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Vorstand

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Molecular studies on *Helicobacter pylori* and the conditions for its toxicity

vorgelegt von

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Introduction

1 General information about *Helicobacter pylori* and its environment

In 1984, a few small colonies appeared on a blood agar plate after a bacterial culture of a human stomach biopsy was forgotten over an Easter holiday in a 10% CO_2 incubator at 37°C. The bacteria forming these colonies will be known as *Campylobacter pylori* (Marshall et al. 1985; Marshall and Warren 1984) . A couple of years later, new sequencing methods re-defined its identity as a new member of a new family of the epsilon-proteobacteria, the *Helicobacteraceae*, and it was named *Helicobacter pylori* (Owen 1998). The forgetful event made it possible for the Australian team to confirm their ongoing hypothesis: gastric pathologies are caused by a bacterial organism in the human stomach. Once *Helicobacter pylori* was grown and identified, the team leader Dr. Barry Marshall, decided to fulfill the Koch's postulates and drank a liquid culture of this bacterium in hope to develop gastritis, which he acquired after a certain time in a mild form (Marshall et al. 1985).This experiment branded *Helicobacter pylori* as the cause of gastric pathologies ranging from mild ones (asymptomatic gastritis) to more severe, like peptic ulceration and gastric cancer, and resulted in awarding the Nobel Prize in Physiology or Medicine to Barry Marshall and Robin Warren in 2005.

The next 30 years of research on this bacterium has revealed new characteristics about it and its relationship with humans:

In the 1990's, through epidemiological studies performed to associate the presence of the bacteria with the severe pathologies, two of its most potent toxins where discovered; the Cytotoxin Associated Gene A (CagA) (Crabtree et al. 1994; Warburton et al. 1998) and the Vacuolating Cytotoxin A (VacA) (Atherton et al. 1995; Xiang et al. 1995; Warburton et al. 1998).

In 2000, it was discovered that CagA enters the host cell and it is modified (phosphorylated) by certain host cell kinases to exert damages in cellular function. The following research established that CagA is injected into the cells by a Type IV secretion system, named Cag Type IV secretion system (Cag-T4SS) (Odenbreit et al. 2000; Backert et al. 2000). The components of this system assemble a molecular machinery capable to inject the CagA toxin

in the host cells. It shares some similarities to the Type 4 Secretion System of *Agrobacterium tumefaciens*, but it differentiates enough to drive a research spanning for over 20 years trying to resolve its function. The main reason for the research on the CagA toxin is its association with high levels of immune cell infiltration in the stomach mucosa of patients colonized by *H. pylori* strains causing chronic inflammation. This phenotype was later explained as a consequence of the increased production of chemokines by epithelial cells from patients infected with bacteria containing the Cag-T4SS and the CagA toxin (Singer and Sansonetti 2004; Crabtree et al. 1994; W Fischer et al. 2001; Stein, Rappuoli, and Covacci 2000).

Since its discovery most of the research has concentrated on the ability of *H. pylori* to cause disease. Several publications highlight the amount of damage caused by it and how to eliminate it. The medical community searched to establish uniform diagnostic parameters and treatment procedures with the only purpose to battle this microbe. These efforts resulted in a standard description of the chain of events and changes in the gastric mucosa leading to gastric cancer (Correa and Piazuelo 2012) (Figure 1). Additionally, eradication programs were placed using different combinations of antibiotics with recommendation to all medical community to eradicate upon discovery.

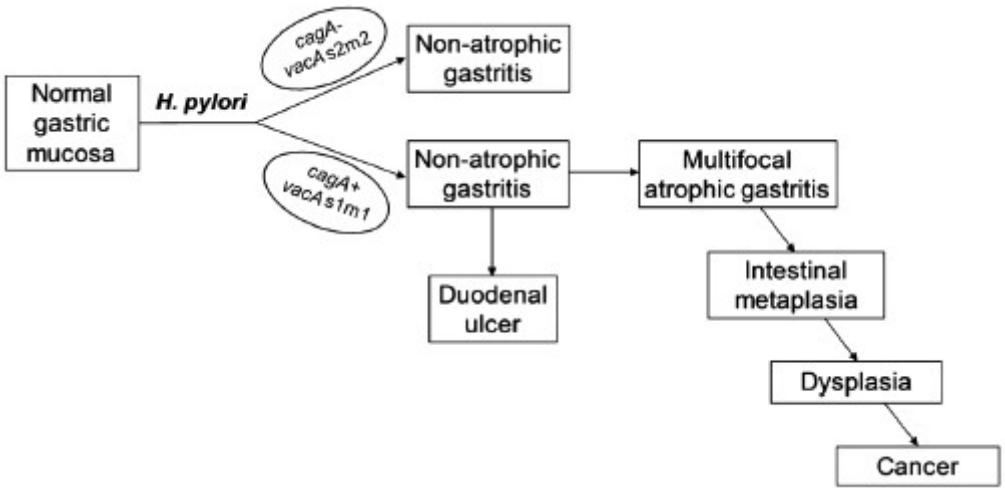

Figure 1. Gastric pathologies evolution related to the presence of *H. pylori*.
From Correa & Piazuelo J Dig Dis. 2012;13:2–9

However, two key questions remained open: How is *H. pylori* transmitted and what or where is the natural host / reservoir of this bacterium? Studies addressing these questions are plenty. There have been assumptions that water, flies and food are sources of contamination and responsible for transmission (Vaira et al. 1998; Engstrand 2001; Linz et al. 2013). While the

way of transmission is still a subject of debate, it has been now accepted that *H. pylori* lives only in the human stomach; and the fact that *H. pylori* is only evolving with humans was confirmed during studies analyzing sequences from *H. pylori strains* isolated from around the world. The data showed a correlation between variations of specific *H. pylori* genes and the actual data from human migrations, which depicts a co-evolution between humans and *H. pylori* and underlines the robust relationship between them (Falush et al. 2003; Linz et al. 2007).

Since its discovery, the medical community agreed that *H. pylori* should be eliminated upon diagnosis, and a cascade of events affecting those who were treated started to unveil a visible trend in developed countries: A higher incidence of esophagus related pathologies. One of the first concrete reports about possible consequences of the eradication of *H. pylori* appeared in the beginning of the 21st century, when the group of Martin Blaser observed lower risk of Barrett's Esophagus in patients infected with *H. pylori* (Vaezi et al. 2000) and the group of Farin Kamangar performed a meta-analysis revealing that the lack of *H. pylori* correlates with an increased risk of developing Barret's esophagus, which can develop in esophagus cancer (Islami and Kamangar 2008; Fassan et al. 2009).

