Cross-sectional study of human coding- and non-coding RNAs in progressive stages of *Helicobacter pylori* infection

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*Helicobacter pylori* infects 4.4 billion individuals worldwide and is considered the most important etiologic agent for peptic ulcers and gastric cancer. Individual response to *H. pylori* infection is complex and depends on interactions between host and environmental factors. The pathway towards gastric cancer is a sequence of events known as Correa’s model of gastric carcinogenesis, a stepwise inflammatory process from normal mucosa to chronic-active gastritis, atrophy, metaplasia and gastric adenocarcinoma. This study examines gastric clinical specimens representing different steps of the Correa pathway with the aim of identifying the expression profiles of coding- and non-coding RNAs that may have a role in Correa’s model of gastric carcinogenesis. We screened for differentially expressed genes in gastric biopsies by employing RNAseq, microarrays and qRT-PCR. Here we provide a detailed description of the experiments, methods and results generated. The datasets may help other scientists and clinicians to find new clues to the pathogenesis of *H. pylori* and the mechanisms of progression of the infection to more severe gastric diseases. Data is available via ArrayExpress.

**Background & Summary**

*Helicobacter pylori* is one of the most successful human bacterial pathogens, infecting 4.4 billion individuals worldwide$^1$. Infection can induce gastric pathologies ranging from chronic gastritis in all infected individuals to peptic ulcers (in 15–20% of patients) and gastric cancer (0.5–1% of patients)$^2$.

Individual response to *H. pylori* infection is complex and depends on a combination of environmental factors, genetic background, host response and strain virulence$^3$. The pathway towards gastric cancer is a sequence of events known as Correa’s model of gastric carcinogenesis, a stepwise inflammatory process from chronic-active gastritis (CAG), atrophy (AT), intestinal metaplasia (IM) and gastric adenocarcinoma$^4$.

This study examines gastric clinical specimens representing different steps of the Correa pathway with the aim of identifying the expression profiles of coding- and non-coding RNAs (microRNAs and small RNAs) that may have a role in Correa’s model of gastric carcinogenesis and, potentially, to develop novel clinical biomarkers.

RNAseq (for microRNAs and non-coding RNAs) and microarrays (for coding RNAs) were used to screen for differentially expressed genes in gastric biopsies (antrum/corpus). The expression of a selection of genes was confirmed in a validation cohort of patients using quantitative real-time PCR (RT-qPCR). The general study design is illustrated in Fig. 1. Here we provide a detailed description of the experiments conducted, methods used and results generated. The datasets may help other scientists and clinicians to find new clues to the pathogenesis of...

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H. pylori and the mechanisms of progression to severe disease states. The transcriptomics data is available in the ArrayExpress database5.

Methods
Patient selection. The Digestive Service has assembled a collection of samples from dyspeptic patients. The study was undertaken in accordance with the Declaration of Helsinki, with the approval of the ethics committee at our institution (code: 2005511; approval date: 2006/1/11).
For histopathological evaluation, sections were stained with haematoxylin-eosin (Fig. 2). Demographic and clinical characteristics of patients can be found in Online only Table 1.

RNA extraction and quality control. Two antrum and two corpus biopsies were used to isolate total RNA. Total RNA was extracted using the mirVana miRNA isolation kit (ThermoFisher, MA, USA) as per the manufacturer’s protocol and stored at −80 °C for downstream analysis. DNase treatment was performed as described in the DNA-free Kit protocol (Cat. No. AM1906, ThermoFisher, MA, USA). Total RNA was quantified with the Qubit® RNA Assay Kit (ThermoFisher, MA, USA). Quality was assessed using Agilent RNA 6000 Nano chips (Cat.No. 5067-5151) on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), including calculation of the RNA integrity number (RIN). The RIN score was 7.72 ± 0.6 (Fig. 3).

mRNA microarrays. Biotin-labeled cRNA samples for hybridization were prepared from 200 ng total RNA using Epicentre TargetAmp Nano-g Biotin-aRNA Labeling Kit for Illumina system (Cat. No. TAN091096; Epicentre, WI, USA). Labeled cRNA was hybridized to the HumanHT-12_V4.0 expression arrays (Cat. No. BD-103-0204; Illumina Inc., San Diego, CA) as described in the protocol/instructions. HumanHT-12 v. 4 Expression arrays were scanned with the iScan system (Illumina Inc., San Diego, CA, USA) and raw data were decoded using GenomeStudio Gene Expression Module (Illumina Inc., San Diego, CA, USA). Intensities were quantile-normalised and differentially detected transcripts were calculated using the Bioconductor limma package⁶.

