microRNA profiling in duodenal ulcer disease caused by *Helicobacter pylori* infection in a Western population.

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SUPPLEMENTARY MATERIAL-References

SUPPLEMENTARY MATERIAL-Methods

Reference Standard determination

Urea breath test (UBT) was performed using UBiTest 100 mg (Otsuka Pharmaceutical Europe Ltd, UK). Determinations were performed in accordance with the manufacturer's specifications. Samples were processed by non-dispersive isotope-selective infrared spectroscopy (POCone™ Infrared Spectrophotometer, Otsuka Pharmaceutical, Japan). In accordance with the literature, an increase in the proportion $^{13}C/^{12}C$ ($\Delta^{13}CO2$ [‰]) of 8.5‰ or more after urea intake was considered as indicative of *H.pylori* infection[1].

Rapid urease test (RUT) was performed after mucosal sampling using the JATROX HP test (CHR Heim Arzneimittel GmbH, Germany).

After a positive RUT test, biopsies were plated on Pylori Agar (bioMérieux SA, Spain) in microaerophilic jars (Jar Gassing System, Don Whitley Scientific Limited, UK). After a maximum of a week, grown *H.pylori* isolates were subcultured on Columbia plates (bioMérieux) and identified by colony morphology, Gram-negativity and positivity for urease, catalase, and oxidase tests. Isolates were stored frozen at -80°C and preserved in *Brucella* broth supplemented with 15% glycerol.

Biopsies for histology were stained with Giemsa and evaluated by a pathologist specializing in digestive diseases.

RNA extraction and quality control

Total RNA was extracted using the mirVana miRNA isolation kit (Ambion) as per the manufacturer's protocol. Total RNA was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quality was assessed by using Agilent RNA 6000 Nano chips on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with calculation of the RNA integrity number (RIN). RIN score for the training group samples was 7.7±0.66 and 7.2 ± 1.24 for the validation group.

High-throughput miRNA microarrays

miRNA profiling was performed with Human v1 MicroRNA expression beadchips (Illumina Inc., San Diego, CA) as described in [2]. Briefly, 200 ng of total RNA were polyadenylated with poly-A polymerase, converted into cDNA using a biotinylated oligo-dT primer with a universal PCR sequence at its 5'-end and hybridized to microRNA-specific oligos (MSO). After washing, the second-strand cDNA synthesis was performed. Amplified products were hybridized to BeadChips and each addressed bead location was read with a BeadArray Reader (Illumina Inc.). The Human v1 miRNA panel targets 735 human ncRNAs (470 human miRNAs from miRBase 9.1 plus 265 other published miRNAs).

Reverse transcription and real-time PCR

miR-9, *miR*-96, *miR*-98, *miR*-137, *miR*-146a, *miR*-155, *miR*-196a*, miR*-204, *miR*-519e and *miR*-650 expression were assessed by miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Denmark). miRBase accession numbers can be found in Supplementary Table S2. Reverse transcriptase (RT) reaction contained 12.5 ng of template total RNA, 1× RT buffer, 1µl of Enzyme Mix, and nuclease free water in a total volume of 10 µl. Reactions were incubated in a AlphaSC thermocycler (Analytik Jena AG, Germany) for 60 min at 42°C, followed by heat-inactivation of RT for 5 min at 95°C and held at 4°C.

Real-time PCR was performed in a 7500 real-time PCR System (Applied Biosystems, USA). PCR reactions were performed with SensiMix dU SYBRGreen Kit (Quantace Ltd, UK). Each PCR reaction contained 1× SensiMix dU Mix (1x Reaction buffer, Heat-Activated DNA Polymerase, dNTPs, dUTP, 4.0mM MgCI2 and ROX), 1x SYBRGreen I,1U of Uracil DNA Glycosylase, 2 µl of LNA microRNA primer mix, and 6.5µl of 1:50 dilution of cDNA template. PCR volume was 20µl. Both water blank and non-reverse transcribed RNA samples were used as negative controls. Reactions were incubated in a 96-well optical plate (4titude, UK) at 37°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60° for 1 min with subsequent melting curve analysis.

All RT-qPCR assays were performed using an Applied Biosystems 7500 real-time PCR System with SensiMix dU SYBRGreen Kit (Quantace).