Subsequent studies have shown that lack of *H. pylori* increases the chances of asthma development in children and young adults (Reibman et al. 2008). This apparently protective effect of *H. pylori* for asthma was corroborated in experimental animal studies using mice (Arnold et al. 2011). Other diseases hallmarked by failure of an adequate immune response can also be linked to the presence of *H. pylori*, as in the case of Multiple Sclerosis, Inflammatory Bowel Syndrome (IBS) and Crohn's disease (Li et al. 2007; Gavalas et al. 2015; Halme et al. 1996).

At the moment, 30 years of research in *Helicobacter pylori*, together with a new perspective on the relevance of the human microbiota in health and disease, might change the view we have of *Helicobacter pylori* from human pathogen to human symbiotic organism.

2 Objective and purpose of this professorial dissertation

Research around microorganisms and their relevance in health and disease has increased since genomic identification has overcome the challenges of the need of cultivation for their identification. At the same time, the scientific and medical communities are accepting that

microbes and their interrelationship on the human surfaces, like the gut, can alter the function of organs, including the brain (Rhee, Pothoulakis, and Mayer 2009).

As such, and because of the studies I have performed in the last years on *H. pylori*, I am inclined to consider *Helicobacter pylori* not a pathogen. I cannot deny that it possesses mechanisms and molecules that can trigger severe damage on the gastric tissue, but let us not forget that "For a fight, two are necessary", and part of this damage is caused by the host´s immune reaction. *H. pylori* can defend itself against an immune response; it has the means and no real known competition in its environment. By investigating the situations or conditions in which the toxins are causing damage, we might be able to find a way to co-exist for other thousands of years with it as we have done until now.

2.1 Objectives

- Studies on cellular parameters needed for the deleterious effects of CagA and VacA on host cells.
- Establishment of a reliable quantification of the amount of CagA injected into the host cells through western blot analysis.
- Ecology of *Helicobacter pylori*: what happens to their toxicity when they have to share the same host?

2.2 Studies on cellular parameters needed for the deleterious effects of CagA and VacA on host cells

Publications: Sewald et al 2011, Jiménez-Soto et al 2009, Kaplan-Türköz B Jiménez-Soto et al 2012, Pham KT et al 2012

Toxins need to reach their target in order to exert their deleterious effect on cells. *Helicobacter pylori* uses different mechanisms to deliver their VacA and CagA toxins. Both toxins are transported across the two-membrane system of the bacterium (inner and outer membrane) thanks to molecular machines termed secretion systems. While VacA is exported to the outer membrane and released to the supernatant through a Type V secretion system (Wolfgang Fischer et al. 2001), CagA is injected directly into the host cell through a machinery denominated the Cag Type IV Secretion system (Cag T4SS)(Odenbreit et al. 2000).

A very important function of VacA is its capacity to regulate the immune response. It achieves it by blocking the release of the cytokine interleukin-2 (IL-2). Part of a normal response from T-cells includes their clonal expansion and this is signaled by the IL-2. Therefore, VacA is able to inhibit the proliferation of T-cells hindering them to adequately respond to a *H. pylori* infection (Gebert et al. 2003). Similar activity is presented by the gamma glutamyl transpeptidase of *H. pylori* (Oertli et al. 2013) Since VacA is released into the environment, it needs to be actively taken up by T-cells upon contact with its receptor, the Integrin Beta 2 (ITGB2) (Sewald et al. 2008). However, binding is not all what is needed and the timing has to be adequate. Therefore, cellular events activating ITGB2 by serine/threonine phosphorylation through cellular PKCs set in motion the uptake of the VacA toxin by endocytosis, which affects the IL-2 production (Sewald, Jimenez-Soto, and Haas 2011). Some details on the internalization steps are still unknown: does the clustering of ITGB2 facilitate the insertion of the toxin into the membrane and its uptake or is it during the normal recycling of activated ITGB2 by the T-cells that the toxin enters the endocytic system and inserts itself into the membrane? Independently of the mechanism, the capacity of VacA to reach its target cells without direct contact makes it ideal for deployment as a long-range effector on the immune response to *H. pylori*.

While VacA may act on remote areas of the gastric mucosa, CagA reaches its target only when bacteria achieve close contact to the target cell. Since it is directly injected into the host cell, the machinery requires a docking place or cellular receptors. One of them is the cellular integral membrane protein Integrin beta 1 (ITGB1) which interacts with four components of the T4SS machinery: CagL (Kwok et al, 2007), CagA, CagI and CagY (Jimenez-Soto et al. 2009).

We have further looked at the mechanism of how these proteins are able to interact with ITGB1. In collaboration with the excellent crystallography team of Laurent Terradot in Lyon (France), the crystal structure of the N-terminal region of CagA is now known and we have located the region of CagA that interacts with ITGB1 within the amino acids 310 and 450 of the protein. A recombinant oligopeptide containing this region competes with the natural Cag T4SS reducing the amount of CagA translocated into the host cells (Kaplan-Turkoz et al. 2012).

Additionally, our studies on two of the proteins interacting with ITGB1, CagI and CagL show that both proteins interact with each other. As a consequence, any mutation in one of them

leads to the instability of the other. These results highlight their importance in the functionality of the Cag T4SS and its associated functions (Pham et al. 2012).

Recent work in our team has discovered that Outer Membrane Proteins, specifically the HopQ, are necessary for the functionality of the Cag T4SS and that other receptors on the cell might be used for the translocation in the cell (Belogolova et al. 2013; Königer et al. 2016; Javaheri et al. 2016). Ongoing work will bring further insights in the mechanisms underlying the efficient translocation of CagA in the host cell.

2.3 Establishment of a reliable quantification of the amount of CagA injected into the host cells through western blot analysis

Publications: Jiménez-Soto et al 2012, Zeitler et al 2016, Jiménez-Soto et al 2016.