miRNA and small RNA sequencing. TruSeq miRNA and small RNA library preparation. Briefly, 3' adapter ligation was performed by incubating 1 µg of total RNA of each sample with the adapter for 2 minutes at
Fig. 3 Quality control of the RNA samples, sequencing libraries and sequencing reads. (a) Agilent Bioanalyzer electropherogram showing total RNA from sample #47 (left) and violin plot of RIN values according to disease state (right). (b) Library Size Selection by resolution of total RNA on 6% Novex TBE PAGE Acrylamide gels. Original acrylamide gel for sample #47 (left), after 145–160 bp miRNA bands (middle) and small-RNA fragments (200–300 bp) (right) were excised. (c,d) Final library QC. Precise library quantification was performed using real-time PCR and size distribution was assessed with Agilent BioAnalyzer High Sensitivity DNA Chips. Upper and lower panels show sample #47 miRNA and small-RNA libraries, respectively. (e) Sequencing of the 192 libraries generated over $9.8 \times 10^8$ raw reads. Mean library counts were $5.9 \times 10^6$ and $4.4 \times 10^6$ for miRNAs and small RNAs, respectively. The panels show the distribution of quality scores per base (upper panels) and the read count per library (lower panels) for both miRNA (left panels) and small-RNAs (right panels).
70 °C. Then 5'-adapter was added alongside using a truncated T4-RNA ligase 2 (Cat. No. M0351S, New England Biolabs, MA, USA) in an incubation at 28 °C for 1 hour. Half of the ligation product was used for the reverse transcription performed with SuperScript II reverse transcriptase (Cat. No. 18064-014, ThermoFisher, MA, USA) in a thermocycler for 1 hour at 50 °C. Next, enrichment of the cDNA was performed using PCR cycling: 98 °C for 30 secs; 11 cycles of 98 °C for 10 secs, 60 °C for 30 secs and 72 °C for 15 secs; a final elongation of 72 °C for 10 mins, and pause at 4 °C. PCR products were resolved on 6% Novex TBE PAGE gels (Cat. No. EC6265BOX, ThermoFisher, MA, USA). microRNA and Small_Non-coding-RNA fragments between 145–160 and 200–300 bp respectively, were cut from the gel. microRNA and Small_Non-coding-RNA libraries were extracted from polyacrylamide gel with the MinElute gel extraction kit (Cat. No. 28604, Qiagen, Germany) using an adapted protocol, in which gel slices were dissolved in a diffusion buffer (0.5 M ammonium acetate; 10 mM magnesium acetate; 1 mM EDTA, pH 8.0; 0.1% SDS) overnight at room temperature plus 3 hours and 30 min at 50 °C. The libraries were visualized on an Agilent 2100 Bioanalyzer with the Agilent High Sensitivity DNA kit (Cat. No. G2938-90320, Agilent Technologies, Santa Clara, CA) and quantified using quantitative PCR with the Kappa Library Quantification Kit (Master Mix and DNA Standards, Cat.No. KK4824, Roche-Kappa, Basel, Switzerland).

Next-generation sequencing (NGS). The libraries were pooled, and 12pM 12xmicroRNA-libraries and 14pM 12xSmall_Non-codingRNA-library pools were sequenced. Multiplexed libraries were hybridized to flow cells on a cBot Cluster Generation System (Illumina, San Diego, CA, USA) using TruSeq SR Cluster Kit v3-cBot-HS (Cat. No. GD-401-3001; Illumina, San Diego, CA, USA). The clustered flow cells were loaded onto a HiScanSQ sequencer. The sequencing was performed using the TruSeq SBS Kit v3-HS (Cat. No. FC-401-3002; Illumina, San Diego, CA, USA) for 50 cycles.