Microarray Data and Statistical analysis

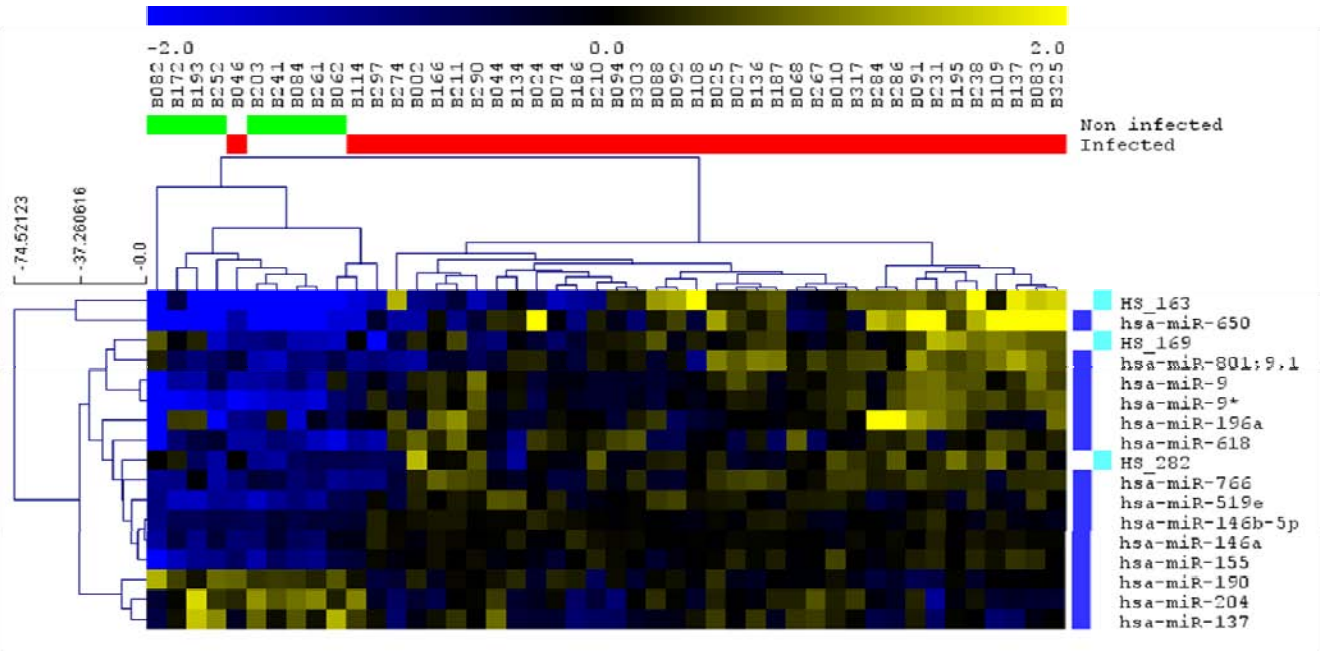
The Illumina Beadchip raw data was imported to BeadStudio v3.2 (Illumina Inc.) and decoded results were subsequently analyzed and normalized using quantile normalization [3]. Differential expression analysis were done using LIMMA package [4]. miRNAs with median intensities greater than 8 were selected for validation analysis. Those miRNAs exhibiting a significant p-value ($p< 0.05$) and a fold change of greater than 1.5 in either direction were chosen for further studies. To account for multiple testing errors, a Benjamini-Hochberg False Discovery Rate (FDR) procedure was applied. We performed globaltest [5] to assess the influence of sex, age (≤45 years vs. >45 years), endoscopic diagnosis or *H.pylori* virulence factors on gene expression.

