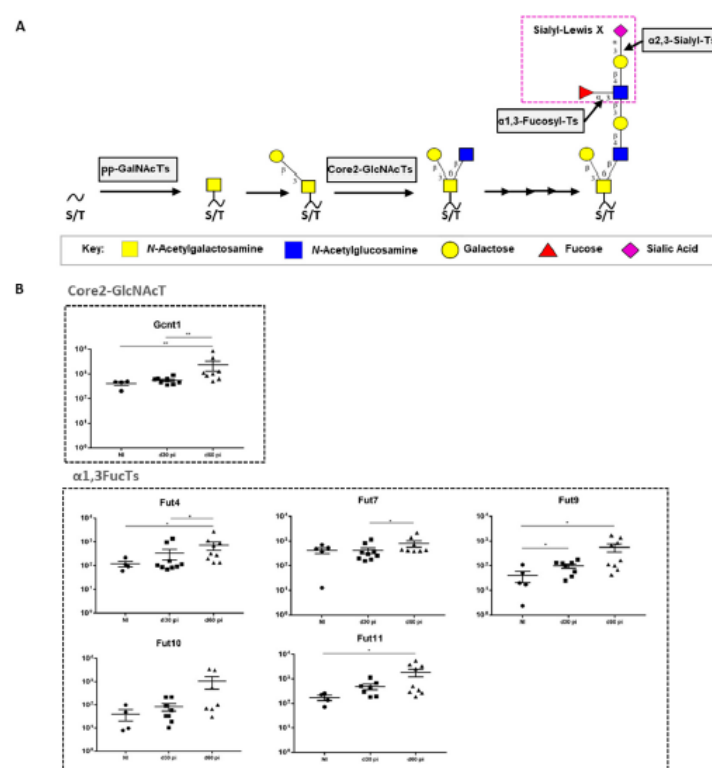


Figure 2. Cont.

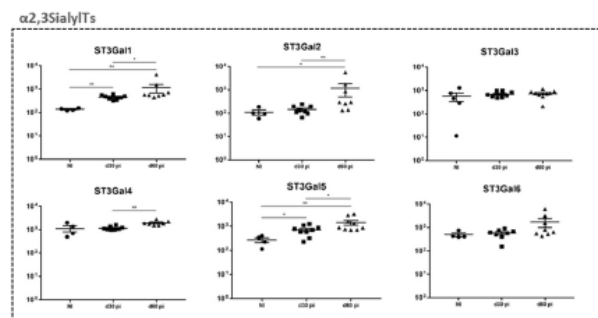


Figure 2. *M. tuberculosis* HN878 infection in C57BL/6 upregulates the transcription of several glycosyltransferase genes involved in the SLeX biosynthetic pathway. (A) Schematic representation of the biosynthetic pathway of SLeX-core 2 O-glycans. (B) The expression of the indicated glycosyltransferases, divided in 3 main categories (Core 2-GlcNAcT, α 2,3SialylTs and α 1,3FucosylTs), was measured by real-time PCR in lungs of C57BL/6 mice infected via aerosol with a low dose of *M. tuberculosis* strain HN878 on days 30 and 60 post-infection. Non-infected animals (NI) are shown as controls. Each dot represents one mouse of a total of 6–8 from 2 independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$. Monosaccharide symbols follow the Symbol Nomenclature for Glycans [38].

3.3. Lack of *Gcnt1* Does not Preclude Increased Lung Sialylation upon *M. tuberculosis* Infection

We previously showed that mice lacking the GlcNAcT *Gcnt1*, a key enzyme for the biosynthesis of SLeX on core-2 O-glycans, display increased TB susceptibility and that both the hematopoietic and the stromal compartments contributed to such susceptibility [15]. That, together with our observation that *Gcnt1* transcription is upregulated upon infection, led us to question the impact of *Gcnt1* deficiency on the expression profile of Lewis antigens in the lung epithelium in naïve or *M. tuberculosis* infected mice. Naïve *Gcnt1*^{−/−} animals displayed a pattern of Lewis antigens expression similar to WT C57BL/6, with absence of type 1 Lewis antigens and type 2 antigens detected on the bronchi and bronchiole epithelium (Figure 3A). Also similar to C57BL/6, the *Gcnt1*^{−/−} showed a more pronounced expression of SLeX as compared to the other Lewis antigens tested (Figure 3A).

Infection of *Gcnt1*^{−/−} animals with *M. tuberculosis* strain HN878 resulted on increased SLeX expression on the lung epithelia during the course of infection, as demonstrated by the automatic quantification of staining, which was more pronounced at day 30 and 90 post-infection (Figure 3B). Similar to what was observed for the C57BL/6 animals, *M. tuberculosis* infection up-regulated the transcriptomic levels of several genes encoding enzymes involved on SLeX biosynthetic pathway on the *Gcnt1*^{−/−} infected animals (Figure 3C). Except for the *Fut4* that was not significantly altered in the *Gcnt1* deficient animals, an up-regulation of the α 1,3-fucosyltransferases, *Fut7*, 9, 10 and 11, was observed upon infection by *M. tuberculosis*, in line with the data obtained for C57BL/6 mice. Regarding the α 2,3-sialyltransferases evaluated, we observed augmented transcripts of *St3Gal1*, 5 and 6 in *Gcnt1*^{−/−} infected mice corroborating the overall increase in terminal α 2,3-sialylation. These data suggest that the remodeling of the lung epithelia sialylated profile upon *M. tuberculosis* infection does not depend on a competent *Gcnt1* enzyme.

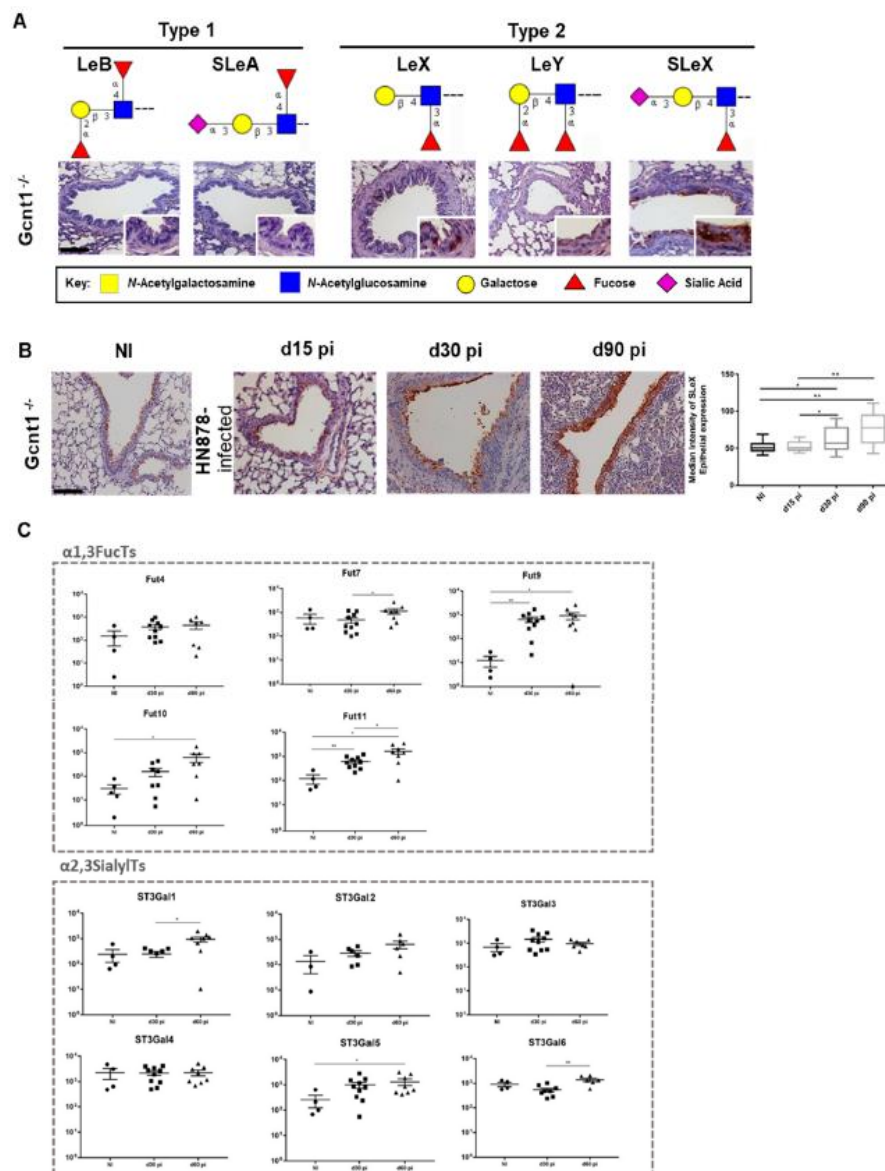


Figure 3. *Gcnt1* null mice infected with *M. tuberculosis* strain HN878 display lung increased SLeX expression concomitant with an up-regulated α2,3-SialylTs and α1,3-FucosylTs gene transcript signature. (A) Expression of type 1 (Lewis B (LeB) and Sialyl-Lewis A (SLeA)) and type 2 (Lewis X (LeX), Lewis Y (LeY) and Sialyl-Lewis X (SLeX)) Lewis antigens on *Gcnt1*^{−/−} mice lung. Magnification 200× and inserts at 400×. (B) SLeX expression on lung epithelium of non-infected (NI) or *M. tuberculosis*-infected *Gcnt1*^{−/−} mice. Mice were infected via aerosol with a low dose of *M. tuberculosis* strain HN878. Lungs were recovered at the indicated time-points post-infection, fixed and lung sections stained for SLeX. Magnification 200×. The panel on the right represents the quantification of epithelial expression of SLeX. (C) The expression of several glycosyltransferases involved on the SLeX biosynthetic pathways (α2,3SialylTs and α1,3FucosylTs) was measured by real-time PCR in lungs of *Gcnt1*^{−/−} on days 30 and 60 post-infection. Non-infected animals (NI) are shown as controls. Each dot represents one mouse of a total of 6–8 from 2 independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test for multiple comparisons. *, *p* < 0.05; **, *p* < 0.01. Monosaccharide symbols follow the Symbol Nomenclature for Glycans [38].

4. Discussion

The role of glycans as chief players in defining pathogen tropism and colonization, as well as in numerous aspects of the immune response to infection, has been increasingly recognized over the last two decades [11,12,40,41]. However, the impact of the host glycosylation machinery, as well as its contribution to host susceptibility in TB is just starting to be unveiled [15,42,43]. Taking into consideration recent studies showing that deficiency in specific glycosyltransferases involved in Lewis antigens biosynthesis affect *M. tuberculosis* infection outcome [15–17], we evaluated how the epithelial expression of these terminal glycan epitopes in the lung was impacted by the infection. For this purpose, we started by analyzing the expression of Lewis antigens in the mouse lung epithelium and observed the exclusive detection of type 2 Lewis antigens and a main expression of the sialylated glycan SLeX. This observation is in accordance with previous reports showing predominance of type 2 Lewis structures and the glycosyltransferases involved in their biosynthesis in different mouse organs [18,27,44]. Furthermore, we observed that, similar to the augmented sialylation described to occur in response to infection by several other pathogens, *M. tuberculosis* infection also resulted in increased levels of SLeX. Interestingly, this increase in SLeX was mostly marked when mice were infected with the hypervirulent strain HN878, in comparison with the laboratory reference strain H37Rv. We have previously shown that SLeX is detected in lung sections of TB patients who were submitted to pulmonary surgery to treat TB [15]. Therefore, our data is in line with recent findings showing that mouse infection with the clinical isolate HN878 recapitulates more closely the pathogenesis of human TB disease, when compared with infection with the laboratory strain H37Rv [36]. In the same line, we found that C3HeB/FeJ mice that display increased susceptibility to *M. tuberculosis* infection presented marked levels of SLeX in the lung epithelium upon infection.

Cellular glycosylation is a complex process with multiple layers of regulation such as: glycosyltransferase gene transcription, availability of nucleotide sugar donors, relative amounts of enzymes competing for identical substrates, Golgi intracellular enzyme trafficking, and glycan turnover at the cell surface [5,45]. Our results reveal that the augmented expression of SLeX in the mouse lung epithelium is accompanied by the transcriptional up-regulation of several glycosyltransferases involved in different enzymatic steps of SLeX biosynthesis. Supporting the differences that we observed regarding SLeX expression when comparing *M. tuberculosis* strains with different virulence features, experimental infection with *M. tuberculosis* H37Rv or HN878 also yield different glycosyltransferase transcriptomic signatures. Whereas infection with the HN878 led to significant lung up-regulation of several α 2,3-sialyl- and α 1,3-fucosyl-transferases, involved in the terminal decoration of SLeX, infection with H37Rv strain displayed fewer significant changes limited to the enzymes involved in the addition of the terminal fucose residues. Noteworthy, comparison of glycosyltransferases expression data in our infected mice with transcriptomic analysis of whole blood from active TB patients [15] reveals a common pattern, with identification of several homologous genes, therefore supporting a common mechanism across species. Furthermore, in line with the up-regulation of SLeX during the course of disease, a recent study showed that urinary levels of sialic acid, the terminal glycan of SLeX structure, discriminate patients with active TB from healthy controls and patients with non-tuberculous pulmonary diseases [46].

Although the molecular signaling pathways leading to up-regulation of glycosyltransferases in response to *M. tuberculosis* infection remain unknown, augmented epithelial sialylation has been previously associated with infection and inflammation [3,47]. A classic example is the up-regulation of terminal sialylated structures by *H. pylori*. These bacteria exploit the host glycosylation machinery to induce the expression of its adhesin's glycan receptors promoting bacterial binding and a successful infection. Remarkably, the tissue expression levels of SLeX are also associated with the *H. pylori* strain pathogenicity, with more virulent strains promoting increased epithelial levels of this sialylated glycan [12,13]. In the case of *H. pylori* infection, it is acknowledged that both glycoproteins

and glycolipids contribute for gastric glycophenotype switch to negatively charged glycans, such as SLeX. However, the carriers of SLeX in the lung epithelium in the context of *M. tuberculosis* infection remain elusive. Additionally, several cytokines, such as TNF and IL-1, have been previously shown to transcriptionally upregulate genes encoding glycosyltransferases [48,49].

Recently, we have shown that *Gcnt1* deficiency is associated with increased susceptibility to *M. tuberculosis* infection [15]. The *Gcnt1* enzyme belongs to the core-2 GlcNAc transferases family, which are responsible for the initiation of the core-2 extension required for the generation of SLeX core-2 O-glycans. Therefore, we decided to explore firstly if *Gcnt1* abrogation would impact Lewis antigen expression in the lung epithelium, and secondly how lack of *Gcnt1* would affect the modulation of SLeX expression upon *M. tuberculosis* infection. We observed that *Gcnt1* deficiency did not change the Lewis antigens lung repertoire, and that *Gcnt1*^{−/−} mice lung epithelium also displayed increased SLeX, supported by up-regulation of terminal α2,3-sialyl- and α1,3-fucosyl-transferases, in response to infection. Given the redundancy in the glycans biosynthetic pathways it is possible that the same enzymatic step may be catalyzed by different enzymes. In mice, two different enzymes *Gcnt1* and *Gcnt3* share core-2 GlcNAc-transferase activity [18]. To test the hypothesis of a *Gcnt3* compensatory activity in *Gcnt1* null mice, we evaluated the transcription levels of *Gcnt3*, upon infection but no significant changes were found when comparing WT and *Gcnt1*^{−/−} mice (data not shown). Therefore, it is tempting to speculate that the overall increased sialylation of the lung may result from SLeX capping of different glycan carriers in C57BL/6 and *Gcnt1*^{−/−} mice. From a biochemical perspective, it will be interesting to structurally analyze the glycan composition of the *Gcnt1*^{−/−} lung to investigate for possible remodeling events. Of note, these remodeling events may involve N-glycans, as well as O-glycans, a hypothesis worth pursuing in the future.

Our findings reveal the modulation of SLeX during *M. tuberculosis* infection and highlight the significance of glycans and their biosynthetic pathways as possible new targets to modulate inflammation and immune response balance in TB.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2076-2607/9/1/99/s1>, Figure S1: Lung bacterial burdens obtained for the different experimental infections used in this study, Figure S2: Transcriptomic analysis of glycosyltransferase-encoding genes on C57BL/6 mice infected with H37Rv *M. tuberculosis* strain.

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Funding: This work was financed by FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through FCT—Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274) and by grants POCI-01-0145-FEDER-028955 (to MS), POCI-01-0145-FEDER-028489 (to AM), and POCI-01-0145-FEDER-016585 (to CAR). CAR acknowledges the Consortium for Functional Glycomics funded by NIGMS-GM62116. This study was funded by the Spanish Government-FEDER Funds through CP13/00174, CPII18/00031 and PI16/01511 grants, and the CIBER Enfermedades Respiratorias Network; and by the Spanish Society of Pneumology and Thoracic Surgery (SEPAR) through grant 16/023. KLF and RM are funded by FCT PhD scholarships SFRH/BD/114405/2016 and SFRH/BD/131159/2017, respectively; MS is funded by FCT through Estimulo Individual ao Emprego Científico. The APC was funded by FCT (POCI-01-0145-FEDER-028489).

Institutional Review Board Statement: All animal experiments were performed in accordance with recommendations of the European Union Directive 2010/63/EU, and were approved by the i3S Animal Ethics Committee and the Portuguese National Authority for Animal Health (# 014811/2016).

07-13) or by the Animal Experimentation Ethics Committee of the Hospital Universitari Germans Trias i Pujol (#B9900005) and the Dept d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Government.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study is contained within this article or supplementary material.

Acknowledgments: The authors thank the excellent support from the i3S scientific platforms, namely Animal facility, and Histology and Electron Microscopy—member of the national infrastructure PPBI—Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122).

Conflicts of Interest: The authors declare no conflict of interest.

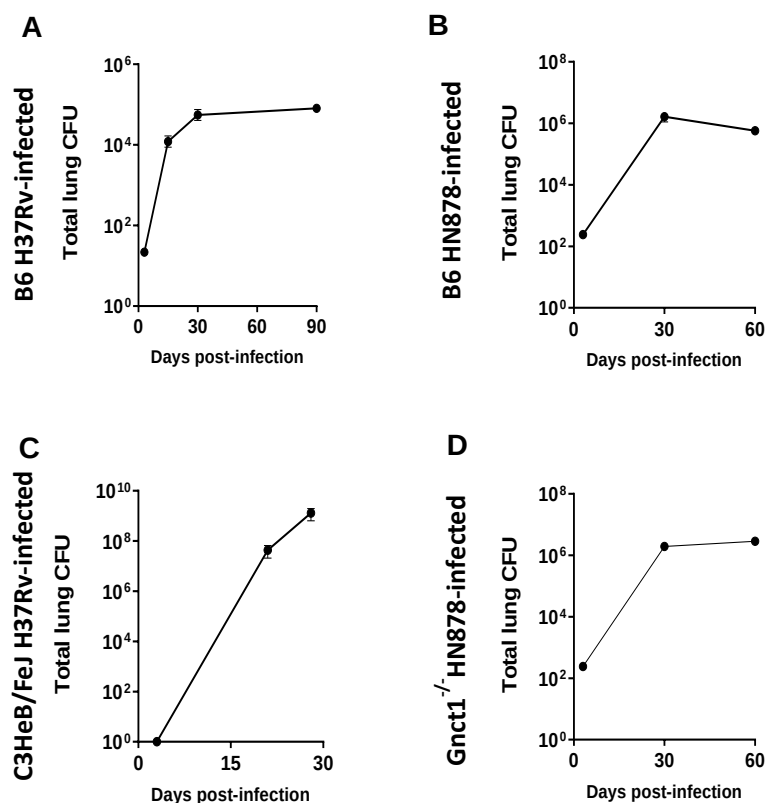
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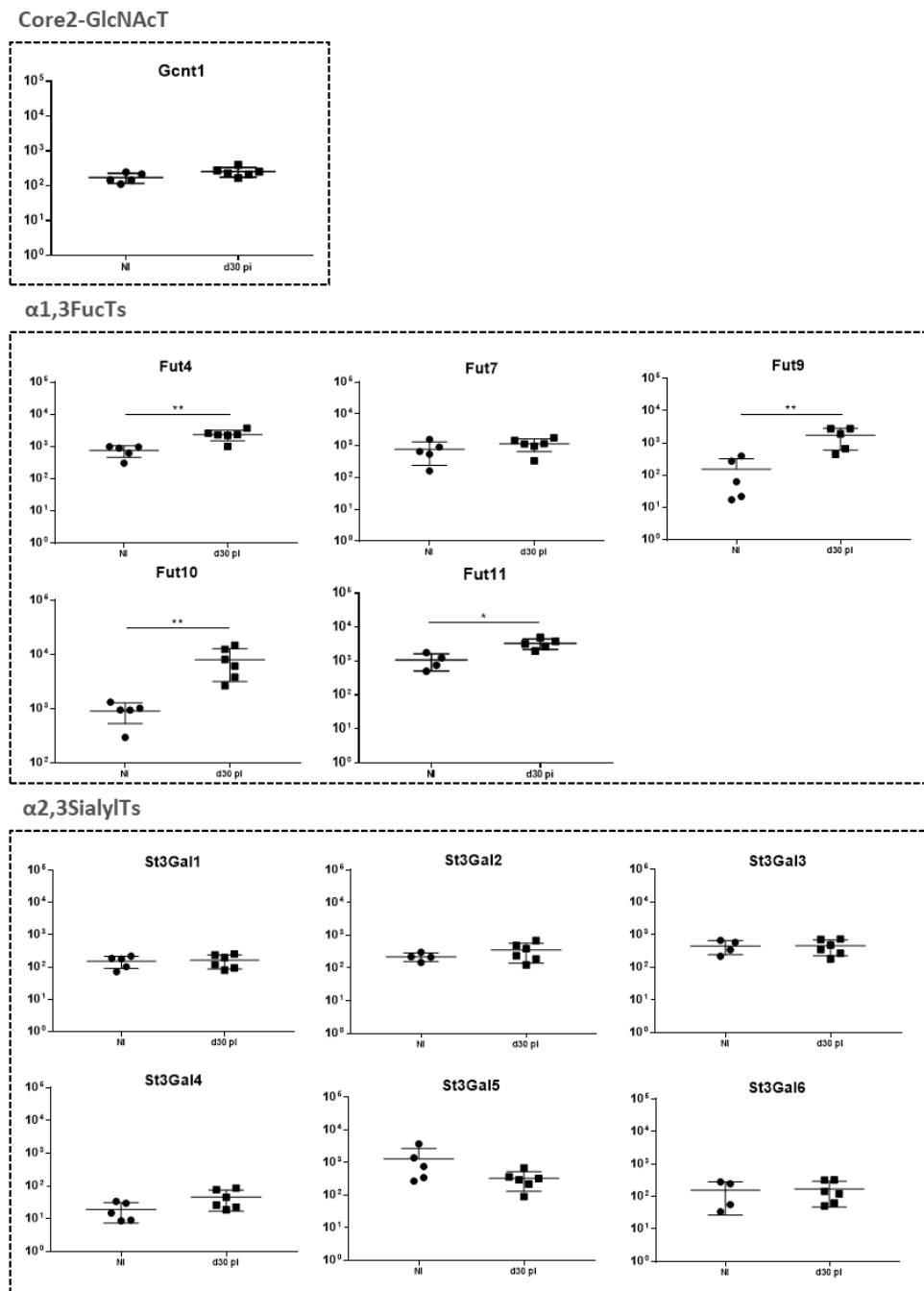
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Supplementary Material



Supplementary Figure 1. Lung bacterial burdens obtained for the different experimental infections used in this study.