Helicobacter pylori is considered a fastidious microorganism which has to be grown on rich media complemented with serum. Independently of the serum's origin (human, ovine, bovine or equine), its animal origin causes variability between production lots and companies. Additionally, the ITGB1 is able to interact with serum proteins making the growth of the bacterium on serum-complemented media a variability factor for our studies on CagA translocation (see above). *H. pylori* is able to extract cholesterol from eukaryotic cells and use it for growth thanks to its cholesterol-trans-glucosidase protein (Ctg) (Wunder et al. 2006). We therefore developed new solid and liquid media with cholesterol and fatty acid complementation instead of serum. The validation of the new media showed that most *H. pylori* strains responded well to the cholesterol supplement and the lack of serum proteins strongly facilitated extraction of secreted bacterial proteins for further studies (Jimenez-Soto et al. 2012).

The effect of cholesterol complemented media on CagA translocation showed us the need to evaluate CagA translocation in a quantitative form. Until that moment, analysis of CagA translocation has been restricted to a yes/no question by evaluating the presence or absence of tyrosine phosphorylated CagA in an infection, since effective CagA translocation causes that CagA is phosphorylated by the host cell kinases Src and Abl on the EPIYA motifs (Mueller et al. 2012; Stein, Rappuoli, and Covacci 2000; Poppe et al. 2007). Recent methods for quantification have been developed in our team using a beta-lactamase fused to the N-terminal domain of CagA (Schindele et al. 2016). Although extremely helpful for studies with high throughput, because of the use of fluorescence as readout, it depends on the enzymatic

capacity of the fused enzyme, which requires a certain amount to reach detection and, at the same time, it can reach a saturation point in different times of an experiment, depending on the experimental conditions and cellular fitness. Therefore we chose to use the phosphorylation signals from the standardized detection method to achieve a quantification of CagA translocation.

Traditional methods used in cell biology studies for semi-quantitative analysis of western blot signals assume that housekeeping protein levels remain constant. Therefore, the signal levels of detection of the protein to be tested are normalized to the signals of the housekeeping proteins (HKP). This quantification system presents conflicts in infection biology, since the treatment with an organism that alters cell processes can change the levels of HKP. Any changes in HKP expression abolishes the purpose of this proteins as normalization factor. To solve this problem, we adapted the use of the Stainfree system for the semi-quantitative evaluation of levels of CagA phosphorylation (Gurtler et al. 2013). The new protocol does not require the use of HKP as normalization factor. Instead it uses the signals of tryptophan-containing proteins that do not overlap with the signals of the bacteria. The validation of this method showed us a more stable quantification of densitometry signals and opened the possibility to evaluate several aspects of infection (Zeitler et al. 2016).

The previously described procedure, together with the development of a method for removal of external bacteria after infection, allowed us for the first time to determine the actual amount of CagA toxin injected in the host cells during the course of an *in vitro* infection. Only 1.5% of the whole CagA present in infection assays is injected into the host cells (Jimenez-Soto and Haas 2016). This came as a surprise considering that CagA is the 4th most produced protein by the bacteria, requiring a high amount of resources (Jungblut et al. 2000). These results are contradictory: very low amounts of the produced CagA are injected in the host cells and are still able to alter the cells. The synthesis of a 140 - 170 kDa protein requires many resources from the bacteria, which opens the possibility to the existence of other functions for CagA that do not include translocation in the host cell. Future studies will be aimed to determine if the CagA found outside of the bacterium plays an active role in infection.

2.3.1 Ecology of *Helicobacter pylori*: what happens to their toxicity when different *H. pylori* have to share the same host?

Publications: Jiménez-Soto et al 2013, Zeitler et al 2017, Rojas-Rengifo et al 2015, Rojas-Rengifo et al (manuscript in preparation).

In nature many kinds of *Helicobacter pylori* strains exist. Based on the presence of CagA and VacA and their variants, the strains were classified as Type I and Type II (Xiang et al. 1995). While more classifications were proposed, at the moment it has been agreed that all strains containing the *cagA* gene are Type I (cagA +) while all lacking the gene are Type II (cagA -) strains.

2.3.1.1 Data from *in vitro* studies

Infection of humans can include several different strains of bacteria. As with any living being, there might be competition for resources, like for iron. At the same time CagA has been implied in the iron homeostasis on *in vitro* assays (Tan et al. 2011). For CagA to be injected in the host cells, it requires cellular receptors, which makes feasible that, under multiple infections, bacteria will compete for them in order to inject CagA. Taking this in consideration, we performed experiments with the purpose to find out if certain Type I strains are more effective in translocation during co- and pre-infections (See Figure 2 for description of experimental setup). We found that regardless of the Type I strains used, if both were added simultaneously (co-infection), both strains were not able to inject the CagA as efficient as when they were alone. But what happens when one of the strains has 60 min advantage? In this case the second strain was unable to inject its CagA (reduction up to 90%). Is it possible that the competition was for the receptor? If yes an isotype mutant lacking the T4SS will have no effect on CagA translocation in both setups. Our data contradicted this hypothesis: Strains without Cag T4SS caused exactly the same effect. After verifying different aspects like binding capacity of both strains, minimum amount of bacteria required for the effect, transfer of strains from one well to another to rule out effect from one bacteria to the other, etc.; we found that: i) the reduction is caused as a response of the cell to its contact with the bacteria, ii) the triggering agent on bacteria is of protein nature, iii) binding of the secondary strain is not affected by the first one, iv) the reduced amount of CagA phosphorylated is not an effect on diminished host kinase activity, v) outer membrane proteins HopQ, HopI, AlpB and BabA play a role in the triggering mechanism in the cell, and vi) very low levels of bacteria

(Multiplicity Of Infection MOI 5) are able to trigger a measurable response from the cell (Jimenez-Soto et al. 2013).