NGS Data analysis. Base calling was performed with the Illumina Real Time Analysis software (RTA, version 1.13.48) and the FASTQ files were generated with CASAVA (version: 1.8.1). Secondary data analysis was done using the sRNAbench package 9. Briefly, reads were aligned to the human genome (UCSC hg19) using Bowtie 1.1.2 10. miRNA annotations were obtained from miRBase 11. Sequencing analysis was done by using the sRNAbench package 8. Briefly, after adapter trimming and unique read grouping, reads were aligned to the human genome (UCSC hg19) using Bowtie 1 allowing for one mismatch. To provide annotations for RNA elements that mapped to the human genome, miRBase 12 was used for mature and pre-miRNA sequences was used and a matrix of counts were created. To process count and to identify differentially expressed miRNAs we use edgeR package 12. Transcripts were considered differentially expressed provided their edgeR FDR-adjusted P value was < 0.05.

Quantitative PCR validation. Twenty-five RNAs were reanalyzed to validate 24 messenger RNAs and 12 miRNAs. The RNAs used were a subset (n = 25) of the aliquots of the same RNA samples we used for sequencing and microarray analysis. Studied genes are summarized in Table 1.

cDNA synthesis. miRNA validation was performed using the miRCURY LNA Universal RT microRNA PCR system (Exiqon, Denmark). miRNAs were reverse transcribed according to the manufacturer’s protocol using 10 ng of total RNA (Cat. No. 203301; miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit II). For coding RNAs, 1.0 µg of total RNA was converted into cDNA using PrimeScript RT Reagent Kit (Cat. No. RR037A, Takara, Japan).

Quantitative PCR. Coding RNAs were amplified using predesigned PrimeTime 5' Nuclease Assays (IDT, Iowa, USA) (assay catalog numbers are in Table 1) and PremixExTaq Probe qPCR mastermix (Cat. No. RR390W; Takara, Japan). miRNAs were quantified using predesigned microRNA LNA PCR Primer sets (Exiqon, Denmark) and SensiMix SYBR Low-ROX Kit (Cat. No. QT625-05, Bioline, UK). Amplification was performed in duplicate on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 384-well plates.

qPCR data analysis. The raw PCR data was exported from QuantStudio Real-Time PCR Software v1.2 (Applied Biosystems) onto a RDML 13 file and imported into LinRegPCR (v2016.1) 14. LinRegPCR was used to determine PCR efficiencies (E) and to calculate the starting concentration per sample (N0). First, the program determines the baseline fluorescence and performs baseline subtraction. Then a Window-of-Linearity for all PCR samples per amplicon is set and then the algorithm determines: the mean PCR efficiency per amplicon (Emean), the quantification cycle (Cq) value per sample and the fluorescence threshold set to determine the Cq (Nq). With these data, N0 is calculated using N0 = Nq / (Emean)^Cq.

Data Records

Individual miRNA and small-RNA FASTQ files and a tab-delimited file for the processed microarray data have been deposited in the ArrayExpress public repository 3. The accession numbers are: E-MTAB-8890 for miRNAs, E-MTAB-8896 for small RNAs and E-MTAB-8889 for mRNAs. The sample metadata records are provided in Online only Table 1.
### Technical Validation

#### Quality control. Sample collection. In order to ensure the collection of biopsy tissue samples would provide high-quality results for microbiology, molecular analysis and histology, a two-round biopsy protocol was followed. During the endoscopy, a first set of biopsy samples was collected for microbiological (in sterile saline) and molecular analysis (in RNAlater) and a second set were fixed in formalin for histopathological examination. By doing this, we ensured that formalin contamination of biopsy forceps did not interfere with the RUT and *H. pylori* culture. Histological examination was performed by a pathologist specialized in digestive diseases. In order to increase the total RNA yield and because intestinal metaplasia is typically present as small mucosal patches, we isolated RNA from two gastric biopsies per anatomical location. The reason is that the biopsy cores examined by the pathologist are different from the biopsy specimens used for molecular analysis. By using two biopsies, we were more confident that if the pathologist reported intestinal metaplasia in the histology specimens, intestinal metaplasia would also be present in the molecular biology cores. Additionally, two biopsies are the minimum recommended by the Updated Sydney System18.