C57BL/6 mice were infected by aerosol with a low dose of *M. tuberculosis* strain H37Rv (A) or HN878 (B). C3HeB/FeJ mice were infected intravenously with H37Rv strain (C). *Gcnt1*^{-/-} mice were infected by aerosol with a low dose of *M. tuberculosis* strain HN878 (D). At the indicated time-points post-infection, the lungs of infected mice were collected, and the bacteria burden determined by CFU



Supplementary Figure 2. Transcriptomic analysis of glycosyltransferase-encoding genes on C57BL/6 mice infected with H37Rv *M. tuberculosis* strain.

(A) The expression of several glycosyltransferases involved on the SLeX biosynthetic pathways (pp-GalNAcT, Core 2-GlcNAcT, $\alpha 2,3$ SialylTs and $\alpha 1,3$ FucosylTs) was measured by real-time PCR in lungs of C57BL/6 mice infected via aerosol with a low dose of *M. tuberculosis* strain H37Rv on days 15, 30 and 90 post-infection. Non-infected animals (NI) are shown as controls. Each dot represents one mouse of a total of 6-8 from 2 independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's test for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$.

Appendix II

Deficiency in the glycosyltransferase Gcnt1 increases
susceptibility to tuberculosis through a mechanism involving
neutrophils

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2020

Mucosal Immunology

<https://doi.org/10.1038/s41385-020-0277-7>



ARTICLE OPEN

Deficiency in the glycosyltransferase Gcnt1 increases susceptibility to tuberculosis through a mechanism involving neutrophils

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Modulation of immunity and disease by glycans is increasingly recognized. However, how host glycosylation shapes and is shaped by tuberculosis remains poorly understood. We show that deficiency in the glucosaminyl (N-acetyl) transferase 1 (Gcnt1), a key enzyme for core-2 O-glycans biosynthesis, drives susceptibility to *Mycobacterium tuberculosis* infection. The increased susceptibility of Gcnt1 deficient mice was characterized by extensive lung immune pathology, mechanistically related to neutrophils. Uninfected Gcnt1 deficient mice presented bone marrow, blood and lung neutrophilia, which further increased with infection. Blood neutrophilia required Gcnt1 deficiency in the hematopoietic compartment, relating with enhanced granulopoiesis, but normal cellular egress from the bone marrow. Interestingly, for the blood neutrophilia to translate into susceptibility to *M. tuberculosis* infection, Gcnt1 deficiency in the stroma was also necessary. Complete Gcnt1 deficiency associated with increased lung expression of the neutrophil chemoattractant CXCL2. Lastly, we demonstrate that the transcript levels of various glycosyltransferase-encoding genes were altered in whole blood of active tuberculosis patients and that sialyl Lewis x, a glycan widely present in human neutrophils, was detected in the lung of tuberculosis patients. Our findings reveal a previously unappreciated link between Gcnt1, neutrophilia and susceptibility to *M. tuberculosis* infection, uncovering new players balancing the immune response in tuberculosis.

Mucosal Immunology (2020) 13:836–848; <https://doi.org/10.1038/s41385-020-0277-7>

INTRODUCTION

The host immune system has evolved to respond to infection whilst avoiding tissue damage. Deregulation of protective immune responses to *Mycobacterium tuberculosis* (Mtb) often associates with tissue pathology.¹ For example, an inadequate recruitment of neutrophils to Mtb-infected lungs may cause severe tissue damage. Exacerbated neutrophilia is thus linked with increased severity of infection in mouse^{2,3} and man.⁴ Understanding the factors that control or disrupt protective immune networks during tuberculosis (TB) is therefore paramount for the design of more efficient strategies and to inform on potential TB-associated side effects of immune therapies to other diseases.

The importance of glycosylation in innate and acquired immune responses is emerging.⁵ Glycosylation regulates leukocyte recruitment from the vasculature to most tissues, through the modulation of interactions between endothelial selectins and their ligands in leukocytes.^{6,7} Deficiency in several

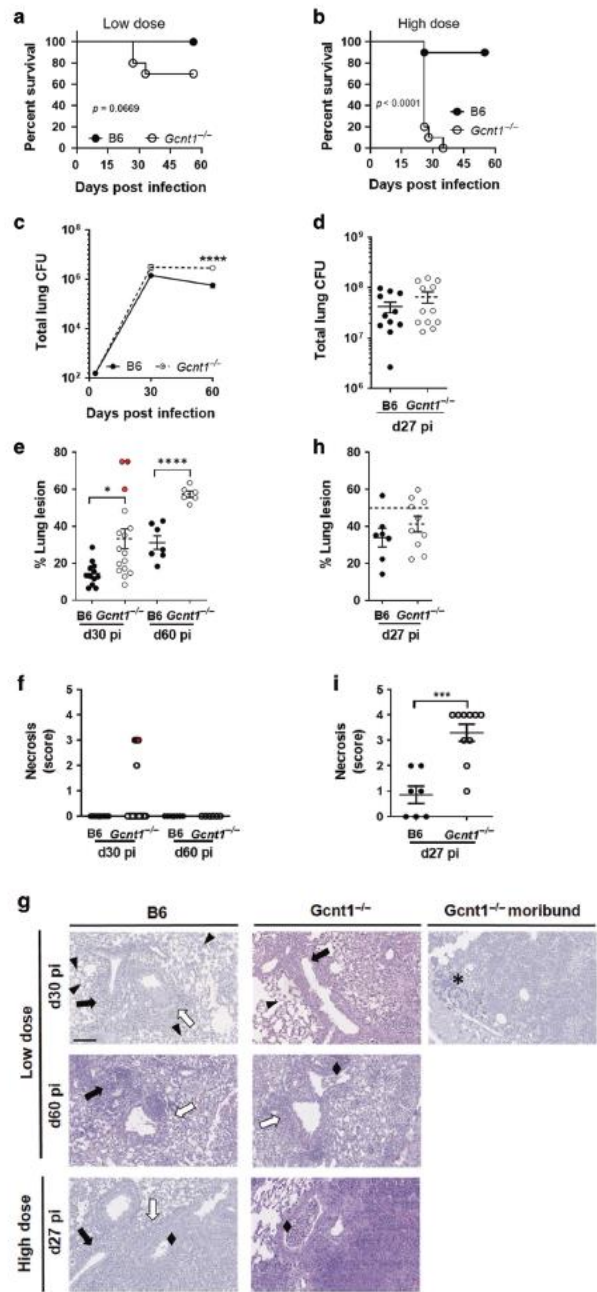
glycosyltransferases involved in the biosynthesis of functional selectin ligands, as *Galnt1*, *Gcnt1*, *B4galnt1*, *Fut4*, *Fut7* and *St3gal4*, results in defective leukocyte recruitment^{7–9} and in homeostatic alterations, namely pronounced blood neutrophilia.^{10,11} Selectin ligands are commonly decorated with O-glycans bearing a terminal sialyl Lewis x (sLe^x) (sialic acid α 2,3Gal β 1–4 (Fuc α 1,3) GlcNAc-R) epitope.⁶ Extensive O-glycosylation is also characteristic of transmembrane or secreted gel-forming mucins,¹² important components of the lung mucus. Mucins play key functions as surface receptors for the binding of several adhesion molecules, mediators of the interaction with pathogens, and local modulators of inflammatory responses.^{13,14} Pathogens, as *Helicobacter pylori*, may modulate the expression, turnover and glycosylation of host mucins.^{15–18} How O-glycosylation contributes to host protection in TB is largely unknown. Deficiency in the α 1,3-fucosyltransferase *Fut7* or *Fut4/Fut7* double deficiency impaired T cell homing to the lymph nodes during Mtb experimental infection, but the traffic of

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Received: 26 July 2019 Accepted: 12 February 2020
Published online: 13 March 2020



effector T cells to the lung was normal.¹⁹ Both *Fut7* deficient (*-/-*) and *Fut4*^{-/-}/*Fut7*^{-/-} mice were initially shown to resist normally to low dose aerosol infection,¹⁹ but a shorter survival of these mice upon infection was reported in a later study.²⁰

Here, we reveal that core-2 GlcNAc transferase *Gcnt1* deficient (*Gcnt1*^{-/-}) mice¹¹ display increased susceptibility to *Mtb* infection, mechanistically mediated by neutrophils and by *Gcnt1* activity in stromal and hematopoietic compartments. Furthermore, we

Fig. 1 Deficiency in *Gcnt1* associates with increased susceptibility to *Mtb* infection. C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) mice were infected by aerosol with *Mtb* strain HN878 with a low (a, c, e–g) or high (b, d, h, i, g) dose of bacteria. a, b The weight of the animals was monitored to determine survival curves, that included 10–13 animals in two independent experiments. Statistical analysis was performed with a log-rank (Mantel-Cox) test for the Kaplan Meyer curve. c, d At the indicated timepoints post-infection, the lungs of infected mice were collected and the bacteria burden determined by CFU enumeration. e, h At the indicated timepoints post-infection, lung pathology defined as the percentage of infiltrate per lobe (Fig. S1A, B), was determined upon H&E staining and morphometric analysis of the right upper lobes of infected lungs. The histopathologic features of the infected lungs were assessed and a relative score attributed (Table 1); the score obtained for necrosis is plotted in f and i. The pictures in g are H&E staining of representative animals within each experimental group. Arrowheads point to intra-alveolar necrotic debris, black diamond point to bronchiolar debris, black arrows point to perivascular lymphocytes, white arrows point to peribronchiolar lymphocytes and asterisks to calcification sites. Scale bar corresponds to 100 µm. In c each dot represents the Mean ± SEM and in d–h each dot represents an individual mouse of 6–12 in at least two independent experiments. In e, f red dots represent moribund *Gcnt1*^{-/-} mice. Statistical analysis was performed using multiple t-test (c) or unpaired t-test (d–i). **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Table 1. Lung histopathology analysis of *Mtb*-infected mice.

	Score	Low dose				High dose	
		day 30		day 60		day 27	
		B6 (12)	<i>Gcnt1</i> ^{-/-} (13)	B6 (7)	<i>Gcnt1</i> ^{-/-} (6)	B6 (7)	<i>Gcnt1</i> ^{-/-} (10)
Perivascular lymphocytes	0						
	1	8.30					
	2	91.7	23.1	28.6		28.6	16.7
	3		76.9	71.4	100	71.4	83.3
Peribronchiolar lymphocytes	0						
	1	44.4	55.6	57.1	33.3	14.3	
	2	55.6	33.3	42.9	50	42.9	66.7
	3		11.1		16.7	42.9	33.3
Bronchiolar debris	0	70.0	66.7	42.9	50	14.3	
	1	30.0	22.2	57.1	33.3	57.1	10.0
	2		11.1		16.7	14.3	20.0
	3					33.3	70.0
Intra-alveolar necrotic debris	0						
	1	16.7	46.2	57.1	16.7	33.3	
	2	66.7	30.8	14.3	50	66.7	
	3	16.7	23.1	28.6	33.3	71.4	100
Necrosis	0	100	92.3	100	100	42.9	
	1					28.6	10.0
	2		7.70			28.6	10.0
	3						20.0
	4						60.0

Lung H&E sections of C57BL/6 (B6) or *Gcnt1*^{-/-} infected animals were assessed and scored for the indicated histopathological features. Indicated is the % of animals with a certain score within the indicated experimental group. Indicated in brackets is the number of animals analysed per group.

report for the first time altered expression of glycosyltransferases associated with sLe^x core-2 O-glycan biosynthetic pathway in whole blood of TB patients. Our findings support the modulation of O-glycosylation during *Mtb* infection, showing that alterations in this pathway compromise the ability of the host to deal with infection in the most competent way.

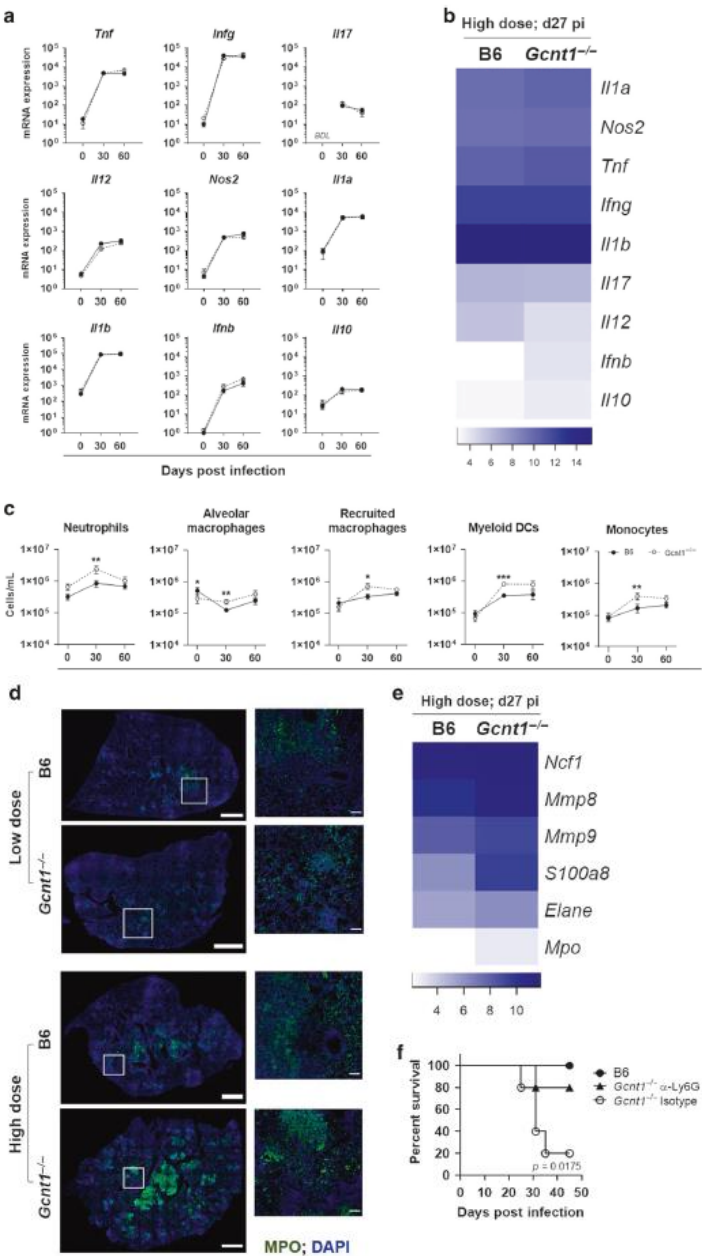
RESULTS

Deficiency in *Gcnt1* is associated with higher susceptibility to *Mtb* infection

Studies with *Fut4*^{-/-} and *Fut7*^{-/-} hinted at a possible role for the host glycosylation machinery in TB.^{19,20} As *Fut4* and *Fut7* catalyse the terminal decoration of glycans, we reasoned that studying the impact of an earlier step of glycan biosynthesis would better reveal its biological effect during *Mtb* infection. Thus,

we used mice lacking *Gcnt1*, a key enzyme for the biosynthesis of core-2 O-glycans.¹¹ C57BL/6 and *Gcnt1*^{-/-} mice were infected via aerosol with *Mtb* strain HN878. Upon a low dose infection, 18.7% of *Gcnt1*^{-/-} mice succumbed around 30 days post-infection, whereas, as expected, all C57BL/6 mice survived (Fig. 1a). Strikingly, all *Gcnt1*^{-/-} mice infected with high doses of *Mtb* succumbed within 40 days, while 90% of C57BL/6 mice survived throughout the 60 days of the experiment (Fig. 1b). *Gcnt1*^{-/-} mice, which survived the low dose of infection, showed a higher lung bacterial burden than C57BL/6, particularly on day 60 post-infection (Fig. 1c). No significant differences in bacterial burdens of C57BL/6 or *Gcnt1*^{-/-} mice infected with high doses of *Mtb* for 27 days were observed (Fig. 1d), despite the different survival rates.

The percentage of lung lesion was quantified to measure lung pathology (Fig. S1A, B). As compared to C57BL/6, *Gcnt1*^{-/-} mice



infected with low doses of *Mtb* showed increased lung pathology, which was mostly pronounced on day 60 post-infection with increased scores for perivascular and peribronchial lymphocytes (Table 1, Fig. 1e and Fig. S1Q). The extent of lung lesion was

particularly high for the *Gcnt1*^{-/-} mice, which succumbed to infection (Fig. 1e, Fig. S1D). Lung histopathologic analysis of the infected mice revealed an overall exacerbation of pathologic features in *Gcnt1*^{-/-} mice, with the moribund mice presenting

Fig. 2 Exacerbated neutrophilia drives increased susceptibility of *Gcnt1*^{-/-} mice to *Mtb* infection. **a–e** At the indicated timepoints post-infection, the lungs of C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) mice infected by aerosol with a low or high dose of *Mtb* strain HN878 were harvested and a cellular suspension prepared. **a, b, e** The relative expression of the indicated genes was determined by real-time PCR. **c** Percentages of the indicated immune cell populations were determined by flow cytometry. The gating strategy is shown in Fig. S2A. **d** Representative images of MPO staining (green) in lung sections of B6 and *Gcnt1*^{-/-} mice infected with low or high doses of *Mtb*. Sections were counterstained with DAPI (blue). Scale bars correspond to 1 mm (left panels) and 100 μm (right panels). In **a, c** each dot represents the Mean ± SEM for 10 animals in two independent experiments. Non-infected animals (day 0) were used as controls. **b, e** Represented are heatmaps of log2 relative expression of the indicated genes in lung samples of B6 and *Gcnt1*^{-/-} mice 27 days after high dose *Mtb* infection. **f** C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} mice were infected by aerosol with a high dose of *Mtb* strain HN878. On day 18 post-infection the *Gcnt1*^{-/-} mice were treated with the neutrophil-depleting anti-Ly6G mAb (black triangles) or an isotype control (open circles). The weight of the animals (5 per group) was monitored to determine survival curves. Statistical analysis was performed per time point with unpaired two-tailed Mann-Whitney test (**a, c**) or with log-rank (Mantel-Cox) test for the Kaplan Meyer curve (**f**). * refer to statistic differences between C57BL/6 or *Gcnt1*^{-/-} mice. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

severe inflammation, pronounced necrosis and calcification (Table 1, Fig. 1e–g). In infections with high doses of bacteria, a higher frequency of infected *Gcnt1*^{-/-} mice showed more than 50% of lung infiltrates as compared to C57BL/6, although globally no significant differences in relative lesion burden were noted (Fig. 1h). The histopathological features observed during infection with low doses of *Mtb* were recapitulated and exacerbated for those with high doses, with infected *Gcnt1*^{-/-} mice presenting extensive lung pathology, particularly extensive necrosis, and histological features of bronchopneumonia (Table 1, Fig. 1i, g and Fig. S1E).