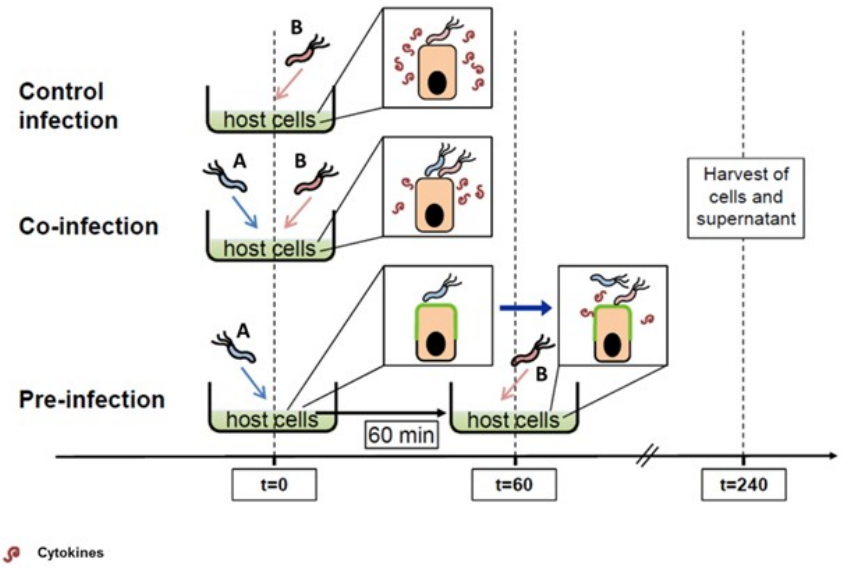

Figure 2 Experimental setup for the co- and pre-infection experiments.
Cells were synchronized and infected simultaneously (co-infection) or with time delays (pre-infections) with two different *H. pylori* strains (Strain A and Strain B in graphic). For each time point where the translocation-capable strain (Strain B) was added, a control infection was done in order to evaluate the normal capacity of the bacteria to translocate CagA in the assay. The amount of translocated CagA by the control infection determines the control value for the semi-quantitative analysis of translocation.

Based on the reduction of CagA translocation, we expected that the effect of resistance to CagA translocation should reduce the damage in the cytoskeleton associated with a CagA phosphorylation. This damage causes a deformation of the cells known as the "Hummingbird" phenotype, characterized by elongation of the cells and loss of contact points deforming the cell in a form reminding a hummingbird in flight. This hypothesis was verified, and the deformation of the cells was strongly reduced and sometimes none existent, even when the values of CagA reduction were only 50%, which could mean that the AGS cells used in our experiments have a certain threshold for the amount of CagA that starts to affect the cytoskeleton and the adhesion to the surface of the plates (data not shown). We also observed that co- and pre-infection experiments reduced the level of IL-8 secretion usually triggered by a functional Cag T4SS, which will hypothetically reduce the quantity of neutrophils recruited to the areas where *H. pylori* is interacting with the host cells. By having less inflammatory cytokines, less damage will be caused by the immune response in the gastric tissue, which

will ensure the survival of the host and the bacterium at the end of the day. The reduction of IL-8 indicates as well that the whole mechanism for CagA translocation has been affected, not only the translocation of CagA, which plays no direct role in the levels of IL-8 induced by *H. pylori* (Sokolova et al. 2013)

All these data were obtained from *in vitro* infections with AGS cells, a gastric adenocarcinoma standard cell line for CagA translocation assays. These cells have epithelia-like characteristics; however, CagA can be translocated as well in immune cells (Odenbreit et al. 2001). Therefore, we performed the same experiments in a human macrophage-like cell line, THP-1, and in primary human blood leukocytes. In both cases, multiple infections reduced the amount of translocated CagA (Figure 3).

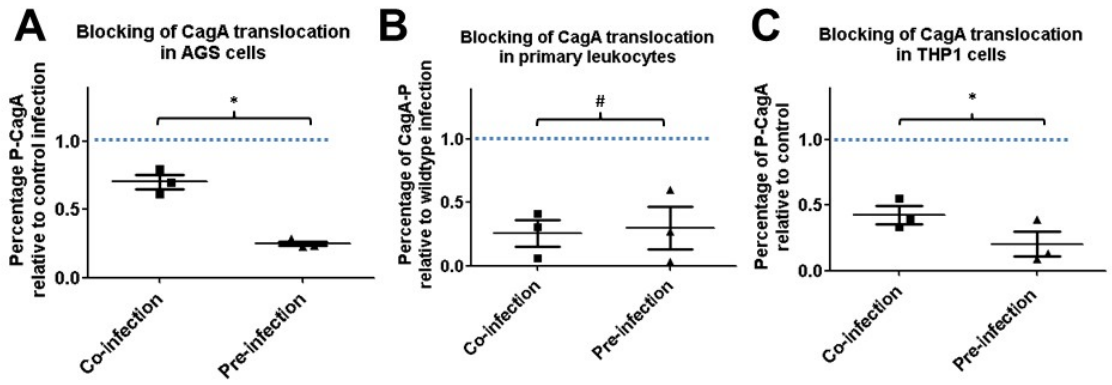

Figure 3. CagA translocation blocking effect in immune cells

In all cell types the presence of two infecting strains reduced the amount of CagA translocated (Control CagA phosphorylation levels are represented by the dotted line (Value 1). However, while in AGS cells (A) there is a strong difference between the co- and pre-infection, in primary leucocytes (B) is no difference observable; and in THP-1 cells, a macrophage-like leukemia cells line, although significant, the difference is strongly reduced compared to that observable in AGS cells.

The differences between co- and pre-infection could be caused by two different responses of the cell or by a progressive and accumulative cellular process. To determine which of both possibilities is the most plausible, we performed a time-lapse evaluation of CagA translocation and additionally IL-8 secretion (Figure 4). As seen here, the most significant effect on CagA translocation during pre-infection occurred suddenly after 50 minutes pre-infection. Early time points are able to reduce, but show no real progressive pattern in the diminished amount of CagA translocation. On the contrary, they are statistically distinguishable from the strong pre-infecting effect after 50 minutes, for which we conclude

that both phenotypes are independent events, for which the cell most probable uses different cellular processes to resist CagA translocation.

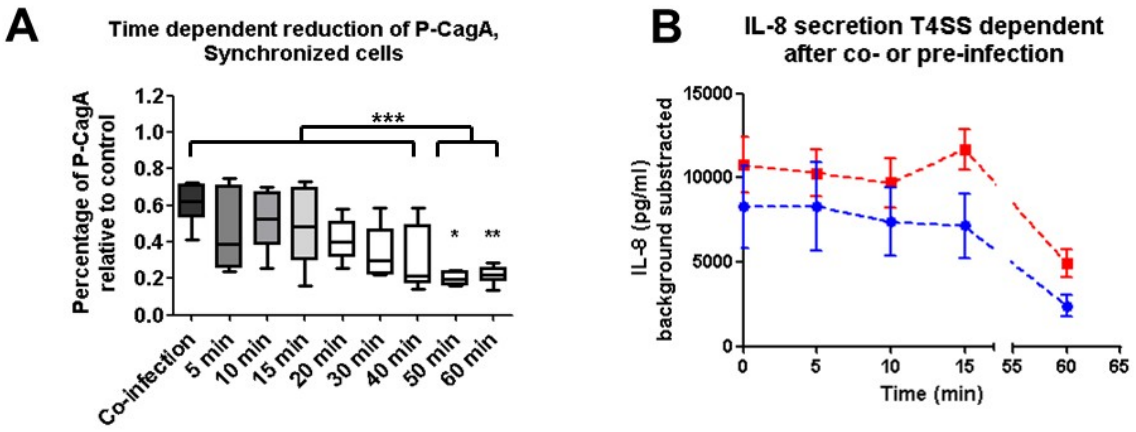

Figure 4. Time lapse evaluation of CagA translocation and IL-8 production during co- and pre-infection experiments. For CagA translocation (A), the co-infection (considered time 0) effect is distinguishable from the strong pre-infection event only after 50 min of pre-infection. In case of IL-8 (B), already after 15 min the pre-infection (blue line) is distinguishable from the control infection (red line) concerning IL-8 levels, and this is constant up to 60 minutes pre-infection.