#### RNA processing. Figure 2 shows the quality control procedures used in this study for RNA integrity, library preparation and sequencing.

| GENE Symbol | Refseq accession | Detects all variants[a] | Exon location[b] | Mean PCR efficiency[b] | Company and catalogue number |
|-------------|------------------|-------------------------|-----------------|------------------------|-----------------------------|
| AGPAT2      | NM_006412(1)     | No                      | 4–5             | 1.86                   | IDT Hs.PT.58.1470724         |
| ACTB        | NM_001101(1)     | Yes                     | 6–6             | 1.86                   | IDT Hs.PT.56a.40703009.g     |
| ANXA13      | NM_0045006(2)    | Yes                     | 10–11           | 1.90                   | IDT Hs.PT.56a.20938889.g     |
| APOB        | NM_000384(1)     | Yes                     | 8–9             | 1.87                   | IDT Hs.PT.56a.19389676       |
| C3          | NM_000064        | Yes                     | 27–28           | 1.90                   | IDT Hs.PT.56a.28400009.g     |
| CDX1        | NM_001804(1)     | Yes                     | 1–2             | 1.89                   | IDT Hs.PT.58.468499         |
| CFH         | NM_001014975(1)  | No                      | 9–10            | 1.89                   | IDT Hs.PT.58.41054235       |
| CPS1        | NM_001122633(3)  | Yes                     | 27–28           | 1.88                   | IDT Hs.PT.58.2708374        |
| CREB1       | NM_134442        | No                      | 3–5             | 1.89                   | IDT Hs.PT.58.4988504        |
| CXCR5       | NM_001716(1)     | No                      | 1–2             | 1.89                   | IDT Hs.PT.56a.1692541       |
| EIF4G2      | NM_001042559(3)  | Yes                     | 3–5             | 1.91                   | IDT Hs.PT.58.6917393        |
| FUT9        | NM_008581        | Yes                     | 2–3             | 1.89                   | IDT Hs.PT.58.22395619       |
| HIPK3       | NM_005734(2)     | Yes                     | 3–4             | 1.89                   | IDT Hs.PT.58.2927056        |
| HNF4G       | NM_004133(1)     | Yes                     | 2–3             | 1.86                   | IDT Hs.PT.58.26955600       |
| IL8         | NM_000584(1)     | Yes                     | 3–4             | 1.82                   | IDT Hs.PT.58.38869678.g     |
| KRT20       | NM_019010(1)     | Yes                     | 5–6             | 1.89                   | IDT Hs.PT.58.39027228       |
| MEG3        | NR_002766(8)     | No                      | 5–10            | 1.86                   | IDT Hs.PT.58.25426100       |
| MMP9        | NM_004994(1)     | No                      | 3–4             | 1.84                   | IDT Hs.PT.58.22814824.g     |
| MTP         | NM_000253(1)     | Yes                     | 18–19           | 1.87                   | IDT Hs.PT.58.94487         |
| MUC2        | NM_002457(1)     | Yes                     | 28–30           | 1.89                   | IDT Hs.PT.58.4321237        |
| POPT1       | NM_015352(1)     | No                      | 6–7             | 1.78                   | IDT Hs.PT.58.19361092       |
| RUNX2       | NM_001024630(3)  | Yes                     | 6–7             | 1.88                   | IDT Hs.PT.56a.19568141      |
| SDHA        | NM_004168(1)     | Yes                     | 3–4             | 1.88                   | IDT Hs.PT.58.41017719       |
| TFF3        | NM_003226(1)     | Yes                     | 1–2             | 1.89                   | IDT Hs.PT.58.1814807        |
| WDR1        | NM_017491(1)     | No                      | 4–5             | 1.88                   | IDT Hs.PT.58.40308614       |
| MIR103A1    | NR_029520.1      | NA                      | NA              | 1.89                   | Exiqon 204063               |
| MIR146A     | NR_029701        | NA                      | NA              | 1.86                   | Exiqon 204688               |
| MIR153–1    | NR_029563        | NA                      | NA              | 1.81                   | Exiqon 204338               |
| MIR155      | NR_030784.1      | NA                      | NA              | 1.90                   | Exiqon 204308               |
| MIR182      | NR_029614.1      | NA                      | NA              | 1.89                   | Exiqon 206070               |
| MIR191      | NR_029690.1      | NA                      | NA              | 1.86                   | Exiqon 204306               |
| MIR192      | NR_029578.1      | NA                      | NA              | 1.91                   | Exiqon 204999               |
| MIR196B     | NR_029911.1      | NA                      | NA              | 1.88                   | Exiqon 204555               |
| MIR199B     | NR_029490.1      | NA                      | NA              | 1.85                   | Exiqon 204450               |
| MIR204      | NR_029621.1      | NA                      | NA              | 1.89                   | Exiqon 206072               |
| MIR215      | NR_029628.1      | NA                      | NA              | 1.87                   | Exiqon 204598               |
| MIR340      | NR_029885.1      | NA                      | NA              | 1.89                   | Exiqon 206068               |