Neutrophils drive the increased susceptibility of *Gcnt1*^{-/-} mice to *Mtb* infection

To investigate the mechanisms underlying the increased susceptibility of *Gcnt1*^{-/-} mice to *Mtb*, we compared the dynamics of the immune response in C57BL/6 or *Gcnt1*^{-/-} mice during infection with low or high doses of bacteria. No significant differences were noted in the dynamics or levels of expression of genes encoding key inflammatory mediators for the immune response against *Mtb* infection with low doses (Fig. 2a). Similarly, no differences were found between non-infected *Gcnt1*^{-/-} or C57BL/6 mice (Fig. 2a). Upon infection with high doses of *Mtb*, we noted an overall higher expression of cytokines in *Gcnt1*^{-/-} animals, as compared to C57BL/6 ones (Fig. 2b). In all, the lung pathology observed in infected *Gcnt1*^{-/-} mice does not seem to result from an uncontrolled cytokine storm.

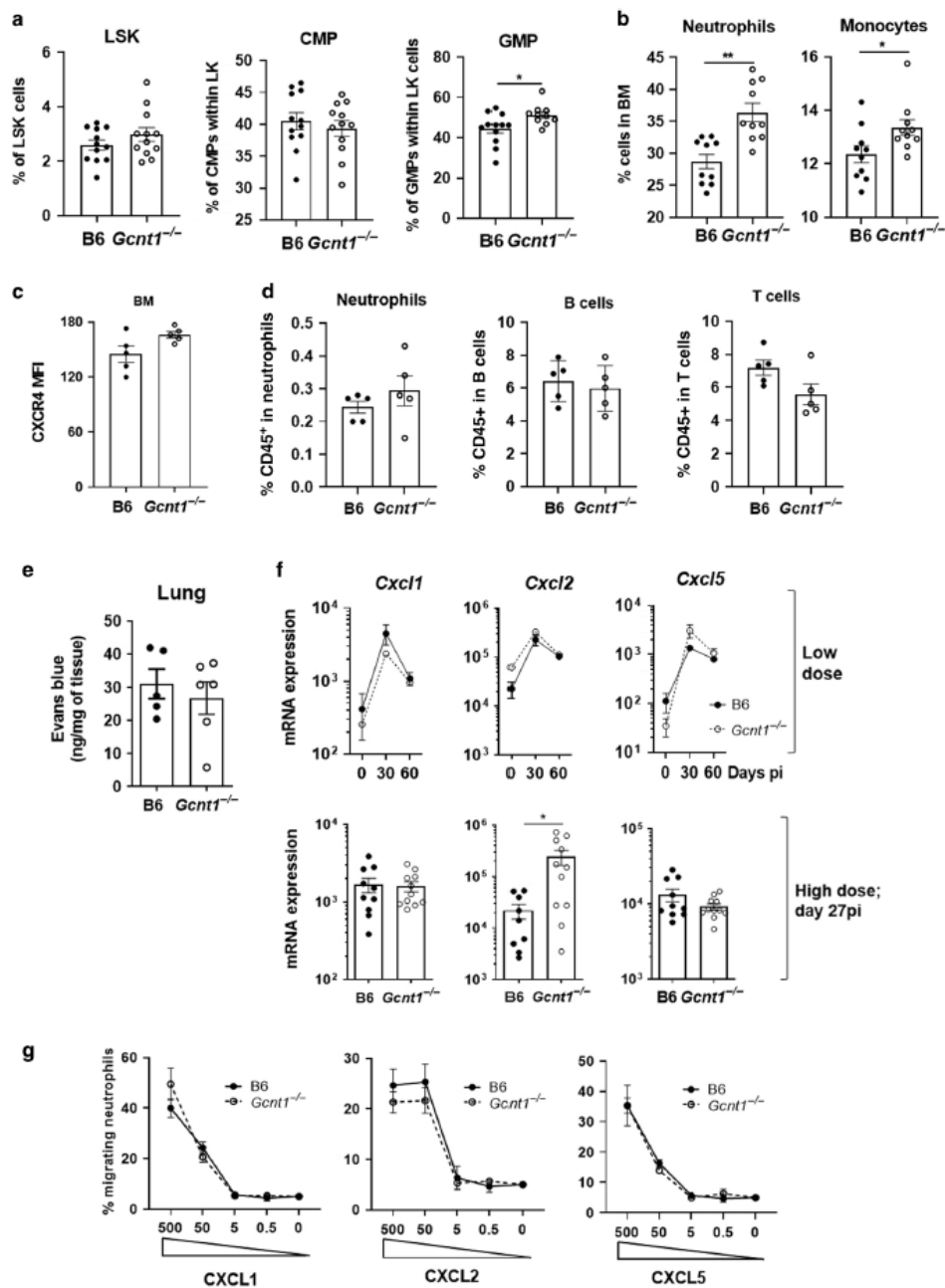
The dynamics of recruitment of myeloid and lymphoid cells to the lung was identical in *Gcnt1*^{-/-} and C57BL/6 mice infected with a low dose of bacteria, but 30 days after infection all tested immune cell populations were significantly higher in the lung of *Gcnt1*^{-/-} as compared to C57BL/6 mice (Fig. 2c and Fig. S2A, B). Moreover, increased numbers of lung neutrophils were detected in *Gcnt1*^{-/-} mice, independently of the infection (Fig. 2c). Because lung infiltrating neutrophils have been associated with increased bacterial burdens, pathology and TB exacerbation,^{1–4} we assessed the contribution of neutrophils to the increased susceptibility of *Gcnt1*^{-/-}. For that, we investigated the distribution of neutrophils in the lungs of C57BL/6 or *Gcnt1*^{-/-} mice infected with low or high doses of bacteria, through detection of myeloperoxidase (MPO) expression by immunostaining. Although neutrophils were present in both mouse strains upon infection, these cells accumulated in the case of infected *Gcnt1*^{-/-} mice (Fig. 2d), in line with the cytometry data. An accumulation of neutrophils in the areas of lesion was particularly observed in infections with high doses of *Mtb*, being more exacerbated in *Gcnt1*^{-/-} mice (Fig. 2d). A similar accumulation was found in the necrotic lesions of moribund *Gcnt1*^{-/-} mice infected with low doses of bacteria (Fig. S2C). Moreover, and in line with the higher presence of neutrophils in the lungs of *Gcnt1*^{-/-} mice infected with high doses of *Mtb*, the expression levels of genes previously associated with a neutrophil-dominated transcriptional module²¹ were increased in these animals, as compared to C57BL/6 ones (Fig. 2e). We next treated

Mtb-infected *Gcnt1*^{-/-} mice with a neutrophil-depleting antibody²² or with an isotype control (Fig. S2D). To assess mouse survival as the readout of this experiment, a high dose of infection was performed. Whereas 80% (*n* = 4) of isotype control-treated *Gcnt1*^{-/-} mice died between 25–35 days post-infection, only 20% (*n* = 1) of those treated with the Ly6G depleting antibody did not survive and all C57BL/6 mice survived infection (Fig. 2f). Therefore, depleting neutrophils largely rescued the increased susceptibility of *Gcnt1*^{-/-} mice to *Mtb* infection. Finally, we compared the response of neutrophils isolated from mice of either genetic background to *in vitro* *Mtb* infection. Although no statistically significant differences were found, we noted an overall enhanced cytokine expression by *Gcnt1*^{-/-} neutrophils upon *Mtb* infection (Fig. S2E), but similar production of reactive oxygen and nitrite species (Fig. S2F). In all, these findings suggest that *Gcnt1* abrogation did not alter the basic functional response of neutrophils to *Mtb*.

Gcnt1 activity modulates granulopoiesis and lung neutrophil recruitment

We next investigated the causes underlying *Gcnt1*^{-/-} mice neutrophilia. Blood neutrophilia was reported for healthy *Gcnt1*^{-/-} mice.¹¹ We here confirmed this finding and further showed its maintenance throughout infection (Fig. S3A,B). To test whether lack of *Gcnt1* may favour granulopoiesis, we analysed the frequency of Lin⁻Sca1⁺cKit⁺ cells (LSK), common myeloid precursors (CMP), granulocytic-monocytic precursors (GMP) and mature cell populations in the bone marrow (BM) of C57BL/6 or *Gcnt1*^{-/-} mice (Fig. S3C,D). No differences were seen in the frequencies of LSK and CMP progenitor cell populations, but an increased frequency of GMPs was noted (Fig. 3a). In line with this increase in GMP, the frequencies of neutrophils and monocytes were higher in the BM of *Gcnt1*^{-/-} mice (Fig. 3b). As for lymphoid populations, a decreased frequency of B cells and a similar frequency of T cells were observed in *Gcnt1*^{-/-} BM, as compared with C57BL/6 ones (Fig. S3E). Thus, the BM and blood neutrophilia observed in *Gcnt1*^{-/-} mice may at least in part result from enhanced BM granulopoiesis.

We next investigated the impact of *Gcnt1* deficiency on the mobilization of the neutrophils between BM and blood. As CXCR4 is a key regulator of neutrophil retention versus egress from the BM,^{23,24} we compared the expression of this receptor in BM neutrophils of each mouse strain. The levels of CXCR4 expressed in BM neutrophils were comparable in C57BL/6 and *Gcnt1*^{-/-} mice (Fig. 3c). Furthermore, to investigate for differences in the egress of neutrophils from the BM in *Gcnt1*^{-/-} mice, we intravenously injected an antibody directed to CD45 and assessed the percentage of CD45⁺ BM sinusoidal cells in either mouse strain. No differences were found in the frequency of CD45 labelled neutrophils, B cells and T cells when comparing the two mouse strains (Fig. 3d). Together, these data suggest that *Gcnt1* deficiency does not alter the hematopoietic cell retention in the BM or egress from the BM to the blood.



We then sought to understand the cause for increased lung neutrophil frequencies in *Gcnt1*^{-/-} mice. We started by testing the permeability of the lung vasculature by measuring the influx of Evans blue injected intravenously. Abrogation of *Gcnt1* did not impact the diffusion of dye into the lung (Fig. 3e), indicating a

similar permeability of the lung vasculature in *Gcnt1*^{-/-} and C57BL/6 mice. Next, we questioned whether the lung neutrophilia of *Gcnt1*^{-/-} mice depended on altered neutrophil chemoattraction. For this, we measured the expression levels of lung *Cxcl1*, *Cxcl2* and *Cxcl5* during infection with low or high doses of *Mtb* (Fig. 3f).

Fig. 3 *Gcnt1* modulates granulopoiesis and CXCL2 expression. The BM of non-infected C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) mice was harvested and the frequency of a LSK, CMP and GMP populations; **b** neutrophils and monocytes or **c** neutrophils expressing CXCR4 determined by flow cytometry. All gating strategies are shown in Fig. S3. **d** Egress of immune cell populations from the BM of in C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) measured as the percentage of CD45⁺ BM sinusoidal cells upon intravenous injection of CD45-PE. **e** Permeability of the lung vasculature in C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) measured through quantification of the amount of Evans Blue extravasated into the tissue upon intravenous injection. **f** The expression of *Cxcl1*, *Cxcl2* and *Cxcl5* in the lungs of mice infected with low or high doses of *Mtb* was determined by real-time PCR, for the indicated timepoints post-infection. **g** Analysis of neutrophils migration through 5 µm transwells towards different concentrations of recombinant CXCL1, CXCL2 and CXCL5. Mean ± SEM of culture triplicates are presented. In **a**, **f** each dot represents a mouse and the Mean ± SEM for 6–15 animals in at least two independent experiments are plotted. Statistical analysis was performed with Student's *t*-test (**a–f**) or unpaired two-tailed Mann-Whitney test for each time point (**g**). * refer to statistical differences between B6 or *Gcnt1*^{-/-} mice. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

Although the transcription of these chemokines in lungs of C57BL/6 or *Gcnt1*^{-/-} mice was similar upon a low dose of infection, the expression of *Cxcl2* was higher in *Gcnt1*^{-/-} deficient mice than in C57BL/6 upon a high dose of infection (Fig. 3f). Finally, we tested the ability of neutrophils from either genetic background to migrate in response to each of these chemokines, using an *in vitro* trans-well system. Independently of the expression of *Gcnt1*, the migration of neutrophils towards CXCL1, CXCL2 or CXCL5 was dose dependent and similar (Fig. 3g). Collectively, these findings support a higher availability of neutrophils in the absence of *Gcnt1*, accompanied by an increased recruitment of neutrophils to the lung of *Gcnt1*^{-/-} infected mice, possibly mediated by the higher expression of CXCL2.

Increased susceptibility to *Mtb* infection requires *Gcnt1* deficiency in both hematopoietic and non-hematopoietic compartments. To investigate if the susceptibility of *Gcnt1*^{-/-} mice to *Mtb* infection was fully dependent on the hematopoietic compartment, we resorted to a mouse BM transplantation model (Fig. 4a and Fig. S4A). When comparing all experimental groups, we observed that both the *Gcnt1*^{-/-}→C57BL/6 and the *Gcnt1*^{-/-}→*Gcnt1*^{-/-} chimeric animals presented significantly higher neutrophils in circulation upon reconstitution and before infection (Fig. 4b), suggesting that hematopoietic deficiency of *Gcnt1* is sufficient to promote neutrophilia. Chimeric mice were then infected with a low dose of *Mtb*. Mice in the *Gcnt1*^{-/-}→*Gcnt1*^{-/-} group presented a different survival curve compared to all other groups, where one animal (representing 14.3%) succumbed to infection after 24 days (Fig. 4c), and also higher lung bacterial burdens 30 days post-infection than any other group (Fig. 4d), paralleling the increased susceptibility to infection of *Gcnt1*^{-/-} mice. Histopathological analysis of the infected chimeric animals showed that although *Gcnt1*^{-/-}→C57BL/6 mice presented edema and larger necrotic areas when compared to the C57BL/6→C57BL/6 group, *Gcnt1*^{-/-}→*Gcnt1*^{-/-} animals presented the most exuberant pathology with severe inflammation, edema, vast necrosis and bronchopneumonia (Fig. 4e and Fig. S4B). Lung flow cytometry analysis of the infected chimeric mice revealed an overall higher frequency of neutrophils in the lungs of *Gcnt1*^{-/-}→*Gcnt1*^{-/-} chimeric mice (Fig. 4f) and similar frequencies of all other cell populations (Fig. S4C). Interestingly, in line with what we had observed in *Gcnt1*^{-/-} infected mice, the expression of *Cxcl2* upon infection was exclusively increased in *Gcnt1*^{-/-}→*Gcnt1*^{-/-} chimeric mice (Fig. 4g).

Thus, although *Gcnt1* deficiency in the hematopoietic compartment is sufficient to deregulate blood neutrophil frequencies in homeostasis, for that to translate into different susceptibilities to *Mtb* infection, deficiency of *Gcnt1* in the stromal compartment is also required.

Mtb infection modulates the host glycosylation machinery at the gene transcription level. Our findings highlight the importance of a competent core 2 O-glycan pathway in regulating neutrophils and immune pathology during TB. Core-2 O-glycans are major carriers of terminal

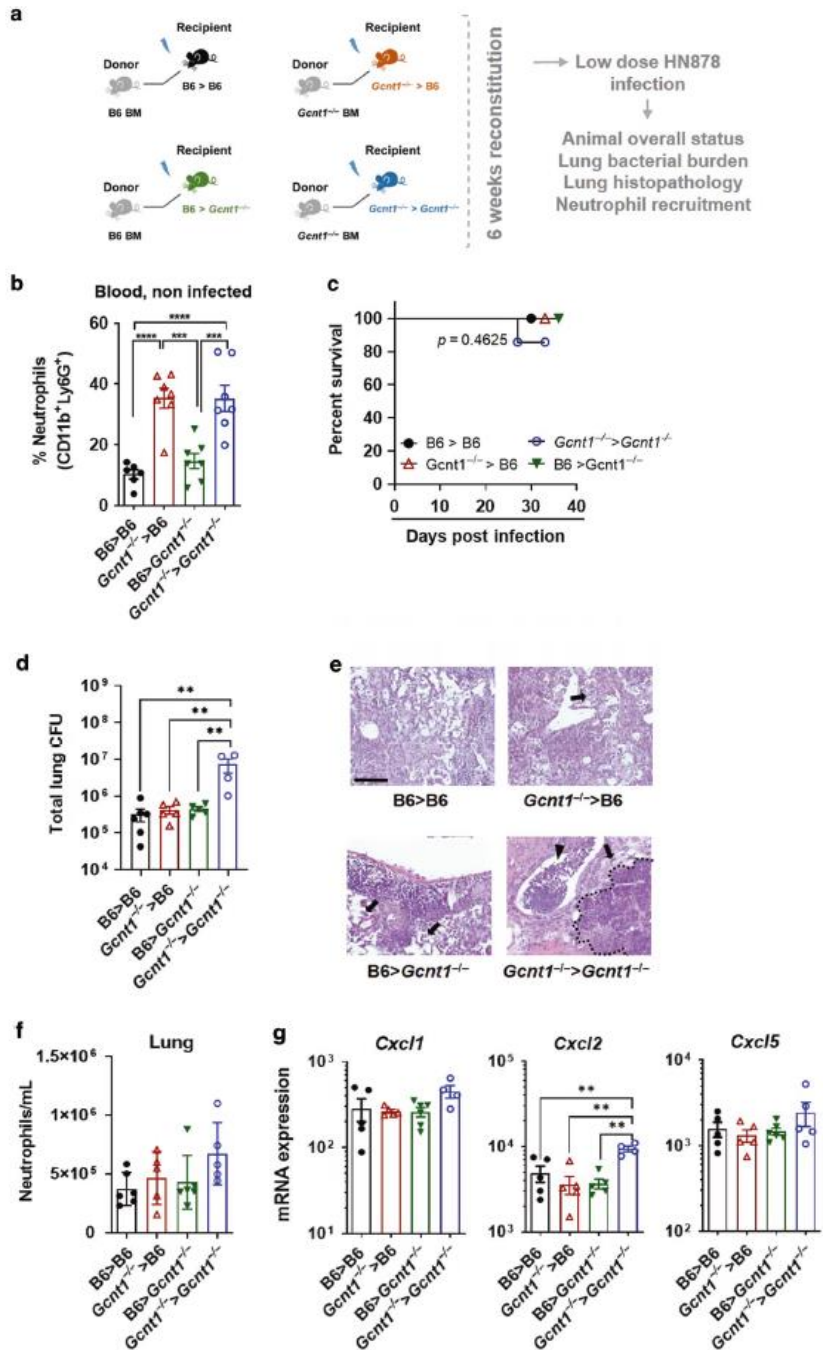
Lewis antigens and, among these, sLe^x is described to decorate the surface of human neutrophils (Fig. 5a). Because a neutrophil-driven signature was observed in the whole blood of TB patients,²⁵ and since our data link *Gcnt1* activity with TB susceptibility through a mechanism involving neutrophils, we next interrogated available human whole blood TB RNA-Seq datasets²⁶ for transcriptional alterations in glycosyltransferase-encoding genes (Table S1). Several glycosyltransferase-encoding genes were significantly up- or downregulated in active TB patients as compared to control or latently infected individuals (Fig. 5b). In particular, significant alterations in genes encoding enzymes of the sLe^x biosynthetic pathway were detected (Fig. 5b). Importantly, a much lower level of variation was observed when latent and controls were compared (Fig. 5b), thus showing that TB disease impacts the glycosyltransferase transcriptomic signature. Lastly, we examined lungs of TB patients for the presence of sLe^x antigens. TB patients (Table 2) were enrolled in a study in Georgia and underwent lung surgery as a therapeutic option, after antibiotherapy failure.²⁷ Independently of the patient's gender and *Mtb* drug susceptibility profile, lung sLe^x expression was detected in epithelial and immune cells (Fig. 5c and Fig. S5).

Altogether, we provide, for the first time, evidence for the modulation of core 2-sLe^x antigens during human TB, through the transcriptional regulation of various glycosyltransferase-encoding genes.