Once established that resistance to CagA translocation is a common response of the cells to multiple infections, we asked ourselves if combinations if different *H. pylori* will cause the same response. Experiments with different combinations of Type I strains and Cag T4SS deficient strains, one mutant (P12ΔT4SSs) and two type II wild strains (X47 and Tx30a) showed that although the general effect was there (Figure 5A), some type I strains responded differently to the pre-infecting strains (Figure 5 (5B to 5C). As we compared these data to the effect on IL-8 secretion Cag T4SS dependent, we observed that the effect on IL-8 induction was only statistically significant in two of the five (5) strains tested (data not shown). As a conclusion, although multiple infections can trigger resistance to CagA translocation and its toxic effects, the amount of IL-8 produced by the cells are not always dependent on the combination of strains used.

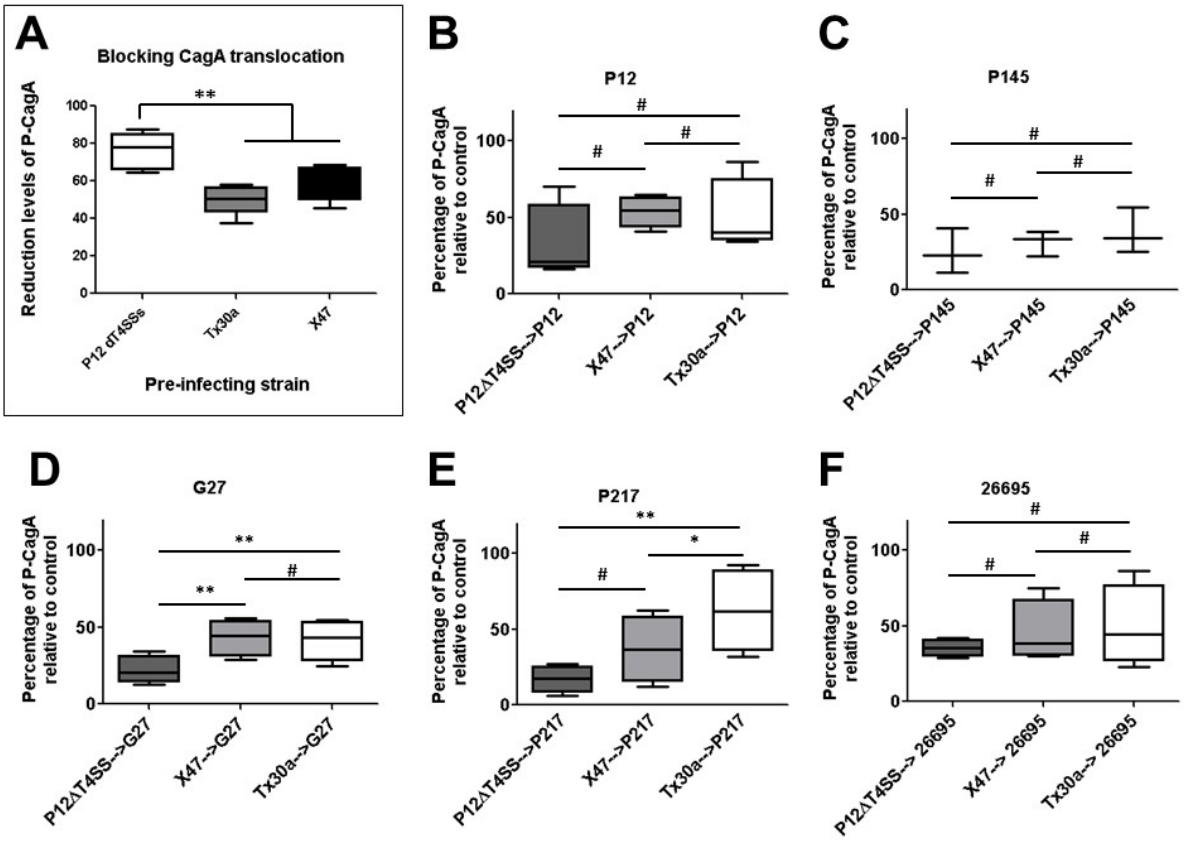

Figure 5. Resistance to CagA translocation by AGS cells in presence of different wild type multiple infections combinations.

Overall (A), co- and pre-infection will always trigger resistance to the translocation of CagA, each combination of pre-infecting strains (see details for each of the strains in publication attached) with translocating strains (P12, P145, G27, P217 and 26995, A-F) caused different levels of blocking. More important, there is a tendency for the wild type type II strains (CagA negative) to block less than the isotype mutant of a type I strain (P12ΔT4SSs).

The results obtained with multiple infections *in vitro* can have great implications for the clinical treatment of *H. pylori* if they could be translated to the human infection/colonization. The distinctive feature of pathologic *H. pylori* infection is the high level of infiltration of neutrophils in the stomach mucosa, producing gastritis, which, if it turns chronic, may lead to atrophic gastritis, intestinal metaplasia and finally gastric cancer. A few previous publications have already observed less damage in stomach tissue when type I and type II strains are present (Figura et al. 1998; Secka et al. 2011), but none of the studies have evaluated two critical aspects: How often does a multiple infection with type I and type II strains takes place? And how does this correlate with the gastric pathology? Therefore, I have started a collaboration with the microbiological laboratory in the Universidad de los Andes and with the gastroenterological department of the hospital Fundacion Santafé de Bogotá to evaluate

both aspects and determine if the *in vitro* effect of multiple infection is relevant in clinical conditions.