LinRegPCR calculations

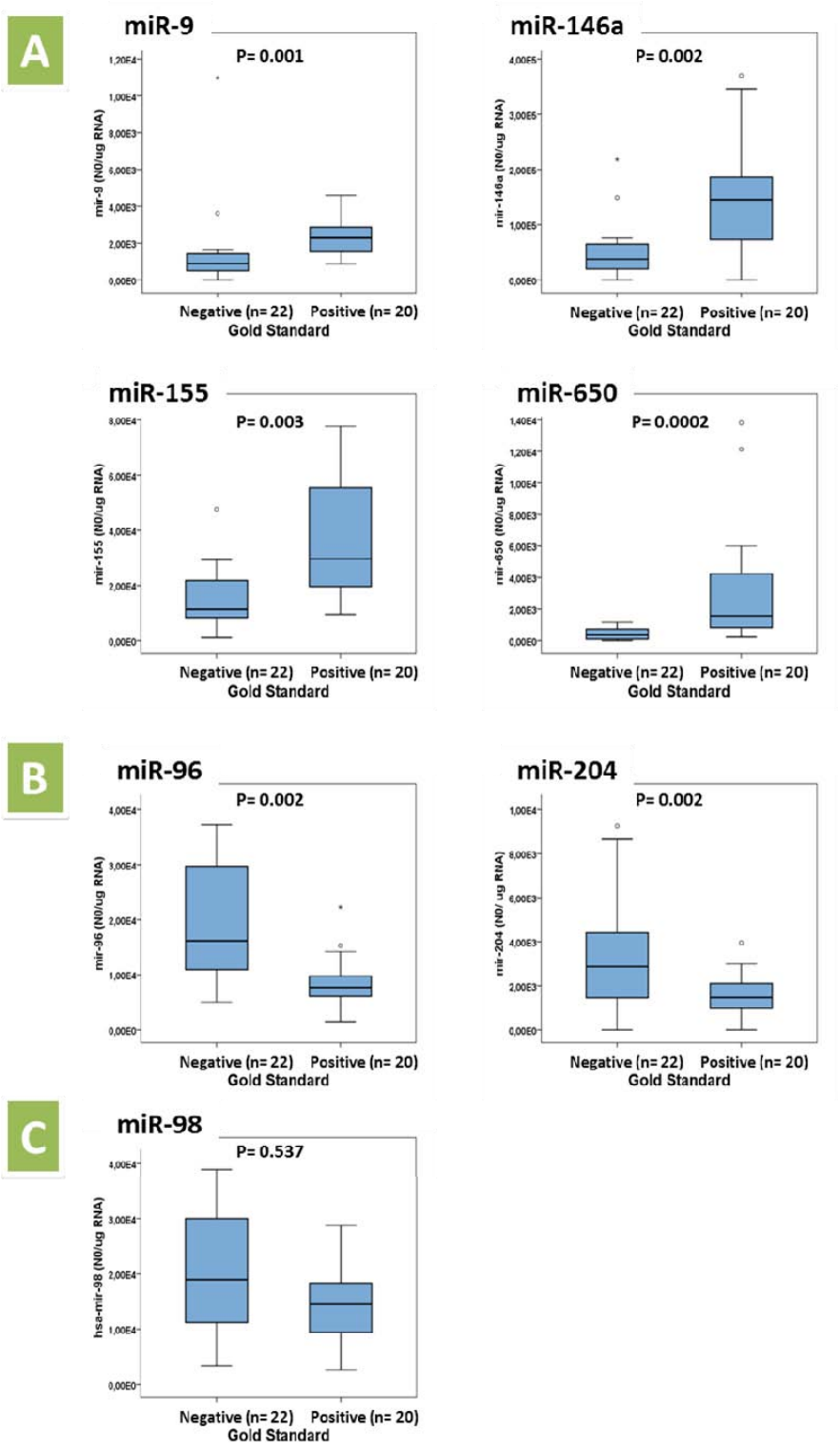
Real time PCR raw data were exported from 7500 Software v2.0 (Applied Biosystems) onto a spreadsheet, by using an Excel macro designed to adapt the data to LinRegPCR requirements. LinRegPCR v12.0 was used to calculate PCR efficiencies and an estimate of the starting concentration per sample (N_0) expressed in arbitrary fluorescence units [6]. First, the program determines the baseline fluorescence and performs baseline subtraction. Then a Window-of-Linearity for all PCR samples is set. LinRegPCR determines i) the mean PCR efficiency per amplicon (E_{mean}), ii) the quantification cycle (C_q) value per sample and iii) the fluorescence threshold set to determine the C_q (N_q). With these data, N_0 is calculated using the formula $N_0 = N_q / (E_{mean})^{Cq}$.