DISCUSSION

Despite the growing significance of glycosylation in immunity,^{5,28} its impact in TB is poorly understood. To address this hitherto unexplored question, we studied the outcome of *Mtb* infection in mice lacking *Gcnt1*, a key glycosyltransferase controlling core 2 O-glycans biosynthesis. *Gcnt1* deficiency impacted bacterial burdens, lung pathology and mouse survival. It also associated with exacerbated BM, blood and lung neutrophilia, already present in naive animals. In keeping with the immune pathological role of neutrophils in TB,^{1–4} the accumulation of these cells was strongly associated with lung necrotic lesions and it was markedly increased in *Gcnt1*^{-/-} infected mice. Further implicating neutrophilia with susceptibility of *Gcnt1*^{-/-} mice to *Mtb* infection, depleting neutrophils from *Gcnt1*^{-/-} mice largely rescued their susceptible phenotype. Interestingly, *Fut4*^{-/-} mice present normal neutrophil counts²⁹ and no phenotype upon *Mtb* infection,^{19,20} whereas *Fut7*^{-/-} mice exhibit blood neutrophilia³⁰ and increased susceptibility to *Mtb* infection.^{19,20} Moreover, our preliminary data show that *St3gal4*^{-/-} mice present normal neutrophil counts in homeostasis and respond to *Mtb* infection similar to C57BL/6 mice.

Blood neutrophilia has been described in *Gcnt1*^{-/-} mice,¹¹ but the underlying mechanisms remain elusive. Here, we show that hematopoietic abrogation of *Gcnt1* was sufficient to cause blood neutrophilia, which may result from enhanced BM granulopoiesis. We have excluded other possible regulatory mechanisms, including a direct modulation of CXCR4 receptor levels in *Gcnt1*^{-/-} neutrophils or a differential egress of immune cells from the BM to



the blood. We also report an increase in lung neutrophil counts in *Gcnt1*^{-/-} mice, which may seem counterintuitive, as absence of *Gcnt1* associates with a partial deficiency of selectin ligands.¹¹ However, the recruitment of neutrophils to the lung may follow an

atypical selectin-independent cascade,^{9,31} as shown during *Streptococcus pneumoniae* infection.³² Also, *Gcnt1* activity has been demonstrated to be dispensable for lung neutrophil recruitment upon LPS stimulation.³³ We show that lung neutrophilia in *Gcnt1*^{-/-}

Fig. 4 Blood neutrophilia of *Gcnt1*^{-/-} mice is promoted by deficiency of this enzyme in hematopoietic cells, but increased susceptibility to *Mtb* infection also requires the non-hematopoietic compartment. **a** Schematic representation of the BM transplantation model used and the experimental groups included. **b** The frequency of neutrophils in the blood of non-infected chimeric mice was determined by flow cytometry, following the gating strategy shown in Fig. S3. **c** Mice in the different chimeric groups were infected with a low dose of *Mtb* strain HN878 and the weight of the animals monitored to determine survival curves. **d** On day 30 post-infection, the bacterial burden in the lungs of the infected mice was determined by CFU enumeration. **e** H&E staining of one representative animal of each experimental group is represented. Black arrows point to edema, black arrowhead spot bronchopneumonia, and dashed black line limits the necrotic areas. Scale bar corresponds to 100 µm. **f** The number of neutrophils present in infected lungs was determined on day 30 post-infection, by flow cytometry. **g** The expression of *Cxcl1*, *Cxcl2* and *Cxcl5* in the lungs of infected mice was determined by real-time PCR, as above. **b, d, f, g** Represented is Mean±SEM, and each symbol represents one mouse. Statistical analysis was performed with a one-way ANOVA using Tukey's test for multiple comparisons (**b, d, f, g**) or with log-rank (Mantel-Cox) test for the Kaplan Meyer curve (**c**). * refer to statistic differences between the indicated chimeric groups. ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

mice involved both hematopoietic and stromal compartments. Whereas the contribution of the hematopoietic deficiency may be related to the increased number of available circulating neutrophils, that of the stroma does not relate with alterations in the permeability of the lung vasculature and may result from an upregulated *Cxcl2* expression. Interestingly, it does not rely on differential expression of *Cxcl5*, a chemokine produced by epithelial cells that drives neutrophil recruitment, contributing to more severe forms of TB.³⁴ In the same context, *Gcnt1*^{-/-} neutrophils showed migratory profiles towards CXCL1, CXCL2 and CXCL5 similar to those of C57BL/6 neutrophils.

Neutrophils are widely described as detrimental in TB,^{1–4} and an interferon-inducible neutrophil-driven blood transcriptional signature was defined in human TB.²⁵ Interestingly, human neutrophils are decorated with sialylated antigens, most notably sLe^x, which biosynthetic pathway involves *Gcnt1* enzymatic activity.⁷ We found alterations in the expression of several glycosyltransferases in whole blood of active TB patients as compared to controls. Furthermore, we detected sLe^x immunostaining in lung sections of TB patients. Of note, previous reports showed that the urinary levels of sialic acid, the terminal glycan of sLe^x structure, discriminate patients with active TB from healthy controls or patients with non-tuberculous pulmonary diseases.³⁵ Also, the regulation of the gene encoding CMAS, the enzyme that provides the substrate for the addition of sialic acid, specifically changed with infection with *Mtb*.³⁶ Thus, modulation of sialylated antigens seems to occur in TB, as described in several pathologies, such as other infectious diseases and cancer.^{6,16,17,37}

Collectively, this study further strengthens the role of glycosylation during immune responses and reveals that regulation of neutrophil homeostasis by host glycosyltransferases impacts TB outcome, a disease connected with high mortality and morbidity. Our findings raise awareness for the possible detrimental impact of targeting glycan dependent interactions, as envisaged for example in anticancer therapies,³⁸ in the context of TB. Additionally, a better understanding of glycans and their functional roles during chronic infection, provides increasing opportunities for discovery and therapeutic intervention in settings like TB.

MATERIAL AND METHODS

Ethics statement

The lung samples study was reviewed and approved by the Ethics Committees of the National Center for Tuberculosis and Lung Diseases of Georgia (IRB00007705-NCTLD-Georgia#1, IORG0006411) and of the Germans Trias i Pujol Hospital (EC:PI-16–171). The blood samples study was reviewed and approved by the Portuguese Comissão de Ética para a Saúde da ARS Norte (#T792). All participants signed informed consents, and all data were anonymized.

Animal experiments followed the 2010/63/EU Directive, and were approved by the I3S Animal Ethics Committee and the Portuguese National Authority for Animal Health (#014811/2016-07-13) or by the Animal Experimentation Ethics Committee of the

Hospital Universitari Germans Trias i Pujol (#B9900005) and the Dept d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Government.

Animals

C57BL/6 wild-type or *Gcnt1*^{-/-} mice were bred and housed at i3S and infected under ABSL3 conditions. The *Gcnt1*^{-/-} mouse was obtained from the Consortium for Functional Glycomics.¹¹ Mice were euthanized with isoflurane or CO₂ inhalation with efforts to minimize suffering. Food and water were *ad libitum*.

Bacteria growth and quantification

Mtb strain HN878 was grown and stored as before.³⁹ Viable bacteria was determined by serial dilution and colony forming unit (CFU) enumeration after 21–28 days of incubation at 37 °C in 7H11 agar plates. Bacterial quantification in infected lungs followed a similar protocol.

Experimental infection and animal monitoring

C57BL/6 and *Gcnt1*^{-/-} (8–12 week old) mice were aerosol-infected with *Mtb* strain HN878 using an inhalation exposure system (Glas-Col), as published.^{39,40} Low and high dose infections delivered <200 or >350 bacteria to the lung, respectively, as determined 3 days post-infection. Infected mice were weighed every week or every two days, and humanely euthanized if 20% of their weight or responsiveness to physical stimulation were lost. Whenever possible, the lungs of moribund animals were harvested for histology assessment.

Organ processing

Lungs were aseptically excised and processed as before.^{39,40} Single-cell suspensions were used for bacterial burden determination, flow cytometry and RNA analysis.

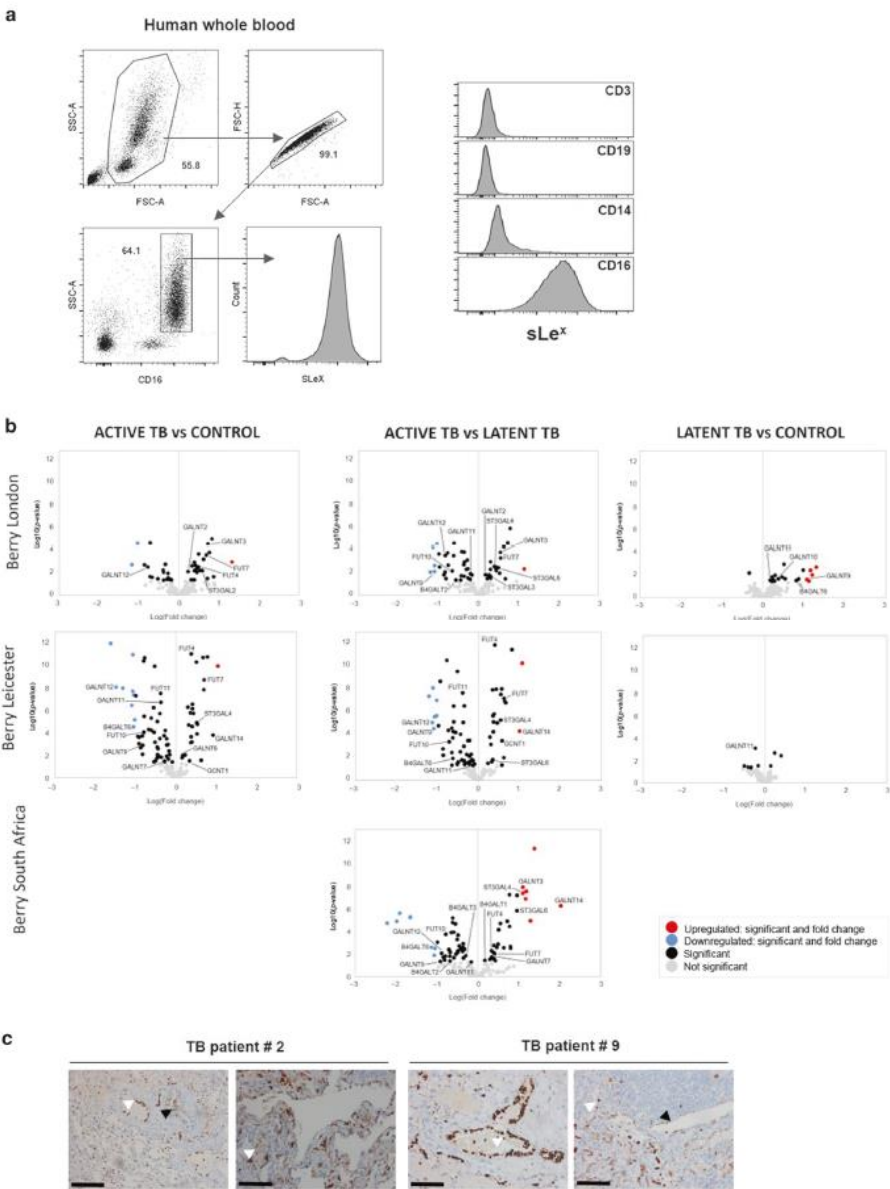
Tissue samples, histology and morphometric analysis

The human lung samples (Table 2) are from the collection obtained within the SH-TBL project, led by the Experimental Tuberculosis Unit (UTE) and conducted in collaboration with the NCTLD; and registered at the ClinicalTrials.gov database under code NCT02715271.

Mouse lungs were fixed in 10% buffered-formalin and paraffin-embedded. Serial sections of 3 µm-thickness were haematoxylin-eosin (H&E) or Ziehl-Neelsen stained or used for immunofluorescence (MPO) analysis.³⁹ Morphometric quantification of lung histology images is detailed in Fig. S1. Pathological scoring analysis of H&E-stained histological sections was performed by a veterinary pathologist blinded to the treatment groups. The histopathological features were scored using the following scale: 0 = absent, 1 = focal, 2 = multiple, 3 = severe, 4 = severe and extensive. The % of animals with a certain score within the indicated experimental group was calculated for group comparison.

MPO immunostaining

Lung sections were stained with a goat anti-mouse antibody against MPO (AF3667, R&D systems; 1:40) overnight at 4 °C



followed by a secondary donkey anti-goat IgG antibody conjugated with Alexa Fluor 568 (A11057, Invitrogen; 1:1000), and DAPI (Biollegend; 1:1000) for 2 h in the dark at RT. Glass coverslips were mounted onto the slides using Vectashield (Sigma). Slides were acquired using the IN Cell Analyzer 2000 (GE Healthcare) and analysed using IN Cell Developer software and ImageJ.^{41,42}

RNA extraction, cDNA synthesis and real-time PCR analysis
Total RNA was extracted from mouse lungs or in-vitro-infected neutrophils, using TRI-Reagent (Sigma), converted to cDNA (NE Biolabs), and subjected to real-time PCR using SYBR green (Applied Biosystems) and specific oligonucleotides (Table S2). Melting and standard curves and RQ values were determined for

Fig. 5 *Mtb* infection impacts the expression of several glycosyltransferase-encoding genes in humans. **a** Neutrophils are the main cells expressing sLe^x in human peripheral blood. Vein blood was collected and stained for CD3, CD14, CD16, CD19 and sLe^x by flow cytometry. Data shown represent one donor out of 25 analysed. **b** Volcano plots displaying the comparisons between Active TB vs Control, Active TB vs Latent TB and Latent TB vs Control regarding the differential expression of annotated coding genes of glycosyltransferases (obtained from Glyco-Enzyme Repository) on the Berry London, Berry Leicester (progressor) and Berry South Africa datasets.²⁶ Labelled genes encode for glycosyltransferases associated with the sLe^x pathway. Dot color indicates if the corresponding gene is upregulated (red, log-fold-change >1 and *p*-value < 0.05), downregulated (blue, log-fold-change < -1 and *p*-value < 0.05), significant (black, log-fold-change > -1 to < 1 and *p*-value < 0.05) and not significant (grey). **c** Lung sections of TB patients who underwent therapeutic surgery were stained for sLe^x as above. Black and white arrowheads point to positive epithelial or immune cells, respectively. Scale bars correspond to 100 µm.

Table 2. Overall characteristics of the participants originating lung samples.

Patient	Gender	Lesion localization ^a	Lesion size (mm)	TB symptoms	Drug sensitivity profile ^b
1	Male	RUL	21, 30	Yes	DS
2	Male	RUL	35	Yes	DS
3	Female	LLL	30	No	MDR
4	Female	LLL	30	No	DS
5	Female	RUL	30	No	DS
6	Female	RLL	42	No	MDR
7	Male	RUL	30, 21	No	MDR
8	Female	LUL	40	No	DS
9	Male	LUL	38	No	MDR
10	Male	LUL	29	No	MDR

All patients were antibiotic-treated before surgery. Highlighted in grey are the patients represented in Fig. 5.
RUL right upper lobe, LLL left lower lobe, RLL right lower lobe, LUL left upper lobe, DS drug sensitive, MDR multi-drug resistant.

each gene. Ubiquitin was used as a reference gene for normalization of target gene abundance.

Flow cytometry

Human or mouse blood, mouse BM and lung were stained for surface antigens (30 min; 4 °C) and fixed for 20 min in 2% paraformaldehyde-PBS after erythrocyte lysis. For the analysis of BM precursors, the mature lineage (Lin) was depleted (Miltenyi Biotec magnetic cell separation system), prior to Lin^{neg} staining with specific antibodies for progenitor markers. Dead cells were always excluded using a viability dye. Cells were acquired on a BD FACS Canto II. Data were analyzed using FlowJo version 10.1.r7. Gating strategies are in Fig. S2 and S3 and the antibodies used in Table S3.

Neutrophil depletion

In vivo depletion of neutrophils from infected *Gcnt1*^{-/-} mice was as described:²² 0.2 mg anti-Ly6G mAb (clone 1A8; BioXCell) or isotype control (clone GL117; R&D Systems) were administered i.p. from day 18 post-infection, every other day for 20 days. To control for the protocol, neutrophils in the lungs of mice treated with anti-Ly6G mAb or isotype control were detected at the end of the experiment by MPO staining (Fig. S2D).

Analysis of BM egress

Mice were intravenously injected with 0.6 µg of anti-mouse CD45 conjugated with Phycoerythrin (PE) (30-F11; Biolegend) in PBS, 2 min prior to euthanasia, to label BM sinusoidal cells.⁴³ Blood and BM cells were collected and analysed by flow cytometry.

Analysis of lung vasculature permeability

Vasculature permeability was assessed using Evans Blue dye as described previously.^{44,45} Mice were intravenously injected with

Evans Blue (1 mg per 30 g mouse, prepared in PBS). Thirty minutes after injection mice were sacrificed and lungs collected. After weight determination, samples were incubated in formamide for ~36 h at 55 °C. The formamide solution was recovered and absorbance was measured at 620 nm and 740 nm to determine the amount of Evans Blue present in the tissue. The presence of heme groups was corrected with the formula: $A_{620}(\text{corrected}) = A_{620} - (1.426 \times A_{740} + 0.030)$.

In vitro infection of neutrophils and chemoattraction assays

For BM neutrophils isolation, BM cells were stained with anti-mouse Ly6G (1A8; Biolegend) followed by purification using anti-biotin magnetic beads and LS columns of MACS cell separation system (both Miltenyi Biotec). For in vitro infection, 10⁵ neutrophils were seeded per well and infected with *Mtb* HN878 at MOI 2 or 10. After 3 h of infection, cells were recovered for RNA analysis or reactive oxygen species detection with DHE and DHR probes. Cells were incubated with DHE or DHR at 37 °C for 10 or 30 min, respectively. Samples were then acquired on a BD FACS Canto II to detect red-fluorescence (DHE) or green fluorescence (DHR) due to probes oxidation. For the chemotaxis assay, 10⁵ neutrophils were seeded on the upper chamber of the 5 µm-pore transwells (96 well plate; Corning) and allowed to migrate for 3 h towards the lower chamber containing media with CXCL1, CXCL2 and CXCL5 (all from Peprotech) at 500, 50, 5 and 0.5 ng/ml or without cytokines. As control for the amount of cells seeded, cells were also seeded in the lower chamber. Cells were then recovered and stained with SAV PE-Cy7 (Biolegend). To quantify the number of migrated cells, counting beads (Biolegend) were added to the cells prior to acquisition in a BD FACS Canto II. All cytometry data were analysed using FlowJo version 10.1.r7.

Generation of BM chimeric mice

Recipient mice were irradiated with doses of 750 rad and reconstituted with 5 × 10⁶ CD3-TCRβ-TCRγ/δ-depleted BM cells. Bactrim (80 mg sulfamethoxazol and 16 mg trimethoprim/250 mL of water, for an estimated dose of 50 mg antibiotic/Kg/day) was administered in drinking water for the first 3 weeks post-reconstitution. A control group, in which BM cells from CD45.1-C57BL/6 mice were transferred into irradiated CD45.2-*Gcnt1*^{-/-} mice, was included to monitor the efficiency of chimerism, 6 weeks post-transplantation (Supplemental Fig. 3A). Chimeras were infected at this stage.

RNA-seq data analysis

Raw paired-end RNA-seq data from whole-blood cohorts Berry London, Berry South Africa and Berry Leicester progressor²⁶ (GSE107995) were processed separately. The reads were quality controlled through FastQC [v0.11.7] (Babraham Bioinformatics) and MultiQC.⁴⁶ Filtering below 20 quality score and below 50 base-pairs, and adapters exclusion was performed using Trimmomatic [v0.38].⁴⁷ Filtered reads were aligned to the human reference genome GRCh38 using HISAT2 [v2.1.0].⁴⁸ StringTie [v1.3.4] was used for the assembly and raw counts matrix generated by prepDE.py.⁴⁹ Differentially expressed genes were analysed in R [v3.5.1] with Bioconductor packages edgeR^{50,51} and limma.⁵² Genes with less than 15 raw counts in all samples were

excluded. 202 genes encoding glycosyltransferases was extracted from the Glyco-Enzyme Repository (<http://glycoenzymes.crc.uga.edu/>). Differentially expressed genes were determined through linear model fitting. Empirical Bayes moderated *t*-statistics test was performed and genes with *p*-value < 0.05 and log2 fold-change < -1 or > 1 were considered significant.

Immunohistochemistry and image analysis

Human lung sections were immunolabeled for sLe^x as described,⁵³ with an antibody directed against CD15s (BD Pharmingen; CSLEX1) and ultraView DAB (Ventana Medical Systems).⁵⁴

Statistical analysis

Data were analysed using GraphPad Prism (version 8.1.0). Student's *t*-test was used to compare two groups and one-way ANOVA for more than two groups, with post-tests as in Figure legends. Data were checked for normality and log normality. Survival curves were analysed using Log-rank (Mantel-Cox) test. Significant differences are as follows: **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.001 and *****p* ≤ 0.0001.