2.3.1.2Clinical Studies

2.3.1.2.1.1How often is a mixed infection present in Colombian patients?

Although some early studies confirm that patients can have multiple *H. pylori* strains in their stomach, to date no study has ever looked for the proportions of type I and type II in them. For this purpose we developed a quick method that allows us to screen 100 isolates per patient to classify them as type I (CagA+) or type II (CagA -) (Rojas-Rengifo et al. 2015). Although Colony Lift Immunoassays had been already reported for proteins found on the surface of bacteria, none where designed for proteins inside the bacteria, as is the case for CagA. While some bacteria have CagA on their surface, the highest amount is found inside. We were successful in establishing the immunoassay, which allowed us to perform the screening of patient isolates, 50 colonies from antrum and 50 from corpus region of the stomach, in a fast and economic way (Rojas-Rengifo et al. 2015).

Our first data revealed that only 26% of the studied population had *H. pylori* colonization, in contrast with reported 69% (Urease Breath Test, UBT) in 1996, 96.3% (Seropositive) in 2000 and 69,1% (histology) in 2003 (Goodman et al. 1996; Bravo et al. 2000, 2003). The low prevalence in our population is in accordance to another study performed in another capital city (Medellin) of Colombia, which revealed a prevalence of 36% (Correa G. et al. 2016).

The results from 92 positive samples show that *H. pylori* colonization with type I and type II is normal in the Colombian population evaluated, with most of the patients presenting mixed infections (57,3%), 26% presenting only type I strains and 16% type II strains. Since it is the first time that this level of detail on the types of *H. pylori* present in patients has been evaluated, it was interesting to realize that strains in patients with mixed population (Type I and Type II) did not present any tropism for the areas of the stomach evaluated (antrum and corpus). Additionally, during biopsy extraction procedures, it was noted by the gastroenterology team that the urease test performed routinely in the hospital directly on the biopsies, showed differences in certain patients by showing urease positive or negative results depending of the origin of the biopsy (antrum or corpus, personal communication with Dr. Belen Mendoza de Molano). As a consequence, the gastroenterological team in the hospital

has adapted the testing procedures in order to improve the diagnosis as a clinical service to the patients, by taking antrum and corpus biopsies for a reliable urease test.

2.3.1.2.1.2 Is there a correlation between Helicobacter pylori infections and pre-cancerous lesions in the population evaluated?

Pelayo Correa has proposed a cascade of events that occur upon infection with *H. pylori* (see Figure 1), where atrophic gastritis (lack of gastric glands in tissue) is the first change observed in the tissue leading towards cancer. This sequence has been accepted by most of the medical community.

Therefore, we compared the presence of atrophic gastritis and intestinal metaplasia changes, evaluated by the pathology department in the Fundación Santafé de Bogotá, with data from the microbiological analysis. To our surprise, there was no relation between any of both pathologies and the *H. pylori* status (OR 0.87). From 400 patients, only age showed to correlate with the presence of both pathologies. This contradicts all what is known about both pathologies and the Colombian population, which is considered to be one of the countries with the highest incidence of gastric cancer (Kudo et al. 2005) and several populations from the southwest coast and mountain regions have been studied previously showing a correlation of *H. pylori* and gastric cancer (Goodman et al. 1996; Torres et al. 2013). However, other factors, like social-economic status have to be considered before comparing the data from the population we analyzed and those previously reported, since the population admitted to the hospital Fundación Santafe de Bogota, attends the socio-economical classes 5 and 6, which represent the higher middle class and high class of the Colombian population.

The data obtained in our study suggests that *H. pylori* is not the cause of severe forms of gastric tissue changes and that other factors, e.g. environmental or social influences might be important in the development of pre-cancerous gastric lesions in the population analyzed.

2.3.1.2.1.3 Does the level of type I and type II strains in a stomach affect the level of inflammation in patients?

Although our results contradict the published relationship of *H. pylori* and pre-cancerous lesions, we decided to evaluate the effect of a mixed infection for the level of neutrophil infiltration in tissue.

Considering that neutrophil infiltration in the tissue causes inflammation, and chronic inflammation can lead to carcinogenesis (Fu et al. 2016), we analyzed the levels of neutrophil

infiltration (neutrophil activity) in the mucosa of all patients with mixed infections, to compare if the percentage of type I vs type II strains in each patient correlates with variations in neutrophil infiltration. We observed no correlation between the percentages of Type I and Type II strains with the level of neutrophils in the tissue analyzed (Figure 6).

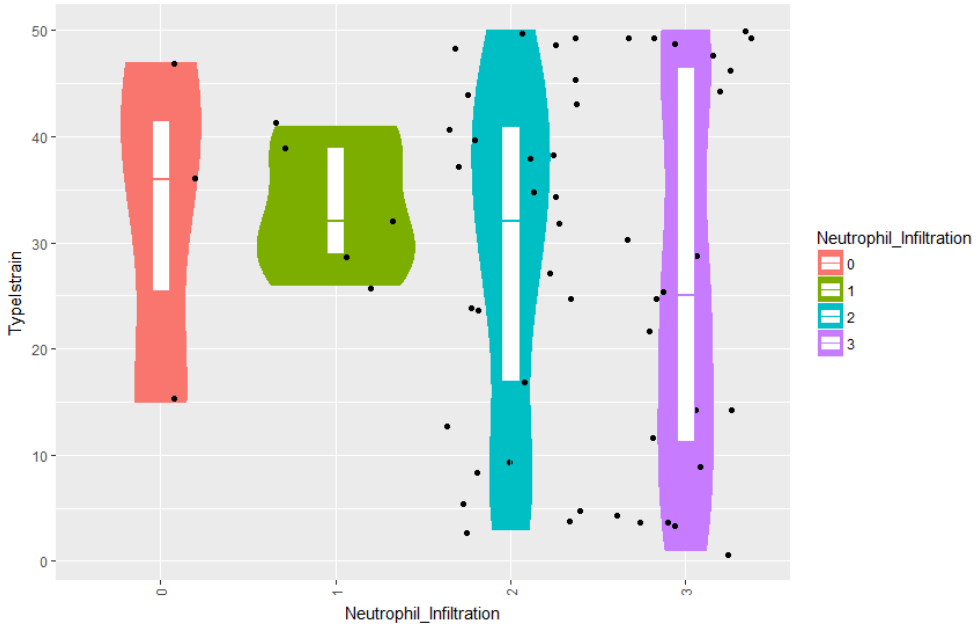

Figure 6. Effect of proportions of type I and type II strains of *H. pylori* in tissue levels of neutrophil infiltration.
Violin plots showing the distribution in percentage of type I strains in mixed infected patients (Y-axis) vs the levels of neutrophil infiltration in tissue (X-axis), ranging from no neutrophil detection (0) in mucosa to high levels of neutrophil detection in the upper mucosa (3). The grading was done in accordance to international standards for diagnostic (Sydney-System)