SUPPLEMENTARY MATERIAL-Figures

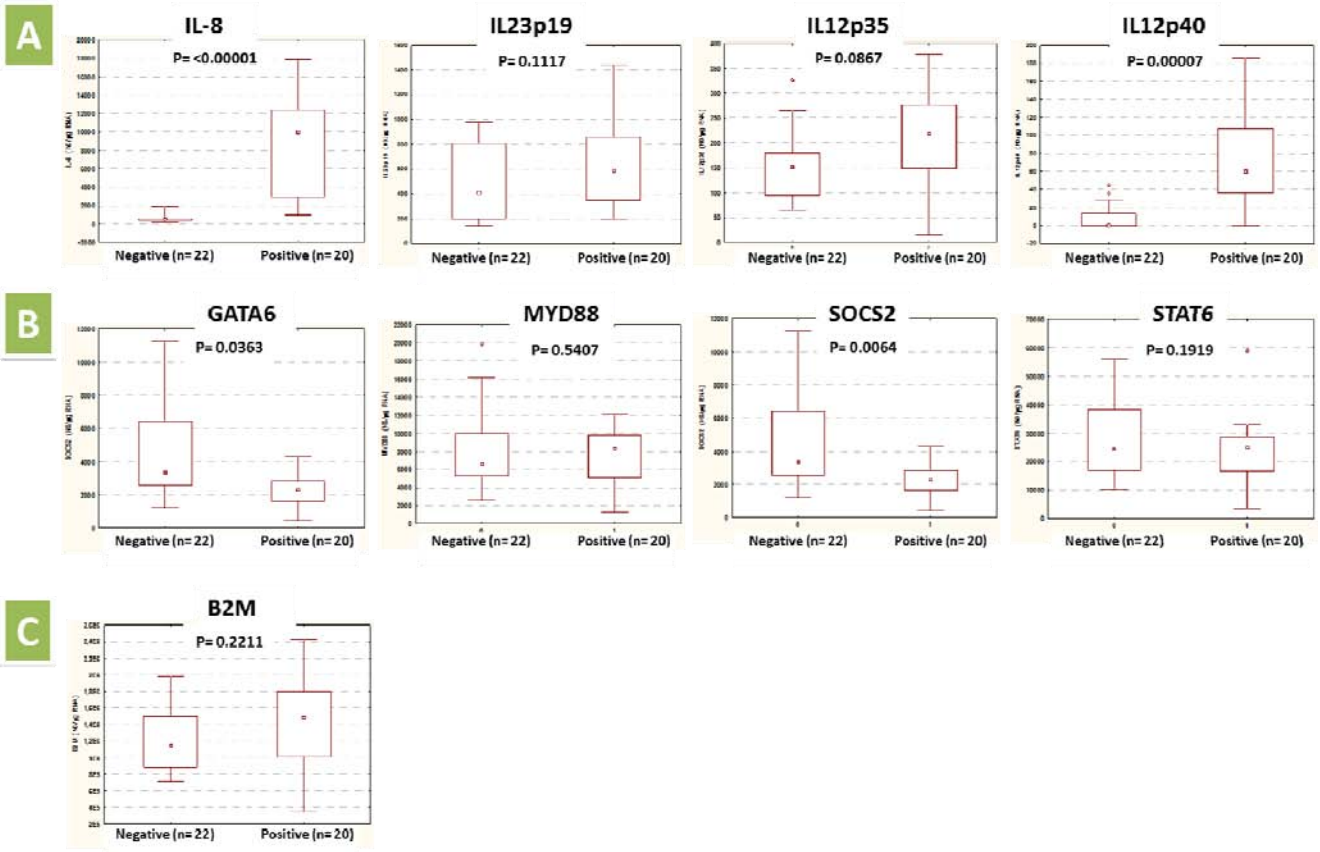
Supplementary Figure S1. Unsupervised hierarchical clustering showing miRNA expression in *H.pylori* non-infected and infected patients.