ACKNOWLEDGEMENTS

The authors thank the excellent support from the I3S scientific platforms, namely Animal facility, Translational Cytometry, Advanced Light Microscopy and BioSciences Screening, member of the national infrastructure PPBI—Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122). This work was financed by grants POCI-01-0145-FEDER-028955 (to M.S.), POCI-01-0145-FEDER-028489 (to A.M.), and POCI-01-0145-FEDER-016585 (to C.A.R.) and by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-000012). C.A.R. acknowledges the Consortium for Functional Glycomics funded by NIH/NIH5-S1046Z1 R01. This study was funded by the Spanish Government-FEDER Funds through CP13/00174, CP118/00031 and PI16/01511 grants, and the CIBER Enfermedades Respiratorias Network; and by the Spanish Society of Pneumology and Thoracic Surgery (SEPAR) through grant 16/023 (to C.V.). K.L.F. and R.M. are funded by FCT PhD scholarships SFRH/BD/114405/2016 and SFRH/BD/131159/2017, respectively; A.M. is funded by FCT; M.S. is funded by FCT through *Estímulo Individual ao Emprego Científico*. The authors thank Anne O'Garra for insightful discussions.

AUTHOR CONTRIBUTIONS

K.L.F., A.R.M., R.M.: performed experiments, analysed data and wrote the manuscript. A.R.M., L.S.C.: analysed the RNA-Seq data. J.S., B.C., L.B., A.L.F., S.M., J.G., G.T., P.R.M., M.M.C.: performed experiments. R.R., S.V., S.G., K.N.: provided the human samples used in the study. R.A., F.G.: analysed the mouse histology samples. C.V.: coordinated the human study. R.A., P.R., C.V., C.A.R.: critically discussed the experimental design and data and wrote the manuscript. A.M., M.S.: coordinated the study, designed the experiments, analysed data and wrote the manuscript. All authors read and approved the manuscript.

ADDITIONAL INFORMATION

The online version of this article (<https://doi.org/10.1038/s41385-020-0277-7>) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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Supplementary Material

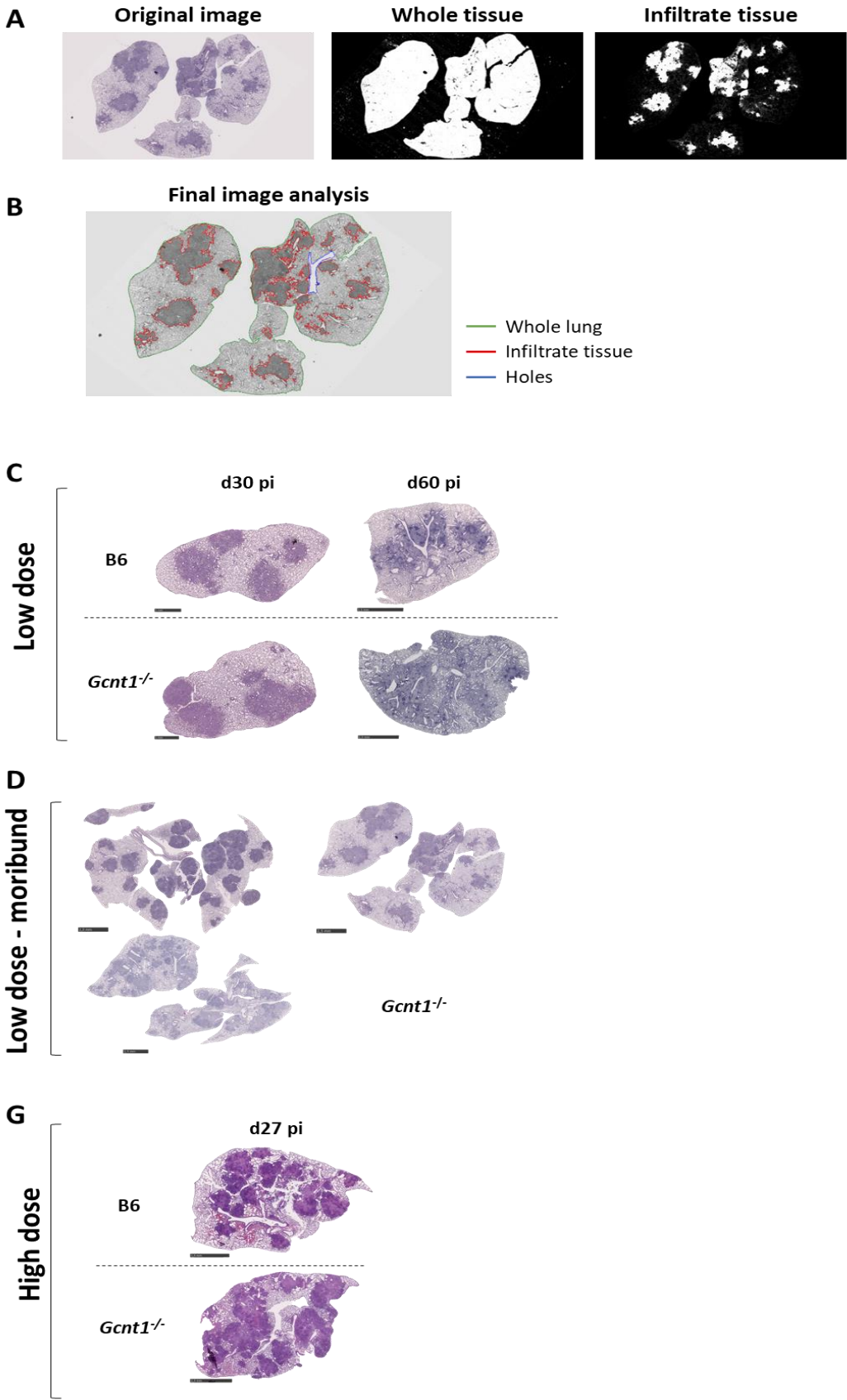


Figure S1

Supplemental Figure 1. Lung pathology in C57BL/6 vs *Gcnt1*^{-/-} mice upon aerosol infection with *M. tuberculosis*. The morphometric analysis was determined using ilastik: Interactive Learning and Segmentation Toolkit (www.ilastik.org/publications.html). (A) Probability maps were obtained for whole lung tissues and for lesion areas and (B) analysed using the CellProfiler Analyst software (version 3.1.5). Whole lung tissue (green line), lung infiltrates (red line) and lung holes (blue line) are identified. Areas were quantified and the percentage of infiltrates per lung calculated. (C, D, E) H&E staining of whole lung sections are shown for mice representative of each experimental group. Scale bar corresponds to 1 mm (for low dose d30pi) and 2.5 mm (for low dose d60pi) (C); 5 mm (low dose moribund) (D); and 2.5 mm (for high dose d27pi) (E).

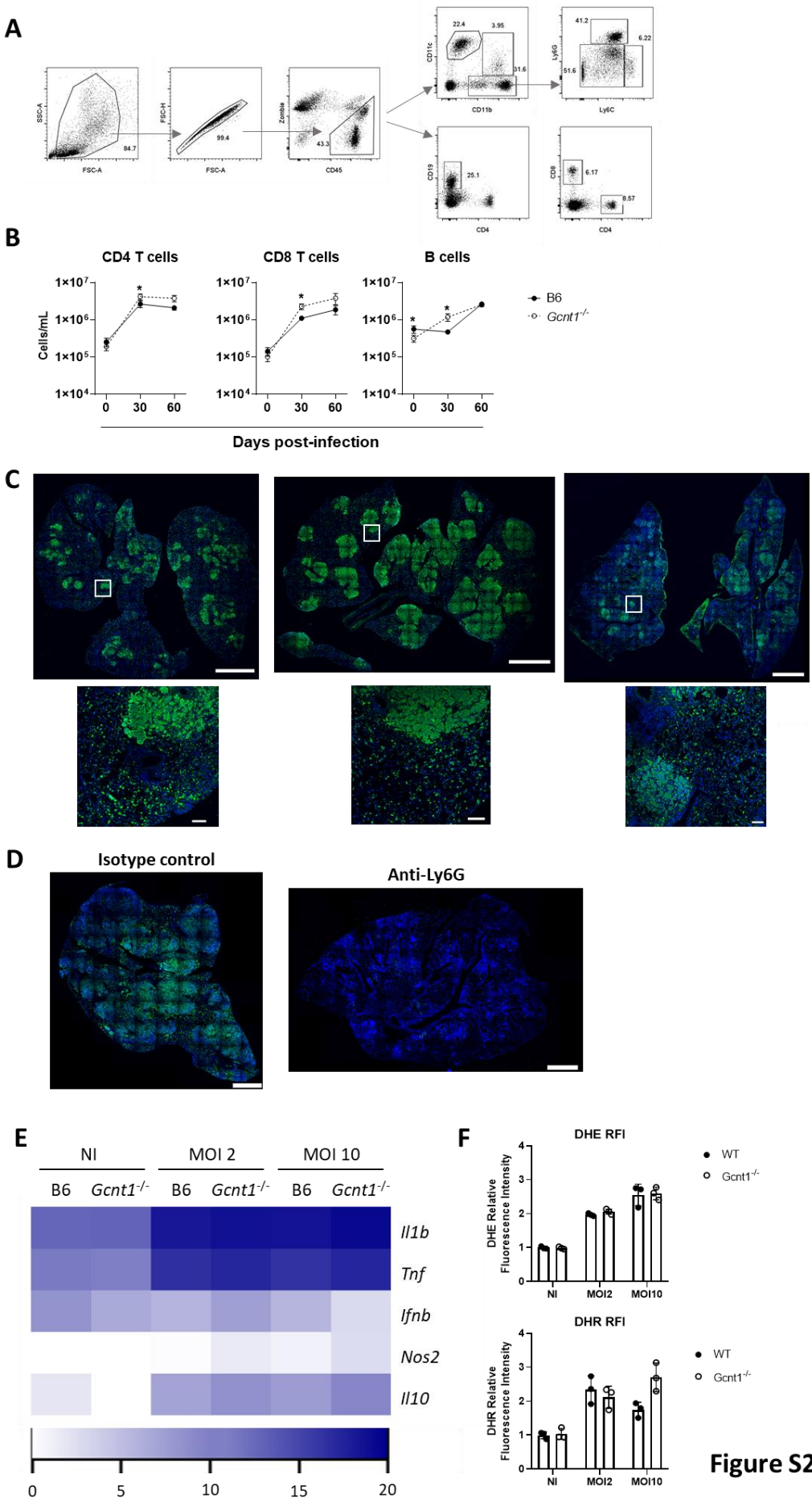


Figure S2

Supplemental Figure 2. (A) Gating strategies to detect immune cell populations in the lung of C57BL/6 and *Gcnt1*^{-/-} mice. (B) At the indicated time-points post-infection, the lungs of C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) mice infected by aerosol with a low dose of *Mtb* strain HN878 were harvested, and a cellular suspension prepared. The indicated immune cell populations were determined by flow cytometry. Each dot represents the Mean $\pm$ SEM for 10 animals in 2 independent experiments. Non-infected animals (day 0) were used as controls. Statistical analysis was performed with unpaired two-tailed Mann-Whitney test for each time point. * refer to statistic differences between B6 or *Gcnt1*^{-/-} mice. *, $p < 0.05$. (C) Immunofluorescence detection of MPO (Vinall LE) in the lungs of *Gcnt1*^{-/-} moribund mice. Sections were counterstained with DAPI (blue). Scale bars correspond to 2.5 mm on whole lung images and 100 μ m on close ups. (D) Representative images of lung sections of infected *Gcnt1*^{-/-} mice that have received isotype control (left) or anti-Ly6G (right) stained with MPO and DAPI. Scale bars correspond to 1 mm. (E, F) Neutrophils were purified from the BM of C57BL/6 (B6) or *Gcnt1*^{-/-} animals and infected in vitro with *Mtb* HN878 at a moi of 2 or 10 as indicated. Four h post-infection, (E) the RNA of infected cells was harvested, converted to cDNA and the expression of the indicated genes measured by real-time PCR. Represented is a heatmap of log₂ relative expression of depicted genes. (F) Detection of reactive oxygen species (ROS) production by neutrophil using dihydroethidium (Aspholm M) and Dihydrorhodamine 123 (Bhella et al.) probes. Represented are the Mean $\pm$ SEM. Each dot represents one well of the experiment.

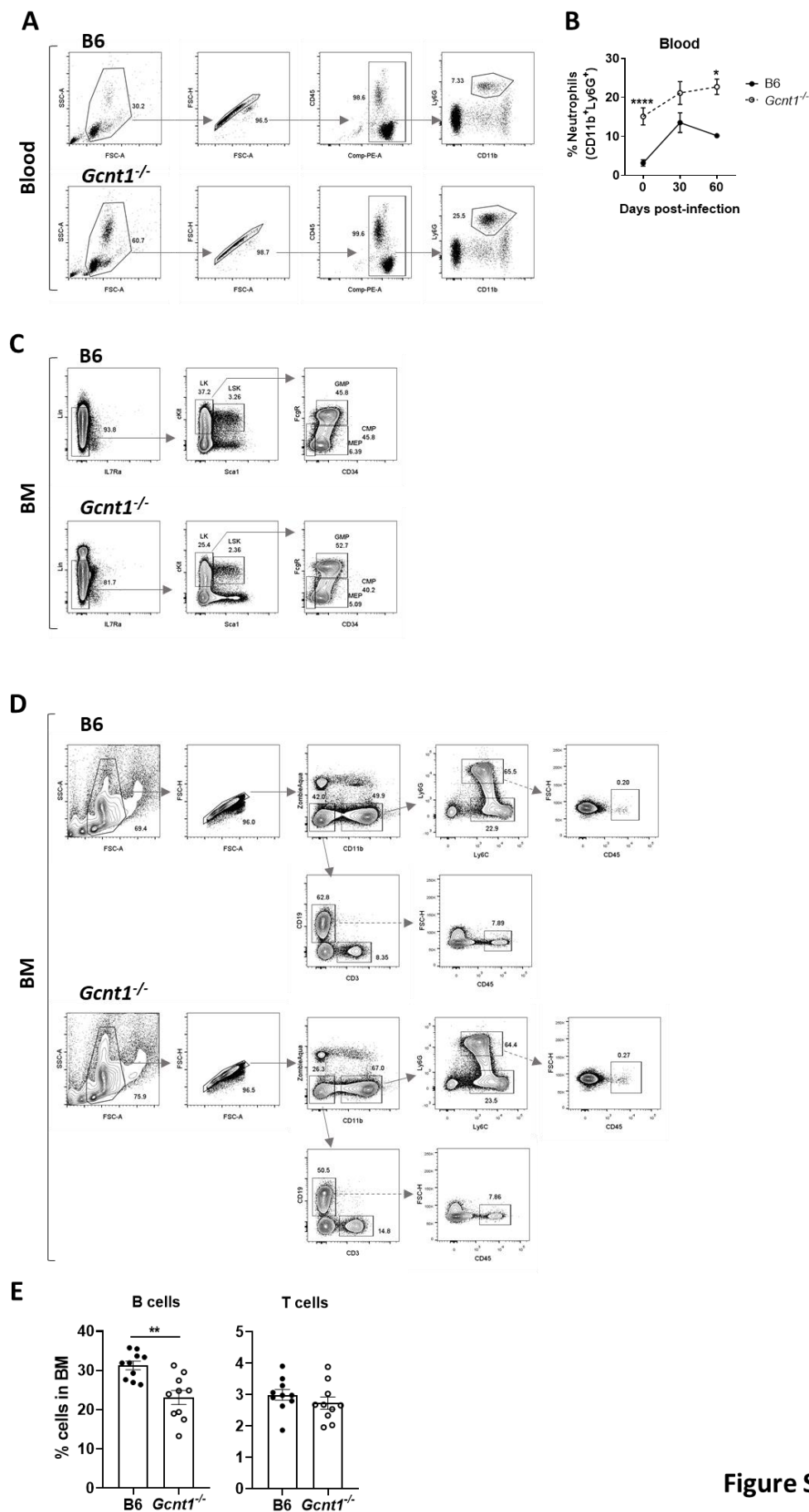
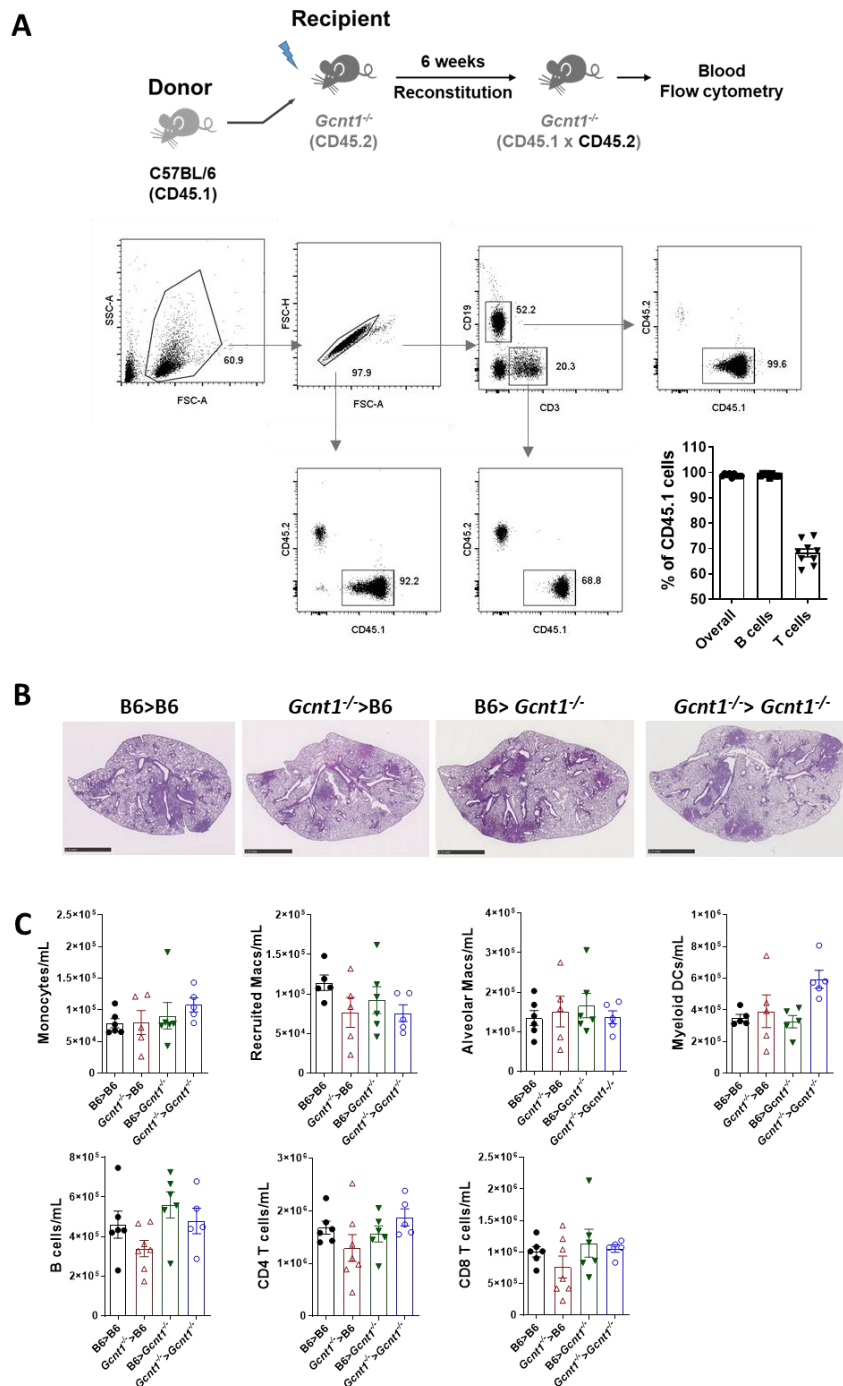


Figure S3

Supplemental Figure 3. (A, C, D) Gating strategies to detect immune cell populations in the blood and BM of C57BL/6 and *Gcnt1*^{-/-} mice. (B) At the indicated time-points post-infection, the blood of C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) mice infected by aerosol with a low dose of Mtb strain HN878 was harvested and the frequency of neutrophils determined by flow cytometry. Non-infected mice were used as controls on day 0. (E) Frequencies of B and T cells present in the BM of uninfected C57BL/6 (B6; black circles) or *Gcnt1*^{-/-} (open circles) mice were determined by flow cytometry. (B, E) Represented are the Mean±SEM. Each dot represents a mouse. Statistical analysis was performed with unpaired two-tailed Mann-Whitney test for each time point (B) or Student's t-test (E). * refer to statistic differences between B6 or *Gcnt1*^{-/-} mice. *, p<0.05; **, p<0.01; ****, p<0.0001.