2.3.1.2.1.4 Effect of multiple infections in adaptation to cytokine induction

From each patient, the strains evaluated in the colony blot assay were stored for further analysis. We selected Type I strains from patients with mixed infections and compared them to the Type I strains from patients presenting only Type I strain infection in their capacity to induce IL-8, an important cytokine in the recruitment of neutrophils to the infected tissue. Our data revealed that the type I strains from mixed infection patients induced less IL-8 than those from type I strains only (Figure 7). This could indicate a selective pressure to develop less gastritis in those patients presenting Type I and Type II strains compared to those with only Type I strains.

Taking advantage of the advances made in recent years on genome sequencing, I am planning to further analyse the genome of these strains and get an idea of the genetic adaptations that

have been taking place in the strains Type I from mixed patients in order to reduce their impact on the immune response of cells.

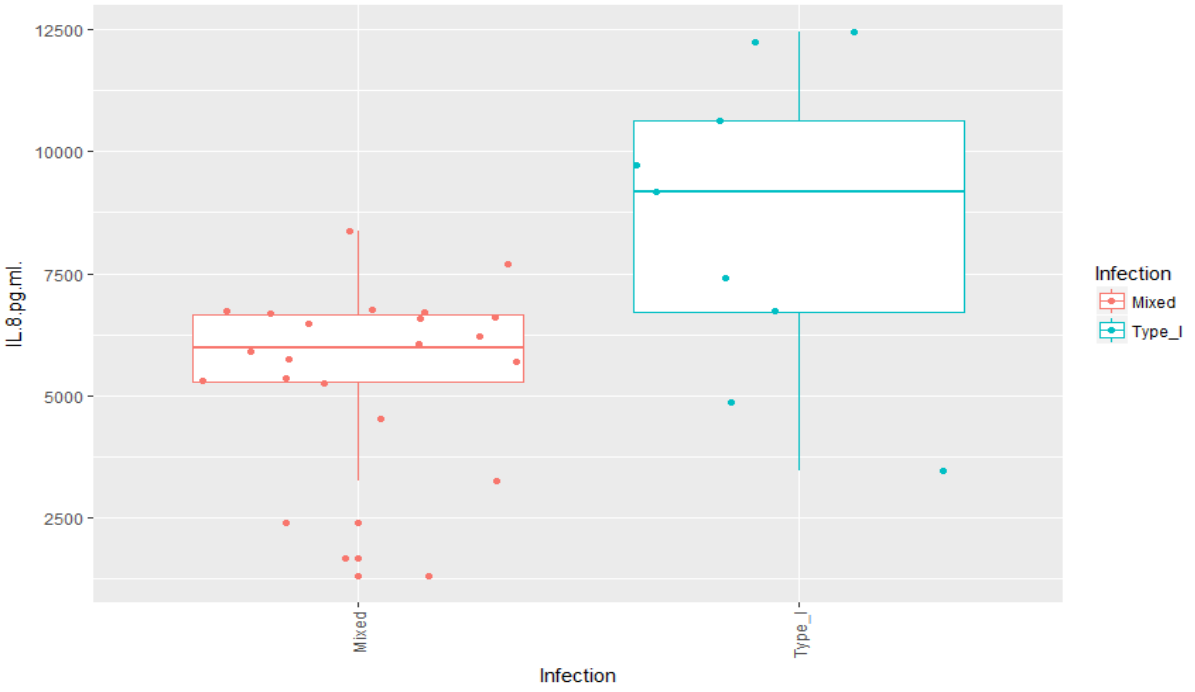

Figure 7. Effect of strain origin in IL-8 induction.

Type I strains from mixed strains-infected patients induce less IL8 production than Type I strains from Type I strain-infected patients. Statistical analysis using a Kruskal-Wallis chi-squared = 6.3712, df = 1, p-value = 0.0116

2.3.1.2.1.5Do strain combinations from patients show the same variability as observed with the lab strains?

We have observed that different combinations of Type I and Type II strains cause variations in the level of blocking CagA translocation. But is this the same in the host? In order to answer this question, we used the isolated strains from patients with mixed infections, and we tested their capacity of CagA translocation and IL-8 induction during co- and pre-infections *in vitro* to evaluate their capacity to block the CagA translocation in AGS cells. At the same time, we wish to observe if the histological data (neutrophil activation and presence and absence of atrophic gastritis) correlate with their *in vitro* behavior. The data confirmed that different combinations of strains cause different effects in CagA translocation (Figure 8) as it was observed previously with the laboratory strains (see above) and that their differences vary depending on the combination of the strains. Remarkable was the data obtained for co-infections with strains from patient 163, which reveal a potentiation of the amount of CagA translocation, in some cases to a double amount of what the single infection control achieved.

This shows that not only can the mixture of strains cause a reduction of toxin in the cells, but as well, certain combinations can promote the toxicity of the bacteria.

Sadly, due to the low number of mixed colonies that survived the shipping from Colombia in order to do the evaluation in Germany, we cannot reach any conclusion regarding the strains' behavior *in vitro* in relation with the histological data obtained. Further efforts will be made in order to develop a safe passage and survival of strains during transport, and therefore increase the amount of strain combinations evaluated in *in vitro* assays.