Table 1. qPCR Primer assays used for mRNA and miRNA validation. aPrimer assays targeting all splicing variants were chosen for validation purposes, and when possible, in the same exon where the Illumina probe was positioned. bPCR efficiency was calculated by LinRegPCR software. Using the raw qPCR data, the algorithm computes iteratively a Window-of-Linearity for a specific amplicon and calculates the Cq and PCR efficiency for each individual reaction and amplicon. NA: not applicable.
Gene expression validation by qPCR. We used LinRegPCR\textsuperscript{18} for calculating individual and mean PCR efficiencies. Amplicons showed high PCR efficiencies, ranging from 1.78 to 1.91. PCR inhibition can be detected using individual PCR efficiency values. Samples showing PCR efficiencies greater than 5% of the PCR mean efficiency per amplicon were excluded. The algorithm also calculates \( N_0 \), \( N_0 \) is the starting quantity of mRNA or miRNA.

Validation of miRNA sequencing results using RT–qPCR

Validation of mRNA microarray results using RT–qPCR

Fig. 4 Validation of miRNAs (a) and messenger RNAs (b) by RT–qPCR. A panel 12 of miRNAs and 24 mRNAs were selected for validation of 25 RNA samples. Aliquots of the same RNA samples were used for sequencing, microarray and qPCR measurements. Raw qPCR data was exported to LinRegPCR software. \( N_0 \) (an estimate of the target starting concentration per reaction) was calculated using the formula \( N_0 = N_\text{r}/E^{10} \) where \( E \) is the amplicon PCR efficiency and \( N_\text{r} \) is the fluorescence threshold set to determine \( C_\text{q} \). The Pearson correlation coefficient \( (R) \), the \( p \)-value and 95% confidence interval are indicated. Additional correlations to genes having multiple probes can be found in ref. \textsuperscript{39}. 

\[ \text{log}_2 (\text{qPCR } N_0) \]
(expressed in arbitrary fluorescence units). Quantitative N₀ values have been used in previous publications²⁸⁻²⁴. Determining N₀ has several advantages over relative quantification. First, the selection of a housekeeping gene is often controversial since the expression of all genes is regulated. Second, the expression of a housekeeping gene varies to a greater or lesser extent under experimental conditions²⁵. Third, to solve this issue a quantitative PCR approach with a correction factor according to the starting amount of RNA used in the reverse transcription has been recommended (i.e. µg of RNA)²⁶ instead of relative quantification.

To evaluate the concordance in gene expression between microarray or RNA-seq and qPCR, we calculated the correlation between normalized microarray/RNA-seq and qPCR log transformed N₀ values (Fig. 4). Overall, high R and low p-values values (R > 0.8, p < 0.001) were observed between microarray and qPCR measurements. Some of them were probe dependent (i.e. C3 probe ILMN_1762260: R = 0.79, p < 0.001, but C3 ILMN_1662523 was not correlated). Five miRNA showed high correlation (R > 0.7, p < 0.001), 4 were poorly correlated (R ~ 0.4, p < 0.05) and 3 were not correlated.

**Usage Notes**

miRNA, small-RNA raw sequencing data (FASTQ) and normalized microarray data can be analysed by a variety of freely accessible packages and platforms, such as R/Bioconductor²⁷. Some R/Bioconductor packages can be used without prior programming knowledge by using the Galaxy platform²⁸.

The authors encourage proper citation of data sources for any work based on this dataset.

Received: 6 April 2020; Accepted: 19 June 2020;
Published online: 08 September 2020

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Acknowledgements
This study was funded by the Fondo de Investigación en Salud (FIS), Instituto de Salud Carlos III, ISCiii (PI12/01802). We are also indebted to A. Salvador for her help with the English.

Author contributions
A.M.A., A.GH and J.L.L processed the microarrays, performed the RNA sequencing and discussed and contributed to writing. J.L. and M.V.C. performed omics data analyses and discussed and contributed to writing. A.B.V. and M.E.Q. performed RNA extraction, QC and qPCR experiments. X.C. designed the study and discussed and contributed to writing. S.L. and M.J.R.L. designed the study, contributed to the patient consent, collection of samples, qPCR data analysis, wrote and edited the manuscript. All authors read and approved the manuscript.

Competing interests
The authors declare no competing interests.

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