Supplemental Figure 4. Global characterization of the cellular content of BM reconstituted mice, pre- and post-infection with *M. tuberculosis*. (A) A control group was included to assess the efficiency of the BM reconstitution model through flow cytometry in whole blood analysis. (B) H&E staining of whole lung sections are shown for one mouse representative of each experimental group. Scale bar corresponds to 2.5 mm. (C) The indicated immune cell populations were determined by flow cytometry for each experimental group. (A, C) Represented are Mean±SEM for each group; individual dots refer to individual mice. Statistical analysis was performed as in Figure 2.

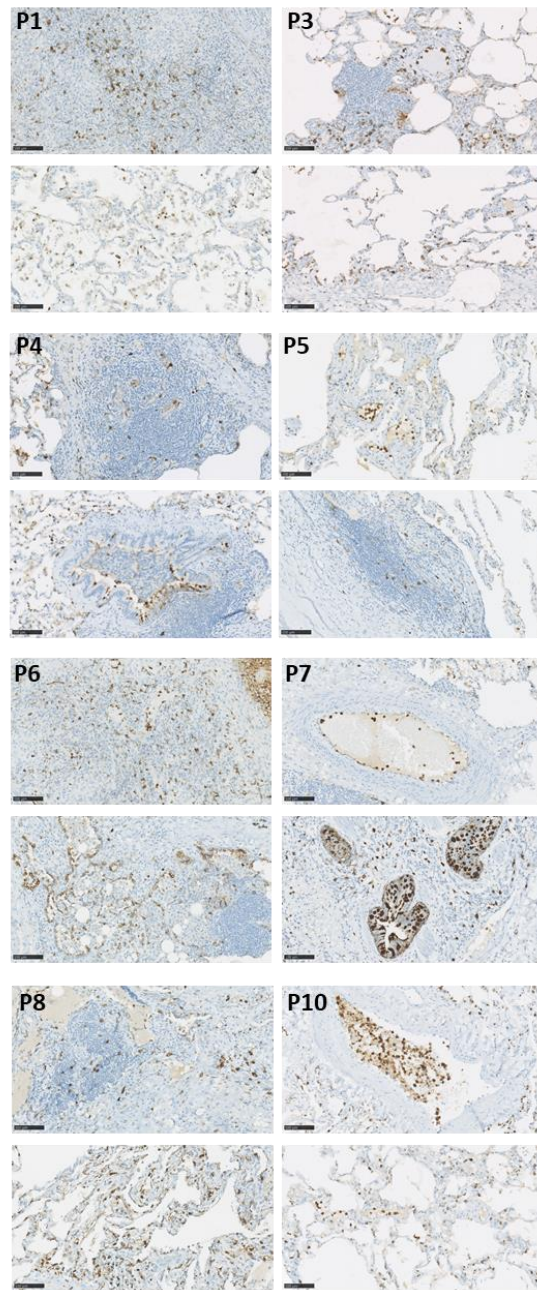


Figure S5

Supplemental Figure 5. sLex detection in lung sections of TB patients. Lung sections of TB patients who underwent therapeutic surgery were stained for sLex as in Figure 5. Scale bar corresponds to 100 μ m.

Table S1. Log fold change and significance values for differential expression of glycosyltransferase codon Leucine and Berry South Africa datasets (Singhania *et al.*, 2018) [#].

GENE NAME	BERRY LONDON								BERRY LEICESTER (progressors)								BERRY SOUTH AFRICA	
	ACTIVE vs CONTROL		ACTIVE vs LATENT		LATENT vs CONTROL		Multiple Testing		ACTIVE vs CONTROL		ACTIVE vs LATENT		LATENT vs CONTROL		Multiple Testing		ACTIVE vs LATENT	
	Log(Wang YK)	P-value	Log(Wang YK)	P-value	Log(Wang YK)	P-value	P-value	FDR	Log(Wang YK)	P-value	Log(Wang YK)	P-value	Log(Wang YK)	P-value	P-value	FDR	Log(Wang YK)	P-value
A4G ALT	0,57039	0,3102	0,35805	0,4541	0,21235	0,7044	0,005	0,6875	0,6256	0,02803	0,57112	0,0457	0,05450	0,8499	0,0507	0,08362	0,70089	0,21311
A4G NT																		
ABO	-0,21185	0,6194	-0,34962	0,3379	0,13778	0,7465	0,622	0,741	-0,0241	0,9177	-0,23454	0,3182	0,21042	0,3771	0,5525	0,61835	0,3078	0,62837
ALG 1	-0,05174	0,7376	-0,20705	0,1198	0,15531	0,655	0,276	0,4354	0,2278	0,0012	-0,25548	0,0003	0,02764	0,6954	0,0003	0,0113	-0,4200	0,00305
ALG 10	-0,21099	0,5251	-0,28090	0,3224	0,06991	0,8329	0,5937	0,7144	-0,3812	0,0794	-0,49217	0,0247	0,11091	0,6151	0,0600	0,09670	0,1095	0,80242
ALG 11	0,34716	0,0999	0,12256	0,4914	0,22460	0,2838	0,2552	0,4141	-0,2790	0,0375	-0,16007	0,2330	0,11893	0,3818	0,1118	0,16413	0,1613	0,32799
ALG 12	0,07734	0,4559	-0,14245	0,1105	0,21980	0,730	0,0843	0,2019	-0,0851	0,1574	-0,10045	0,0972	0,01535	0,8018	0,1976	0,6531	-0,2233	0,0294
ALG 13	-0,24349	0,0373	-0,17424	0,0789	-0,06925	0,5465	0,0727	0,1833	0,5506	0,0000	-0,35439	0,0010	0,19624	0,0700	0,0000	0,0001	0,1918	0,06613
ALG 14	0,29644	0,1920	0,04360	0,8206	0,34004	0,1354	0,2932	0,4534	0,2510	0,1697	0,32257	0,0797	0,07156	0,7002	0,1795	0,24473	0,4608	0,13651
ALG 2	0,36290	0,1167	-0,17607	0,3686	0,53898	0,0214	0,0690	0,1777	0,4561	0,0087	-0,29345	0,0914	0,16267	0,3546	0,0286	0,05102	-0,0267	0,84910
ALG 3	-0,10849	0,2244	-0,13669	0,0749	0,02820	0,7506	0,1792	0,3285	0,1559	0,0170	0,01403	0,8294	0,14191	0,0329	0,0328	0,05731	0,2939	0,01365
ALG 5***																		
ALG 6	0,01220	0,9420	-0,01123	0,9375	0,02343	0,8890	0,9900	0,9929	-0,1388	0,2912	-0,18949	0,1524	0,05065	0,7052	0,3287	0,40474	0,3850	0,02606
ALG 8	-0,18025	0,0939	0,02318	0,7981	-0,20343	0,0595	0,1392	0,2797	-0,0436	0,4744	-0,14382	0,0200	0,10016	0,1088	0,0595	0,09598	-0,2095	0,05970
ALG 9	-0,38475	0,0455	-0,62166	0,0202	0,23691	0,2131	0,0012	0,0126	-0,9415	0,0002	-0,51653	0,0399	0,42501	0,0949	0,0009	0,0260	1,0470	0,00303
B3G ALN T1	0,73749	0,1094	1,14617	0,0044	0,40868	0,3712	0,0157	0,0684	-0,0852	0,7244	-0,17320	0,4764	0,08794	0,7213	0,7754	0,81628	0,7647	0,13222
B3G ALN T2	-0,12022	0,6558	0,03000	0,8961	-0,15022	0,5778	0,8493	0,9013	-0,8716	0,0021	-0,33448	0,2358	0,53715	0,0614	0,0084	0,01750	-0,5961	0,15558

B3G ALT 1																		
B3G ALT 2	- 0,508 49	0, 24 65 9	- 0,814 26	0, 03 20 6	0,305 77	0, 48 42 7	0, 09 54 8	0, 21 87 3	- 0,9 142 4	0,0 009 2	- 0,866 22	0, 00 17 6	- 0,048 02	0, 86 20 4	0, 00 09 5	0,0 02 52	- 1,0 642 3	0,0 12 71
B3G ALT 4	0,385 56	0, 00 11 0	0,089 40	0, 35 32 9	0,296 16	0, 01 06 1	0, 00 40 3	0, 02 82 4	0,5 093 9	0,0 000 0	0,438 63	0, 00 00 0	0,070 76	0, 33 64 4	0, 00 00 0	0,0 00 00	- 0,0 076 9	0,9 38 35
B3G ALT 5																		
B3G ALT 6	- 0,023 12	0, 83 85 5	- 0,098 12	0, 31 28 3	0,075 00	0, 50 95 0	0, 57 95 3	0, 70 30 2	- 0,0 585 4	0,3 584 5	- 0,156 84	0, 01 52 1	0,098 30	0, 13 13 5	0, 04 98 2	0,0 82 25	- 0,3 719 4	0,0 05 67
B3G AT1	- 0,215 42	0, 58 62 1	- 0,263 92	0, 43 48 8	0,048 49	0, 90 23 5	0, 71 54 8	0, 80 83 7	- 0,7 869 0	0,0 000 0	- 0,626 73	0, 00 01 9	- 0,160 16	0, 33 68 0	0, 00 00 1	0,0 00 03	- 0,5 848 0	0,0 20 72
B3G AT2	0,225 01	0, 34 12 5	0,529 72	0, 01 04 6	- 0,304 71	0, 19 89 8	0, 03 60 7	0, 11 75 1	0,1 137 6	0,6 220 0	- 0,078 31	0, 73 56 1	- 0,035 45	0, 88 01 7	0, 87 95 0	0,9 03 55	0,2 272 1	0,4 71 74
B3G AT3	0,004 70	0, 96 73 0	- 0,208 00	0, 03 70 5	0,212 71	0, 06 77 2	0, 06 71 4	0, 17 46 4	0,0 674 1	0,3 492 3	- 0,003 01	0, 96 68 2	0,070 41	0, 33 75 9	0, 54 97 5	0,6 15 95	- 0,3 593 2	0,0 01 22
B3G LCT	- 0,120 21	0, 56 74 4	- 0,434 35	0, 01 80 1	0,314 14	0, 13 84 1	0, 05 28 1	0, 14 93 7	- 0,5 390 9	0,0 000 9	- 0,554 46	0, 00 00 6	0,015 38	0, 91 06 2	0, 00 00 3	0,0 00 11	- 0,2 531 1	0,4 34 59
B3G NT2	0,223 54	0, 07 97 4	0,241 91	0, 02 74 3	- 0,018 37	0, 88 39 0	0, 05 92 2	0, 16 09 3	0,4 430 4	0,0 026 8	- 0,227 78	0, 12 06 1	- 0,215 26	0, 14 79 1	0, 01 08 3	0,0 21 79	0,5 106 0	0,0 00 07
B3G NT3	- 0,219 72	0, 65 54 7	- 0,584 14	0, 16 75 3	0,364 41	0, 46 00 1	0, 37 76 1	0, 53 43 5	- 0,5 422 4	0,0 393 7	- 0,200 38	0, 44 60 7	- 0,341 86	0, 20 07 6	0, 11 46 6	0,1 67 64	- 0,8 241 7	0,1 00 67
B3G NT4	0,409 45	0, 49 43 3	0,646 40	0, 20 81 1	- 0,236 95	0, 69 20 6	0, 44 40 4	0, 59 18 6	0,1 099 1	0,7 046 6	0,282 93	0, 33 23 6	- 0,173 02	0, 55 85 4	0, 62 08 9	0,6 80 92	0,6 228 7	0,3 75 79
B3G NT5	0,363 12	0, 28 64 8	0,097 39	0, 73 62 8	0,265 72	0, 43 43 1	0, 55 88 7	0, 68 65 1	0,1 160 4	0,6 291 7	0,451 42	0, 06 30 4	- 0,335 38	0, 17 22 1	0, 15 78 7	0,2 19 60	1,2 840 9	0,0 00 01
B3G NT6																		
B3G NT7	- 0,487 48	0, 08 09 9	- 0,757 12	0, 00 20 4	0,269 64	0, 32 98 5	0, 00 75 9	0, 04 25 2	- 1,3 172 8	0,0 000 0	- 0,974 72	0, 00 00 2	- 0,342 56	0, 12 45 1	0, 00 00 0	0,0 00 00	- 1,6 511 6	0,0 00 01
B3G NT8	0,381 23	0, 04 21 4	0,444 10	0, 00 62 7	- 0,062 88	0, 73 28 6	0, 01 54 7	0, 06 75 0	0,6 926 8	0,0 000 0	0,644 93	0, 00 00 0	0,047 75	0, 68 58 0	0, 00 00 0	0,0 00 00	0,3 967 7	0,0 26 11
B3G NT9	0,073 67	0, 62 07 6	- 0,075 28	0, 55 34 3	0,148 95	0, 31 87 6	0, 59 55 4	0, 71 57 9	0,1 261 5	0,3 631 3	0,030 27	0, 82 79 1	- 0,156 42	0, 26 90 5	0, 49 89 0	0,5 69 29	0,1 558 1	0,2 70 87
B3G NTL 1	0,139 32	0, 29 48 1	0,117 10	0, 30 17 6	0,022 22	0, 86 66 5	0, 46 65 3	0, 61 05 1	0,2 298 8	0,0 093 5	0,364 06	0, 00 00 6	- 0,134 19	0, 13 39 0	0, 00 02 3	0,0 00 70	0,1 321 4	0,3 79 43
B4G ALN T1																		
B4G ALN T2																		
B4G ALN T3	0,289 08	0, 51 81 3	- 0,069 39	0, 85 54 3	0,358 47	0, 42 34 1	0, 71 25 4	0, 80 61 6	0,2 321 2	0,4 135 0	0,350 77	0, 21 96 0	- 0,118 65	0, 68 16 1	0, 45 45 0	0,5 27 10	- 0,1 616 4	0,7 74 11
B4G ALN T4	- 0,048 61	0, 94 81 3	0,457 94	0, 47 34 2	0,409 32	0, 58 43 6	0, 74 46 3	0, 82 95 8	- 0,3 616 5	0,2 639 6	- 0,647 50	0, 04 76 0	0,285 86	0, 38 61 2	0, 13 75 1	0,1 95 58	- 0,2 093 5	0,7 33 31

B4G ALT 1**	0,062 10 4	0, 41 01 4	0,068 18	0, 28 99 3	- 0,006 08	0, 93 55 2	0, 52 26 8	0, 65 72 4	- 0,0 341 4	0,4 581 5	0,028 87	0, 53 24 0	- 0,063 01	0, 18 01 6	0, 40 50 3	0,4 80 23	0,1 740 1	0,0 35 42
B4G ALT 2	- 0,411 95	0, 20 50 3	- 0,563 34	0, 04 44 5	0,151 39	0, 63 92 9	0, 11 87 1	0, 25 25 6	0,0 419 7	0,7 086 3	- 0,164 57	0, 14 62 2	0,122 60	0, 28 51 3	0, 32 32 3	0,3 99 62	0,6 786 7	0,0 40 87
B4G ALT 3	0,062 56	0, 66 27 0	- 0,092 74	0, 44 90 5	0,155 30	0, 28 09 8	0, 52 75 3	0, 66 13 8	0,1 086 5	0,1 753 4	- 0,024 35	0, 76 18 6	0,133 00	0, 10 41 8	0, 22 00 0	0,2 90 16	0,3 510 1	0,0 21 78
B4G ALT 4	0,185 23	0, 20 54 6	0,168 51	0, 17 72 3	0,016 72	0, 90 83 5	0, 29 95 6	0, 45 1 1	0,0 075 4	0,9 279 2	- 0,006 68	0, 93 64 9	0,014 22	0, 86 71 5	0, 98 60 4	0,9 88 97	0,3 415 6	0,0 69 36
B4G ALT 5	0,614 51	0, 00 05 9	0,634 65	0, 00 00 5	- 0,020 14	0, 90 52 7	0, 00 00 6	0, 00 18 3	0,7 840 4	0,0 000 0	0,819 22	0, 00 00 0	- 0,035 18	0, 74 92 4	0, 00 00 0	0,0 00 00	1,1 845 0	0,0 00 00
B4G ALT 6	0,344 15	0, 35 29 5	- 0,466 55	0, 14 21 2	0,810 70	0, 03 14 8	0, 08 19 0	0, 19 78 9	- 1,0 528 5	0,0 000 3	- 0,625 01	0, 01 19 7	0,427 84	0, 08 81 1	0, 00 01 4	0,0 00 45	1,1 399 0	0,0 02 54
B4G ALT 7	- 0,295 53	0, 03 75 5	- 0,266 84	0, 02 80 3	- 0,028 69	0, 83 69 0	0, 04 12 0	0, 12 76 3	0,0 166 2	0,8 736 1	0,048 74	0, 64 26 8	0,032 12	0, 76 30 3	0, 89 51 7	0,9 16 53	- 0,4 844 3	0,0 00 22
B4G AT1	- 0,721 69	0, 00 00 2	- 0,613 47	0, 00 00 2	- 0,108 22	0, 48 75 9	0, 00 00 1	0, 00 04 1	- 0,7 750 3	0,0 000 0	- 0,771 89	0, 00 00 0	- 0,003 14	0, 97 70 6	0, 00 00 0	0,0 00 00	- 0,7 929 9	0,0 15 09
C1G ALT 1	0,273 02	0, 31 97 7	0,362 83	0, 12 33 9	- 0,089 81	0, 74 24 8	0, 28 25 6	0, 44 23 2	0,3 698 5	0,0 007 5	0,233 43	0, 03 24 0	0,136 42	0, 21 54 3	0, 00 28 9	0,0 06 77	0,8 013 8	0,0 02 99
C1G ALT 1C1	1,329 03	0, 00 10 3	0,172 62	0, 59 99 4	1,156 41	0, 00 38 8	0, 00 27 9	0, 02 22 2	- 0,0 648 3	0,7 941 9	0,054 12	0, 82 84 9	0,118 94	0, 63 88 5	0, 89 50 6	0,9 16 50	0,1 218 3	0,8 14 29
CER CAM	0,228 58	0, 61 69 4	0,242 17	0, 53 45 7	- 0,013 60	0, 97 62 3	0, 79 57 1	0, 86 43 3	- 0,3 246 6	0,0 557 9	0,019 19	0, 90 99 2	- 0,343 85	0, 04 70 5	0, 07 99 8	0,1 23 51	- 0,6 806 2	0,0 71 09
CHP F	- 0,100 04	0, 62 23 7	- 0,158 52	0, 36 14 3	0,058 48	0, 77 32 6	0, 65 27 4	0, 76 05 0	0, 0 836 2	0,4 335 4	- 0,223 33	0, 03 86 0	0,139 72	0, 20 00 9	0, 11 33 2	0,1 65 95	- 0,8 121 1	0,0 33 80
CHP F2	0,409 53	0, 00 21 1	0,187 44	0, 08 90 2	0,222 10	0, 08 58 0	0, 00 79 6	0, 04 40 1	0,2 986 4	0,0 000 0	0,345 71	0, 00 00 0	0,047 07	0, 41 89 4	0, 00 00 0	0,0 00 00	0,0 285 6	0,7 70 65
CHS Y1	0,440 67	0, 00 42 9	0,397 77	0, 00 26 1	0,042 90	0, 77 30 1	0, 00 26 8	0, 02 16 6	0,1 246 8	0,3 467 2	0,156 18	0, 24 13 5	- 0,031 50	0, 81 54 1	0, 45 84 8	0,5 30 85	0,9 583 9	0,0 00 00
CHS Y3																	0,0 834 6	0,8 64 22
COL GAL T1	0,161 90	0, 06 08 0	0,042 14	0, 56 15 6	0,119 76	0, 16 24 5	0, 16 45 9	0, 31 10 3	0,3 169 3	0,0 000 2	0,356 02	0, 00 00 0	- 0,039 09	0, 59 47 0	0, 00 00 0	0,0 00 01	- 0,1 226 2	0,2 19 92
COL GAL T2	- 0,705 91	0, 05 96 2	- 0,974 14	0, 00 29 4	0,268 24	0, 46 80 6	0, 00 95 9	0, 04 95 4	- 1,0 447 6	0,0 000 0	- 1,019 18	0, 00 00 0	- 0,025 58	0, 88 98 9	0, 00 00 0	0,0 00 00	- 0,8 587 1	0,0 96 43
CSG ALN ACT 1	0,144 54	0, 51 45 3	0,110 27	0, 55 96 8	0,034 27	0, 87 69 6	0, 76 22 5	0, 84 16 3	- 0,6 729 5	0,0 000 5	- 0,335 93	0, 03 98 7	- 0,337 02	0, 04 20 6	0, 00 02 6	0,0 00 79	0,1 199 0	0,5 96 43
CSG ALN ACT 2	0,324 79	0, 00 23 3	0,301 80	0, 09 09 9	0,022 99	0, 82 21 7	0, 00 10 1	0, 01 13 0	- 0,2 096 5	0,0 903 1	- 0,045 57	0, 71 29 6	- 0,164 08	0, 19 27 1	0, 20 77 9	0,2 76 74	0,5 516 6	0,0 00 02
DPM 1	- 0,111 35	0, 34 69 7	0,220 20	0, 03 20 1	- 0,331 55	0, 00 65 6	0, 01 42 4	0, 06 40 2	0, 1 676 1	0,1 279 5	- 0,167 27	0, 13 07 1	- 0,000 34	0, 99 75 9	0, 21 17 3	0,2 81 30	0,3 933 0	0,0 01 72
DPY 19L1	0,186 83	0, 24 85 7	- 0,037 29	0, 78 59 4	0,224 12	0, 16 74 3	0, 35 94 4	0, 51 77 6	0,1 633 3	0,1 819 3	- 0,268 72	0, 02 96 9	0,105 39	0, 39 74 0	0, 08 88 0	0,1 35 12	0,2 942 2	0,0 29 46