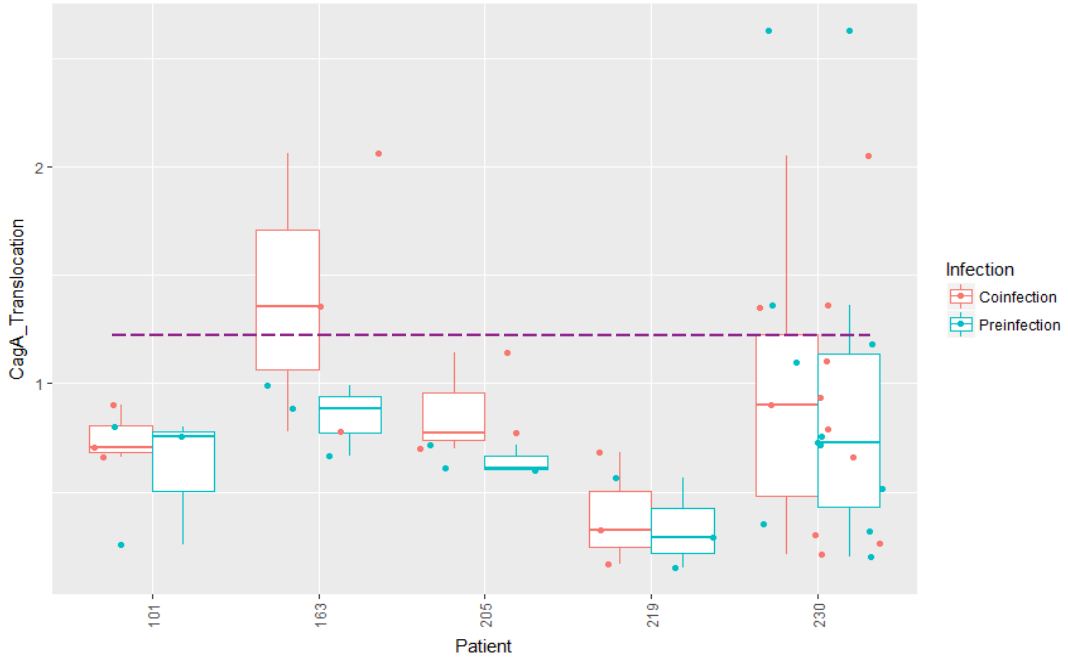

Figure 8. Blocking of CagA translocation in co and pre-infection assays by type II strains with their corresponding type I strains from mixed infected patients.

Type I and Type II strains from five (5) patients (Patient numbers 101,163, 205, 219 and 230, X axis) with mixed infections were used for co- and pre-infection experiments (red and blue boxes, respectively) and the amount of CagA translocated evaluated in each condition relative to the infection controls (Y axis, fold normalized to control infection (single infection with CagA positive strain)) that represent a value of 1 and it is represented by the dotted line.

3 Concluding remarks and Outlook of the research

Research on *Helicobacter pylori* as a pathogen has shed light on the pathogenesis mechanisms that this bacterium has developed in order to survive in the human stomach. The last 30 years have brought light especially to the way the major toxins CagA and VacA are able to modify their stomach environment and control the immune response of the host. At the same time, the insight we have gained through this research has revealed a novel aspect of its relationship

with humans: *H. pylori* has been part of the human stomach since before humans migrated out from Africa (Linz et al. 2007).

We cannot deny that the use of toxins by *H. pylori* can be deleterious to the gastric tissue. Our studies performed with the purpose of learning more about the molecular and cellular requirements to cause disease are important in order to diminish their effect. However, our data on the resistance to CagA toxin effects by co- and pre-infections have revealed that the ecology of *H. pylori* can have an important effect on the toxicity of this oncogenic protein and therefore in their relationship with its host.

At the same time, considering that recent research shows that exposure to *H. pylori* reduces the incidence of diseases related to imbalanced immune responses, we should rethink the eradication policy applied upon its discovery on gastric tissue and consider other possibilities to reduce the capacity of the bacteria to trigger damage in the human stomach, and this can be achieved through basic research on its biology and its interaction with other microorganisms present in the host, independent if they are transient or resident flora. All can have an impact on the behavior of *H. pylori*

For now, if our *in vitro* data on multiple *H. pylori* strains infecting the same host can be confirmed in human patients, we would not only have found an explanation for disagreements previously published about the correlation of CagA presence and disease, but we could consider the use of Type II strains as a method to bring back the balance to the ecosystem in the stomach and to reduce the immune response causing chronic inflammation. This idea should be approached carefully. Our data has revealed that although most of the *H. pylori* strain combinations reduced the effect of CagA toxin and the levels of proinflammatory cytokine IL-8 induced *in vitro*, it shows as well that a personalized evaluation for the best combination of bacteria will be necessary, in order to obtain the best results in a therapy of gastritis using inoculation of adequate *H. pylori* strains.

It is my wish to follow a field of research that combines recent discoveries concerning the effect of microbiota and disease and our knowledge of *H. pylori* to re-evaluate the treatment of gastric diseases related to *H. pylori* colonization. And in order to do so, we have to start to consider *H. pylori* not as a pathogen, but as commensal bacteria, and to treat the associated gastric diseases as a consequence of dysbiosis.

Table of Figures

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From a very early age, since visiting my grandparents farm, I developed an immense love for nature and a strong curiosity for its processes. As a result, I possess a strong appreciation for life and the interactions of all living beings. As microbiologist I decided to concentrate in the interactions of VERY small living beings. I have learned so much from them and their role in nature in my years of research, and an integral part of my research I have been able to communicate further all the marvelous tricks and details my work has taught me about cells, bacteria and viruses. But all could not have been possible without very important people in my life. The first one I want to thank is my husband. He is my strongest sponsor and he has been my greatest support during my life as scientist. But this career could not have been possible without the support and open mindedness of Rainer Haas, my mentor and complice in curiosity about *Helicobacter pylori*. He has supported my unconventional view of life and allowed me to follow many ideas. Many of them have not made it to this document, as it is normal in scientific research, but all of them have taught me more about myself and the people around me.

I want to thank Walther Mothes for opening me the door to research and the marvelous world of cell biology from the microbial view. Here I learned that in order to get to know the cells, I have to follow the steps of the experts: Bacteria and Viruses.

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- Co-infection experiments for CagA translocation. Protocols.io. Jun 08, 2017. DOI: dx.doi.org/10.17504/protocols.io.hjpb4mn

Eidesstattliche Versicherung

Ich, Dr. Luisa Fernanda Jiménez Soto, erkläre hiermit an Eides statt,

dass ich die vorliegende Habilitationsarbeit mit dem Thema

Molecular studies on *Helicobacter pylori* and the conditions for its toxicity

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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München, 6.November, 2017

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Dr. Luisa F. Jiménez Soto

Erklärung

Ich, Dr. Luisa Fernanda Jimenez Soto, erkläre hiermit, dass

- ich nicht schon zweimal ein Habilitationsverfahren im gleichen Fach ohne Erfolg beendet habe, und
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München, 6.November, 2017

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Dr. Luisa F. Jiménez Soto