DPY 19L2	- 0,839 79	0, 12 71 3	- 0,743 40	0, 11 36 0	- 0,096 39	0, 85 95 6	0, 18 36 5	0, 33 39 8									0,4 631 7	0,4 30 40
DPY 19L3	0,688 90	0, 06 16 5	0,671 95	0, 03 33 9	0,016 96	0, 96 27 3	0, 05 98 8	0, 16 20 5	- 0,2 095 9	0,4 503 8	- 0,124 65	0, 65 49 9	- 0,084 94	0, 76 40 1	0, 74 81 3	0,7 92 52	0,2 353 7	0,3 22 67
EOG T	0,032 07	0, 83 87 5	- 0,052 15	0, 69 81 4	0,084 22	59 34 8	85 25 8	90 35 5	- 0,2 659 9	0,1 116 3	- 0,406 03	0, 01 62 6	0,140 04	0, 40 99 5	0, 04 92 4	0,0 81 43	0,1 763 2	0,5 05 12
EXT 1	0,307 01	0, 04 14 5	0,289 86	0, 02 45 7	0,017 15	90 76 1	0, 04 00 1	0, 12 52 8	0,4 110 4	0,0 000 0	0,370 58	0, 00 00 1	0,040 46	0, 61 84 6	0, 00 00 0	0,0 00 00	0,4 936 9	0,0 01 35
EXT 2	0,100 55	0, 35 00 9	- 0,015 54	0, 86 50 2	0,116 09	28 12 9	0, 02 88 6	0, 66 24 4	- 0,0 456 0	0,4 632 9	- 0,008 30	0, 89 42 7	- 0,037 30	0, 55 61 4	0, 73 94 5	0,7 85 38	- 0,4 267 2	0,0 03 66
EXT L1																		
EXT L2	0,713 69	0, 03 14 7	- 0,266 22	0, 33 86 1	0,979 91	0, 00 37 1	0, 01 35 0	0, 06 17 8	- 0,9 127 9	0,0 002 5	- 0,519 54	0, 03 52 1	- 0,393 25	0, 11 48 8	0, 00 11 2	0,0 02 90	- 0,6 846 1	0,0 15 20
EXT L3	0,053 19	0, 73 99 0	0,013 22	0, 92 29 1	0,039 98	0, 80 29 3	0, 94 47 4	0, 96 38 5	- 0,0 076 1	0,9 576 6	0,057 30	0, 69 07 8	- 0,064 90	0, 65 68 9	0, 88 81 0	0,9 10 92	0,0 013 5	0,9 93 31
FUT 1																		
FUT 10	- 0,256 17	0, 35 27 8	- 0,656 67	0, 00 67 0	0,400 50	0, 14 85 4	0, 02 36 1	0, 08 90 9	- 0,7 950 7	0,0 000 9	- 0,715 82	0, 00 04 2	- 0,079 25	0, 69 44 4	0, 00 00 9	0,0 00 31	- 0,7 837 7	0,0 00 18
FUT 11	- 0,075 00	0, 52 37 9	- 0,184 95	0, 06 88 9	0,109 94	0, 35 10 6	0, 18 59 3	0, 33 64 0	- 0,3 788 1	0,0 000 0	- 0,385 41	0, 00 00 0	0,006 60	0, 92 05 8	0, 00 00 0	0,0 00 00	- 0,2 283 7	0,0 56 68
FUT 2	0,126 04	0, 68 18 4	- 0,480 96	0, 07 04 9	0,607 00	0, 05 21 0	0, 08 49 5	0, 20 27 7	- 1,0 938 4	0,0 000 0	- 1,018 39	0, 00 00 0	- 0,075 44	0, 71 93 0	0, 00 00 0	0,0 00 00	- 1,9 843 8	0,0 00 01
FUT 3																		
FUT 4	0,490 05	0, 00 28 9	0,209 53	0, 12 37 9	0,280 53	0, 07 98 2	0, 01 10 4	0, 05 43 0	0,3 817 0	0,0 000 0	0,401 48	0, 00 00 0	- 0,019 78	0, 70 78 5	0, 00 00 0	0,0 00 00	0,3 347 0	0,0 05 10
FUT 5																		
FUT 6																		
FUT 7	0,683 46	0, 00 02 5	0,554 47	0, 00 04 5	0,128 98	0, 46 26 7	0, 00 01 6	0, 00 34 1	0,7 004 7	0,0 000 0	0,626 31	0, 00 00 0	0,074 16	0, 50 81 9	0, 00 00 0	0,0 00 00	0,4 114 2	0,0 09 05
FUT 8	- 0,384 64	0, 00 88 6	- 0,167 61	0, 17 12 5	- 0,217 03	0, 13 15 5	0, 03 06 9	0, 10 57 5	- 0,3 979 2	0,0 003 2	- 0,437 20	0, 00 00 9	0,039 28	0, 72 22 7	0, 00 00 8	0,0 00 27	- 0,3 611 4	0,0 02 62
FUT 9																		
GAL NT1	- 0,017 43	0, 88 82 4	0,123 94	0, 24 41 2	- 0,141 37	0, 25 70 7	0, 39 45 7	0, 54 88 2	- 0,2 366 9	0,0 608 9	- 0,123 89	0, 32 70 5	- 0,112 79	0, 37 88 9	0, 17 11 0	0,2 35 05	0,1 459 5	0,2 44 10
GAL NT1 0	0,215 99	0, 18 00 9	- 0,160 12	0, 24 29 8	0,376 11	0, 02 16 0	0, 06 86 3	0, 17 70 1	0,1 080 3	0,3 693 9	- 0,021 93	0, 85 59 5	- 0,086 09	0, 48 27 1	0, 64 04 1	0,6 98 51	- 0,2 117 2	0,4 06 75
GAL NT1 1	0,007 76	0, 92 02 1	- 0,164 67	0, 01 52 1	0,172 43	0, 02 93 3	0, 02 44 2	0, 09 09 3	- 0,3 716 2	0,0 000 0	- 0,136 12	0, 04 83 7	- 0,235 50	0, 00 08 8	0, 00 00 0	0,0 00 01	- 0,1 600 8	0,0 49 63
GAL NT1 2	- 0,779 94	0, 00 29 3	- 0,830 87	0, 00 02 7	0,050 93	0, 83 97 4	0, 00 04 6	0, 00 68 4	- 1,4 832 5	0,0 000 0	- 1,125 16	0, 00 00 1	- 0,358 10	0, 15 06 1	0, 00 00 0	0,0 00 00	- 0,8 826 3	0,0 08 38
GAL NT1 3																		

GAL NT1 4	0,419 80	0, 40 69 3	0,492 95	0, 25 48 0	- 0,073 15	0, 88 47 5	0, 48 42 4	0, 62 54 4	0,9 243 5	0,0 001 6	1,004 24	0, 00 00 5	- 0,079 89	0, 74 32 2	0, 00 00 3	0,0 00 13	2,0 268 1	0,0 00 00
GAL NT1 5																		
GAL NT1 6																		
GAL NT1 7																		
GAL NT1 8																		
GAL NT2	0,226 85	0, 00 76 8	0,176 29	0, 01 46 0	0,050 57	0, 53 99 9	0, 01 07 2	0, 05 32 6	- 0,0 752 6	0,3 784 1	0,053 05	0, 53 65 0	- 0,128 31	0, 14 16 8	0, 33 42 4	0,4 10 36	- 0,1 601 0	0,2 37 58
GAL NT2 0																		
GAL NT3	0,725 56	0, 00 00 2	0,550 03	0, 00 01 4	0,175 53	0, 27 06 6	0, 00 00 2	0, 00 09 1	0,1 013 1	0,5 903 0	0,294 44	0, 12 08 4	- 0,193 13	0, 31 46 9	0, 29 04 2	0,3 65 78	1,1 016 3	0,0 00 00
GAL NT4	0,182 84	0, 39 64 7	0,262 47	0, 15 59 0	- 0,079 63	0, 71 11 7	0, 34 99 1	0, 50 84 6	- 0,1 342 1	0,3 398 3	- 0,018 03	0, 89 83 7	- 0,116 18	0, 41 75 1	0, 58 89 0	0,6 51 02	- 0,0 338 0	0,8 91 25
GAL NT5	0,880 69	0, 08 72 5	- 0,040 20	0, 92 60 9	0,920 89	0, 07 41 0	0, 15 30 5	0, 29 77 1										
GAL NT6	0,150 39	0, 25 44 1	0,028 24	0, 80 07 9	0,122 15	0, 35 36 3	0, 50 21 0	0, 64 00 0	0,1 723 3	0,0 107 7	0,087 55	0, 19 39 7	0,084 78	0, 21 48 1	0, 03 82 4	0,0 65 47	- 0,2 050 7	0,1 21 06
GAL NT7	0,039 15	0, 75 96 7	0,174 54	0, 11 37 1	- 0,135 39	0, 29 23 6	0, 00 04 3	0, 41 94 3	- 0,2 804 2	0,0 336 2	- 0,178 50	0, 17 66 0	- 0,101 92	0, 44 59 4	0, 09 73 0	0,1 45 94	0,3 390 5	0,0 17 73
GAL NT8																		
GAL NT9	- 0,029 27	0, 95 02 4	- 1,102 56	0, 00 76 1	1,073 30	0, 02 52 6	0, 01 41 6	0, 06 38 2	- 0,8 257 7	0,0 013 0	- 1,091 80	0, 00 00 3	0,266 03	0, 30 23 1	0, 00 00 7	0,0 00 24	- 0,9 156 5	0,0 44 03
GBG T1	0,761 06	0, 00 01 5	0,294 61	0, 06 96 4	0,466 45	0, 01 54 8	0, 00 06 9	0, 00 90 0	0,6 738 3	0,0 000 0	0,567 05	0, 00 00 0	0,106 78	0, 26 12 8	0, 00 00 0	0,0 00 00	0,2 321 1	0,1 85 74
GCN T1	- 0,059 48	0, 88 28 6	0,147 79	0, 66 79 4	- 0,207 27	0, 60 80 3	0, 85 22 6	0, 90 33 1	0,3 388 9	0,0 319 8	0,576 88	0, 00 03 4	- 0,237 99	0, 13 80 9	0, 00 14 6	0,0 03 66	0,3 683 9	0,2 87 09
GCN T2A																		
GCN T2B																		
GCN T2C																		
GCN T3																		
GCN T4	- 1,035 47	0, 00 00 2	- 0,772 10	0, 00 01 6	- 0,263 37	0, 24 32 8	0, 00 00 2	0, 00 09 1	- 1,6 173 8	0,0 000 0	- 1,208 42	0, 00 00 0	0,408 96	0, 05 56 4	0, 00 00 0	0,0 00 00	- 0,1 689 9	0,3 99 70
GCN T7	0,869 43	0, 02 21 5	0,001 05	0, 99 73 6	0,868 38	0, 02 23 0	0, 04 11 3	0, 12 74 4	0,2 682 5	0,2 620 5	0,621 91	0, 01 03 4	- 0,353 66	0, 14 75 6	0, 03 65 7	0,0 63 00	0,5 013 7	0,3 16 80
GLT 1D1	0,451 21	0, 89 7	0,427 49	0, 00 39 4	0,023 72	0, 88 73 1	0, 00 52 9	0, 03 36 6	0,4 024 1	0,0 000 3	0,546 12	0, 00 00 0	- 0,143 70	0, 13 14 5	0, 00 00 0	0,0 00 00	0,7 770 9	0,0 00 04
GLT 6D1																		

GLT 8D1	- 0,190 08	0, 04 28 5	- 0,097 09	0, 21 96 8	- 0,092 99	0, 31 50 0	0, 11 74 9	0, 25 06 5	- 0,1 027 5	0,0 885 6	- 0,048 55	0, 42 17 2	- 0,054 20	0, 37 66 1	0, 23 33 4	0,3 04 90	- 0,0 825 0	0,3 45 26
GLT 8D2																		
GTD C1	0,080 22	0, 56 02 8	0,158 94	0, 17 89 0	- 0,078 72	0, 56 76 2	0, 40 19 2	0, 55 38	- 0,2 373 8	0,0 675 3	- 0,005 84	0, 96 41 4	- 0,231 54	0, 08 01 8	0, 11 88 8	0,1 72 97	0,2 462 5	0,1 74 14
GXY LT1	0,163 70	0, 15 60 6	0,115 72	0, 23 83 6	0,047 98	0, 67 50 5	0, 29 76 9	0, 45 77 5	- 0,1 001 3	0,4 434 5	0,028 88	0, 82 58 3	- 0,129 01	0, 33 31 3	0, 59 36 1	0,6 55 75	0,3 828 5	0,0 07 14
GXY LT2																		
GYG 1	0,828 90	0, 00 00 1	0,794 15	0, 00 00 0	0,034 74	0, 83 81 8	0, 00 00 0	0, 00 01 0	1,0 442 5	0,0 000 0	1,070 54	0, 00 00 0	- 0,026 28	0, 86 43 9	0, 00 00 0	0,0 00 00	1,3 822 6	0,0 00 00
GYG 2																		
GYS 1	0,116 29	0, 30 06 4	- 0,134 89	0, 16 09 0	0,251 18	0, 02 79 8	0, 07 77 8	0, 19 15 9	0,0 129 9	0,8 726 8	0,106 54	0, 19 22 2	- 0,093 55	0, 25 86 2	0, 36 91 6	0,4 46 06	- 0,6 507 4	0,0 00 13
GYS 2																		
HAS 1	0,897 36	0, 12 45 4	0,948 52	0, 05 83 4	- 0,051 16	0, 92 94 8	0, 12 05 1	0, 25 53 4	0,2 044 6	0,4 831 3	0,073 02	0, 80 31 2	0,131 44	0, 65 82 2	0, 77 73 0	0,8 17 75	0,8 816 1	0,1 16 00
HAS 2																		
HAS 3	- 0,526 56	0, 03 34 5	- 0,329 12	0, 11 56 5	- 0,197 44	0, 41 70 3	0, 07 95 0	0, 19 41 7	- 0,4 492 0	0,0 126 4	- 0,179 19	0, 31 82 3	- 0,270 01	0, 13 89 1	0, 04 28 9	0,0 72 28	- 0,4 068 9	0,1 80 72
KDE LC1	0,694 94	0, 12 71 5	- 0,503 83	0, 19 34 0	1,198 76	0, 00 98 9	0, 03 42 4	0, 11 34 4									0,5 256 5	0,3 72 98
KDE LC2	- 0,150 81	0, 43 73 8	- 0,288 13	0, 08 52 0	0,137 31	0, 47 93 1	0, 22 41 7	0, 37 94 3	- 1,0 217 6	0,0 000 1	- 0,649 18	0, 00 37 5	- 0,372 58	0, 09 78 9	0, 00 00 3	0,0 00 11	- 0,6 747 0	0,0 06 62
LAR GE1	- 0,725 44	0, 02 14 1	- 1,143 12	0, 00 00 5	0,417 68	0, 17 83 4	0, 00 02 4	0, 00 44 3	- 1,0 726 7	0,0 000 0	- 1,111 24	0, 00 00 0	0,038 57	0, 83 45 3	0, 00 00 0	0,0 00 00	- 1,6 557 3	0,0 00 01
LAR GE2	- 0,863 30	0, 00 18 9	- 1,039 20	0, 00 00 2	0,175 89	0, 50 89 1	0, 00 00 6	0, 00 18 4	- 1,0 674 1	0,0 000 0	- 0,930 03	0, 00 00 0	- 0,137 38	0, 35 43 2	0, 00 00 0	0,0 00 00	- 1,9 105 6	0,0 00 00
LFN G	- 0,171 97	0, 15 28 4	- 0,351 08	0, 00 10 1	0,179 11	0, 03 67 9	0, 00 42 9	0, 02 94 2	- 0,2 629 6	0,0 000 6	- 0,344 05	0, 00 00 0	0,081 09	0, 21 42 3	0, 00 00 0	0,0 00 00	- 0,4 528 1	0,0 01 87
MFN G	- 0,395 84	0, 00 56 3	- 0,174 69	0, 14 17 3	0,221 15	0, 11 32 2	0, 02 01 7	0, 08 00 9	- 0,1 847 9	0,0 219 1	- 0,217 22	0, 00 75 4	0,032 43	0, 69 07 6	0, 01 48 3	0,0 28 73	- 0,4 910 9	0,0 06 75
MGA T1	0,493 01	0, 00 02 0	0,263 49	0, 01 55 4	0,229 51	0, 06 89 9	0, 00 06 9	0, 00 89 7	0,4 110 3	0,0 000 0	0,384 95	0, 00 00 0	0,026 09	0, 73 83 7	0, 00 00 0	0,0 00 00	0,0 562 0	0,6 46 62
MGA T2	0,025 21	0, 81 13 4	0,026 30	0, 03 05	- 0,001 09	0, 99 18 0	0, 95 02 5	0, 96 75 8	- 0,2 308 0	0,0 126 5	- 0,210 26	0, 02 35 8	- 0,020 54	0, 82 59 5	0, 02 17 1	0,0 39 97	0,0 572 8	0,5 13 80
MGA T3	0,061 70	0, 83 39 7	- 0,200 67	0, 42 53 1	0,262 37	0, 37 43 7	0, 60 36 1	0, 72 16 5	- 0,4 633 4	0,0 019 1	- 0,441 38	0, 00 32 2	- 0,021 95	0, 88 35 1	0, 00 21 5	0,0 05 20	0,4 197 4	0,0 60 22
MGA T4A	- 0,186 77	0, 46 41 9	- 0,445 81	0, 04 37 8	0,259 05	0, 31 10 6	0, 12 71 8	0, 26 36 9	- 0,6 114 4	0,0 000 0	- 0,602 02	0, 00 00 1	- 0,009 41	0, 94 30 4	0, 00 00 0	0,0 00 01	- 0,1 600 8	0,3 95 29
MGA T4B	0,096 60	0, 41 36 7	0,114 81	0, 25 59 4	- 0,018 21	0, 87 71 7	0, 48 78 8	0, 62 85 3	0,3 598 2	0,0 000 0	0,280 59	0, 00 01 6	0,079 23	0, 28 32 9	0, 00 00 0	0,0 00 01	0,0 820 4	0,4 77 52

MGA T4C																			
MGA T4D																			
MGA T5	0,161 10	0, 67 37 0	- 0,293 50	0, 36 97 1	0,454 60	0, 23 73 3	0, 45 05 5	0, 59 74 2	- 0,1 333 4	0,4 234 2	- 0,195 89	0, 24 26 1	0,062 55	0, 71 24 5	0, 48 62 9	0,5 57 27	- 0,3 591 3	0,2 68 10	
MGA T5B																			
OGT	- 0,129 94	0, 21 56 3	- 0,114 11	0, 20 24 9	- 0,015 83	0, 87 92 5	0, 32 92 9	0, 48 89 2	- 0,4 615 0	0,0 000 1	- 0,286 49	0, 00 53 4	- 0,175 01	0, 09 07 5	0, 00 00 4	0,0 00 16	- 0,0 262 6	0,8 38 36	
PIG A	0,463 33	0, 00 28 8	- 0,047 71	0, 70 80 5	0,511 04	0, 00 11 1	0, 00 26 0	0, 02 12 0	- 0,3 610 1	0,0 154 4	- 0,349 13	0, 01 97 2	- 0,011 88	0, 93 71 1	0, 02 23 4	0,0 40 97	- 0,5 589 8	0,0 77 00	
PIG B	- 0,054 44	0, 70 93 9	- 0,017 14	0, 89 04 8	- 0,037 30	0, 79 84 2	0, 93 20 7	0, 95 62 1	0,0 603 0	0,5 844 8	0,101 09	0, 36 21 6	- 0,161 39	0, 15 23 1	0, 35 03 7	0,4 26 92	0,2 239 6	0,0 92 10	
PIG M	0,110 14	0, 37 32 4	- 0,044 67	0, 67 11 1	0,154 80	0, 21 23 2	0, 45 29 0	0, 59 95 5	0,1 448 4	0,0 583 3	- 0,094 78	0, 21 62 6	0,239 62	0, 00 23 4	0, 00 89 0	0,0 18 32	- 0,0 041 5	0,9 74 68	
PIG V	0,015 27	0, 92 77 3	- 0,072 98	0, 61 17 1	0,088 25	0, 60 06 4	0, 82 86 5	0, 88 77 2	0,0 797 4	0,3 468 0	0,136 40	0, 11 03 3	- 0,056 67	0, 51 16 7	0, 27 33 5	0,3 47 80	0,0 663 9	0,6 97 31	
PIG Z	- 0,237 03	0, 33 75 1	- 0,225 65	0, 28 48 0	- 0,011 37	0, 96 31 4	0, 48 16 4	0, 62 35 0	- 0,1 811 2	0,2 552 2	- 0,233 46	0, 14 51 3	0,052 34	0, 74 66 2	0, 30 53 4	0,3 81 09	- 0,8 457 4	0,0 18 52	
POF UT1	- 0,010 37	0, 90 72 7	- 0,079 32	0, 29 86 8	0,068 95	0, 44 00 7	0, 54 32 4	0, 67 41 9	- 0,2 273 6	0,0 003 3	- 0,176 72	0, 00 51 6	- 0,050 65	0, 42 39 5	0, 00 08 0	0,0 02 15	- 0,1 743 2	0,0 85 10	
POF UT2	0,026 76	0, 76 53 3	- 0,197 87	0, 01 18 8	0,224 62	0, 01 47 0	0, 01 42 1	0, 06 39 4	0,2 893 6	0,0 001 8	- 0,122 75	0, 10 71 5	- 0,166 61	0, 03 16 4	0, 00 08 5	0,0 02 27	0,1 327 7	0,1 71 11	
POG LUT 1	- 0,274 41	0, 01 08 6	- 0,132 05	0, 14 26 2	- 0,142 36	0, 17 70 3	0, 03 52 8	0, 11 58 8	0,5 164 8	0,0 006 9	- 0,650 07	0, 00 00 3	0,133 59	0, 38 07 9	0, 00 05 5	0,0 00 17	- 0,1 256 7	0,4 61 43	
POM GNT 1	- 0,209 63	0, 08 98 2	- 0,304 42	0, 00 47 7	0,094 79	0, 43 83 1	0, 01 60 0	0, 06 91 0	0,3 975 8	0,0 000 0	- 0,371 89	0, 00 01 1	- 0,025 69	0, 75 38 4	0, 00 00 0	0,0 00 00	- 0,5 444 3	0,0 00 02	
POM GNT 2	- 0,222 79	0, 21 42 1	- 0,363 16	0, 01 96 9	0,140 37	0, 43 19 6	0, 06 24 2	0, 16 67 6	- 0,0 410 1	0,6 824 4	0,040 62	0, 68 68 6	- 0,081 62	0, 42 48 2	0, 72 64 9	0,7 74 51	- 0,5 763 3	0,1 31 97	
POM T1	- 0,270 69	0, 02 76 8	- 0,421 29	0, 00 01 2	0,150 60	0, 21 36 8	0, 00 05 3	0, 00 74 9	- 0,5 246 5	0,0 000 0	- 0,514 85	0, 00 00 0	- 0,009 80	0, 89 92 2	0, 00 00 0	0,0 00 00	- 0,6 161 1	0,0 00 02	
POM T2	- 0,084 01	0, 52 62 0	- 0,368 56	0, 00 17 9	0,284 54	0, 03 50 7	0, 00 54 3	0, 03 42 0	- 0,1 522 3	0,0 088 5	- 0,113 09	0, 05 18 5	- 0,039 15	0, 50 46 2	0, 02 41 2	0,0 43 83	- 0,5 332 2	0,0 09 91	
PYG B	- 0,081 19	0, 43 02 6	- 0,106 10	0, 22 85 1	0,024 92	0, 80 82 5	0, 45 99 4	0, 60 51 4	0,0 574 5	0,3 210 0	0,000 20	0, 99 72 7	0,057 25	0, 33 20 4	0, 52 64 9	0,5 94 49	- 0,3 269 1	0,0 03 60	
PYG L	0,527 76	0, 00 59 7	0,733 34	0, 00 00 2	- 0,205 58	0, 27 02 7	0, 00 00 8	0, 00 21 3	0,5 354 1	0,0 000 2	0,666 53	0, 00 00 0	- 0,131 13	0, 28 77 7	0, 00 00 0	0,0 00 00	1,1 689 2	0,0 00 00	
PYG M	0,389 46	0, 00 63 3	0,283 57	0, 01 86 7	0,105 88	0, 44 36 7	0, 01 07 2	0, 05 32 6	0,3 772 7	0,0 002 3	0,460 45	0, 00 00 1	- 0,083 18	0, 41 55 0	0, 00 00 1	0,0 00 05	0,4 641 0	0,0 02 62	
RFN G	0,207 47	0, 09 76 7	0,098 37	0, 35 29 9	0,109 09	0, 37 95 5	0, 24 37 6	0, 40 18 1	0,4 364 4	0,0 000 0	0,305 21	0, 00 07 1	0,131 23	0, 14 52 0	0, 00 00 1	0,0 00 03	- 0,0 820 8	0,5 53 79	
RTF DC1																			
ST3 GAL 1	- 0,067 77	0, 31 60 8	- 0,025 28	0, 65 98 0	- 0,042 49	0, 52 85 3	0, 60 22 2	0, 72 06 0	- 0,0 916 0	0,0 401 3	- 0,093 45	0, 03 72 9	0,001 85	0, 96 73 0	0, 05 62 2	0,0 91 35	0,0 589 4	0,4 94 54	

ST3 GAL 2	0,584 92 7	0,00 63 7	0,247 84	0,16 45 2	0,337 08	0,10 79 6	0,02 29 4	0,08 74 3	0,0 920 4	0,5 188 2	0,326 60	0,02 38 2	- 0,234 56	0,10 80 8	0,06 76 7	0,1 07 24	0,6 354 0	0,0 02 79
ST3 GAL 3	0,143 51 5	0,35 84 5	0,312 80	0,02 14 5	- 0,169 28	0,27 94 2	0,06 91 0	0,17 78 3	0,0 507 8	0,6 712 2	0,095 83	0,42 59 0	- 0,146 61	0,23 03 7	0,47 53 3	0,5 46 88	0,0 706 7	0,6 89 27
ST3 GAL 4	0,323 49 0	0,06 34 0	0,451 53	0,00 30 3	- 0,128 04	0,45 65 7	0,01 00 1	0,05 09 4	0,5 188 3	0,0 000 1	0,545 73	0,00 00 1	- 0,026 90	0,81 87 8	0,00 00 0	0,0 01	1,1 023 2	0,0 00 00
ST3 GAL 5	0,150 03 5	0,21 77 5	0,026 69	0,79 57 5	0,123 34	0,30 98 8	0,44 47 8	0,59 27 0	0,1 228 8	0,1 349 8	0,133 81	0,10 56 2	- 0,010 93	0,89 58 7	0,19 35 9	0,2 60 94	- 0,0 273 0	0,7 99 38
ST3 GAL 6	0,180 82 5	0,34 99 5	0,500 57	0,00 34 0	- 0,319 75	0,10 11 6	0,01 23 3	0,05 81 0	0,2 579 3	0,0 716 6	0,349 83	0,01 55 2	- 0,091 91	0,52 71 1	0,04 12 5	0,0 69 93	0,9 571 4	0,0 00 00
ST6 GAL 1	- 0,340 75	0,00 16 7	- 0,360 54	0,00 01 4	0,019 80	0,84 84 7	0,00 02 1	0,00 41 5	- 0,9 924 4	0,0 000 0	- 0,734 36	0,00 00 4	- 0,258 09	0,14 49 1	0,00 00 0	0,0 00	- 0,5 055 5	0,0 00 51
ST6 GAL 2																		
ST6 GAL NAC 1	0,221 58 6	0,67 88 6	- 0,339 65	0,45 75 4	0,561 24	0,29 63 4	0,54 68 6	0,67 71 3	- 0,8 297 6	0,0 080 9	- 0,861 09	0,00 63 0	0,031 33	0,92 09 8	0,00 77 5	0,0 16 24	- 0,9 615 1	0,1 32 16
ST6 GAL NAC 2	0,231 39 2	0,19 68 2	0,278 70	0,07 03 5	- 0,047 32	0,79 03 6	0,16 41 3	0,31 04 7	0,3 946 2	0,0 006 6	0,539 03	0,00 00 1	- 0,144 41	0,21 41 4	0,00 00 1	0,0 00 06	0,4 298 3	0,0 38 22
ST6 GAL NAC 3	0,644 10 2	0,06 87 2	0,245 55	0,41 02 2	0,398 55	0,25 56 6	0,18 78 1	0,33 83 6	- 0,0 526 2	0,8 153 4	- 0,023 58	0,91 70 7	- 0,029 03	0,89 94 2	0,97 29 6	0,9 78 63	0,2 675 8	0,2 52 00
ST6 GAL NAC 4	- 0,008 31	0,96 91 9	0,283 38	0,12 62 7	- 0,291 68	0,17 85 1	0,23 09 7	0,38 71 9	0,5 257 0	0,0 001 2	0,127 95	0,34 19 0	0,397 76	0,00 40 0	0,00 03 6	0,0 01 07	- 0,0 063 4	0,9 72 50
ST6 GAL NAC 5																		
ST6 GAL NAC 6	- 0,313 21	0,12 23 1	- 0,283 49	0,10 14 8	- 0,029 72	0,88 22 0	0,16 87 6	0,31 61 2	- 0,6 084 9	0,0 000 6	- 0,615 36	0,00 00 5	0,006 87	0,96 35 3	0,00 00 2	0,0 00 08	- 0,4 954 8	0,0 05 47
ST8S IA1	- 0,672 67	0,02 46 9	- 0,990 75	0,00 02 0	0,318 08	0,27 96 6	0,00 07 7	0,00 96 0	0,8 343 7	0,0 001 9	- 1,069 96	0,00 00 0	0,235 59	0,29 08 5	0,00 00 0	0,0 00 02	- 0,9 861 7	0,0 00 89
ST8S IA2																		
ST8S IA3																		
ST8S IA4	0,304 01 9	0,06 45 9	0,307 18	0,02 95 2	- 0,003 16	0,98 44 3	0,05 65 0	0,15 60 9	0,0 765 6	0,4 978 0	0,101 71	0,37 05 0	- 0,025 16	0,82 69 4	0,64 30 8	0,7 00 81	0,7 192 7	0,0 00 01
ST8S IA5									0,2 412 0	0,3 798 1	0,394 69	0,15 36 8	- 0,153 48	0,58 33 4	0,35 21 1	0,4 28 45		
ST8S IA6	- 0,088 24	0,86 55 3	- 1,206 44	0,00 85 2	1,118 20	0,03 54 4	0,01 80 0	0,07 45 5	- 0,5 017 1	0,0 316 0	0,005 04	0,98 27 4	- 0,506 74	0,03 32 1	0,04 79 5	0,0 64	- 0,8 824 5	0,0 69 67
STT3 A	- 0,068 12	0,34 98 6	0,021 36	0,73 02 1	- 0,089 48	0,22 07 8	0,46 02 6	0,60 55 0	- 0,1 964 8	0,0 011 1	- 0,218 78	0,00 03 2	0,022 30	0,71 18 7	0,00 03 7	0,0 01 07	- 0,0 650 1	0,3 53 42
STT3 B	- 0,077 54	0,59 50 6	0,009 10	0,94 16 5	- 0,086 64	0,55 27 3	0,82 05 9	0,88 17 7	0,3 980 3	0,0 137 6	- 0,187 72	0,24 41 0	- 0,210 31	0,19 84 8	0,04 75 9	0,0 79 16	0,0 643 3	0,5 46 55

Appendix – Chapter 7

UGC G	0,125 98 2	0, 29 50	0,298 60	0, 00 48 0	- 0,172 62	0, 15 30 8	0, 01 77 2	0, 07 38 2	0,1 483 5	0,2 952 5	0,205 10	0, 15 06 6	- 0,056 74	0, 69 41 3	0, 32 75 4	0,4 03 65	0,7 745 9	0,0 00 00
UGG T1	0,279 07 5	0, 05 55	0,098 64	0, 42 11 0	0,180 43	0, 21 14 0	0, 15 73 2	0, 30 25 9	0, 0 485 0	0,5 237 5	- 0,012 48	0, 87 02 7	- 0,036 02	0, 64 22 6	0, 80 44 6	0,8 41 47	0,1 733 0	0,0 74 40
UGG T2	0,360 25 3	0, 14 49	0,246 13	0, 24 12 8	0,114 12	0, 64 13 7	0, 28 59 8	0, 44 57 8	0,0 567 6	0,7 486 2	0,260 95	0, 14 40 6	- 0,317 71	0, 07 99 8	0, 17 38 1	0,2 38 22	0,7 977 4	0,0 02 11
UGT 1A1																		
UGT 1A10																		
UGT 1A3																		
UGT 1A4																		
UGT 1A5																		
UGT 1A6																		
UGT 1A7																		
UGT 1A8																		
UGT 1A9																		
UGT 2A1																		
UGT 2A3																		
UGT 2B10																		
UGT 2B11	- 0,274 21	0, 49 96 9	- 0,866 15	0, 01 47 6	0,591 94	0, 14 80 8	0, 04 57 8	0, 13 63 2									- 0,0 175 1	0,9 74 13
UGT 2B15																		
UGT 2B17																		
UGT 2B28	0,201 01	0, 61 91 1	- 1,099 19	0, 00 22 3	1,300 20	0, 20 6	0, 15 7	0, 01 50 7										
UGT 2B4																		
UGT 2B7																		
UGT 3A1																		
UGT 3A2																		
UGT 8	- 1,178 15	0, 00 17 8	- 0,836 64	0, 00 84 0	- 0,341 51	0, 34 55 8	0, 00 29 7	0, 02 30 9	- 0,4 726 6	0,0 187 0	- 0,055 50	0, 78 16 7	- 0,417 16	0, 04 13 6	0, 03 88 5	0,0 66 39	- 2,2 154 0	0,0 00 02
WBS CR1 7																		
XXY LT1	0,003 77	0, 97 84 5	0,022 04	0, 85 29 1	- 0,018 28	0, 89 56 9	0, 98 11 7	0, 98 66 8	0, 0 344 8	0,6 590 9	- 0,014 25	0, 85 60 6	- 0,020 23	0, 79 95 4	0, 90 63 9	0,9 25 29	- 0,7 071 3	0,0 03 96
XYL T1	- 0,220 64	0, 03 76 9	- 0,263 15	0, 00 43 0	0,042 51	0, 68 32 3	0, 01 11 1	0, 05 44 2	- 0,7 991 2	0,0 000 0	- 0,604 52	0, 00 00 0	- 0,194 60	0, 09 10 0	0, 00 00 0	0,0 00 00	0,5 881 0	0,0 00 12
XYL T2	- 0,163 48	0, 07 88 4	- 0,279 35	0, 00 07 0	0,115 87	0, 20 97 4	0, 00 29 6	0, 02 30 6	0,1 662 1	0,0 042 3	- 0,136 73	0, 01 87 2	- 0,029 47	0, 61 43 0	0, 00 92 0	0,0 18 88	- 0,6 138 2	0,0 00 01

* **Abbreviations:** FC, Fold Change; FDR, False Discovery Rate

** Bold indicates the genes encoding enzymes participating on the sLex biosynthetic pathway

*** Genes with expression levels below the threshold in the datasets

Singhania, A., R. Verma, C.M. Graham, J. Lee, T. Tran, M. Richardson, P. Lecine, P. Leissner, M.P.R. Berry, R.J. Wilkinson, K. Kaiser, M. Rodrigue, G. Woltmann, P. Haldar, and A. O'Garra. 2018. A modular transcriptional signature identifies phenotypic heterogeneity of human tuberculosis infection. *Nat Commun* 9:2308.

Table S2. Oligonucleotide sequences used in this study.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Tnf</i>	GCCACCACGTCTTCTGTCT	TGAGGGTCTGGGCCATAGAAC
<i>Il1g</i>	CCTGGTGCACAACATCGCC	AATCAAACAGAGGCCGCATGC
<i>Il17</i>	CTCAGACTACCTCAACCGTTCCA	TTCCCTCCGCATTGACACA
<i>Il10</i>	ATTTGAATTCCTGGGTGAGAAG	CACAGGGGAGAAATCGATGACA
<i>Il1b</i>	GCACTGGGTGGAATGAGACT	AGTGGAGAGCAGTTGAGGACA
<i>Il1a</i>	GGGAAGATTCTGAAGAAGAG	GAGTAACAGGATATTTAGAGTCG
<i>Il1b</i>	ACCTTCCAGGATGAGGACATGA	AACGTCACACACCAGCAGGTTA
<i>Il12</i>	CAAATTACTCCGGACGGTTC	AGAGACGCCATTCCACATGTC
<i>Nos2</i>	CTGGGAGCGCTCTAGTGAAG	CGATGCACAACTGGGTGAAC
<i>Cxcl1</i>	AGCTTGAAGGTGTTGCCCTCAG	AGCTTCAGGGTCAAGGCAAGC
<i>Cxcl2</i>	CCCAGACAGAAAGTCATAGCCAC	CTTCCGTTGAGGGACAGCAG
<i>Cxcl5</i>	GAGCTGCGTTGTGTTTGCTT	TAGCTTTCTTTTGTCACTGCCC
<i>Elane</i>	ATGTCAGCAGCCCACTGTGT	ATCCGCTGCACAGAGAAGGT
<i>Ncf1</i>	CTATCTGGAGCCCCCTTGACA	ACAGGGACATCTCGTCTCTTT
<i>Mmp8</i>	TGGTGATTTCTTGCTAACCCC	TACACTCCAGACGTGAAAAGC
<i>Mmp9</i>	GGACCCGAAGCGGACATTG	CGTCGTCGAAATGGGCATCT
<i>Mpo</i>	TGTCCGTGTCAAGTGGCTGT	AGTGGGGCTTCGTCTGTTGT
<i>S100a8</i>	TGAGTGCCTCAGTTTGT	CCAACACAAGGAACTCTTCG
<i>Ubiquitin</i>	TGGCTATTAATTATTCGGTCTGCAT	GCAAGTGGCTAGAGTGCAGAGTAA

Table S3. Antibodies used in this study.

Flow cytometry - Human blood		
Target	Clone	Company
CD3	UCHT1	BD Pharmigen
CD14	61D3	Invitrogen
CD15s	CSLEX1	BD Pharmigen
CD19	H1B19	eBioscience
CD16	eBioCB16	eBioscience
Flow cytometry - Mouse blood, bone marrow and lung		
Target	Clone	Company
CD3	145-2C11	eBioscience
CD4	RM4-5	eBioscience
CD8	5H10-1	Biolegend
CD11b	M1/70	Biolegend
CD11c	N418	Biolegend
CD19	eBio1D3	eBioscience
CD45	30-F11	Biolegend
CXCR4	L276F12	Biolegend

Ly6C	AL-21	BD Pharmigen
Ly6G	1A8	Biolegend
Flow cytometry - Mouse bone marrow precursors (Lin⁺)		
Target	Clone	Company
B220	RA3-6B2	Biolegend
CD3	145-2C11	Biolegend
CD4	A161A1	Biolegend
CD8	53-6.7	Biolegend
CD11b	M1/70	Biolegend
CD11c	HL3	Biolegend
CD19	6D5	Biolegend
Gr1	RB6-8C5	Biolegend
NK1.1	PK136	Biolegend
Ter119	TER119	Biolegend
Flow cytometry - Mouse bone marrow progenitors (Lin⁻)		
Target	Clone	Company
CD34	RAM34	eBioscience
c-Kit	2B8	Biolegend
FcyR	24G2	BD Pharmigen
Flt3	A2F10	Biolegend
IL-7R α	A7R34	Biolegend
Sca-1	E13-161.7	BD Pharmigen
Intravenous injection / Flow cytometry - Mouse bone marrow		
Target	Clone	Company
CD45 (PE conjugated)	30-F11	Biolegend
Intraperitoneal injection - Neutrophils depletion		
Target	Clone	Company
Ly6G	1A8	BioXcell
Immunofluorescence – Mouse lung		
Target	Clone	Company
MPO	AF3667	R&D
Anti-goat	A-11057	Invitrogen
Immunohistochemistry – Human lung		
Target	Clone	Company
CD15s (sLe ^x)	CSLEX1	BD Pharmigen