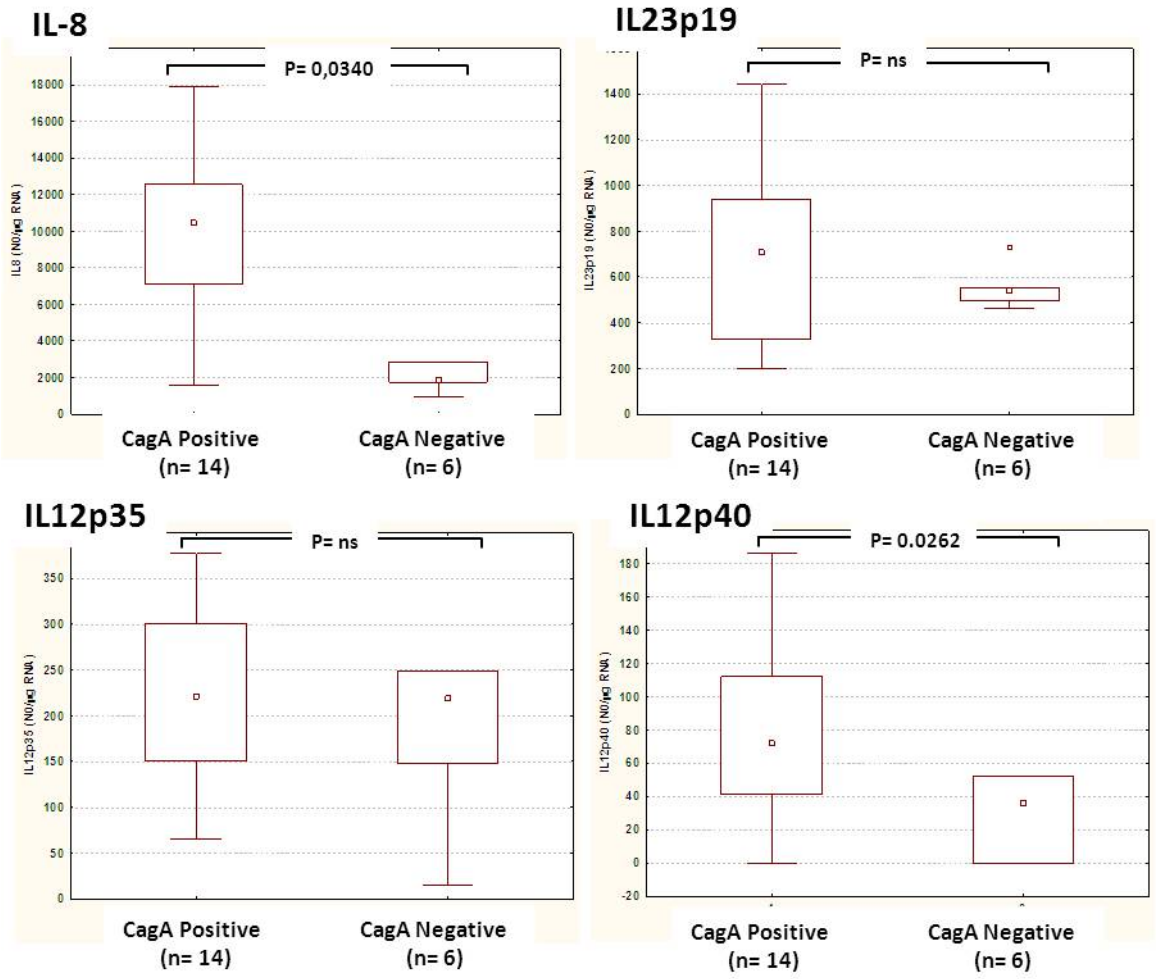
Supplementary Figure S2. Expression profile of the selected miRNAs in the validation set of dyspeptic patients according to the reference standard for *H.pylori* infection. (A) Upregulated miRNAs – *miR*-9, *miR*-146a, *miR*-155 and *miR*-650 – in H.pylori infected patients. (B) Downregulated miRNAs – *miR*-96 and *miR*-204 – in *H.pylori* infected patients. (C) *miR*-98 expression was unchanged and was used as a "housekeeping" miRNA. Mann-Whitney U Test. Box plots show the 25th, 50th (median), 75th, and range (whiskers).



Supplementary Figure S3. Expression profile of the selected mRNAs in the validation set of dyspeptic patients according to the reference standard for *H.pylori* infection. (A) Selected interleukins (B) Signalling molecules (C) "Housekeeping" β2-microglobulin. Mann-Whitney U Test. Box plots show the 25th, 50th (median), 75th, and range (whiskers).



Supplementary Figure S4. Gene expression of selected interleukins in relation to cagA status. Units are: N_0/µg RNA. Mann-Whitney U test.



SUPPLEMENTARY MATERIAL-Tables

Supplementary Table S1. Primers used for *H.pylori* genotyping

Gene	*Region*	*Primer name*	*Sequence 5'-3'*	*Product size (bp)*	*Allele*	*Reference*
vacA	**Signal**	VA1-F	ATGGAAATACAACAAACACAC	259	s1	[7]
		VA1-R	CTGCTTGAATGCGCCAAAC	286	s2	
	Middle	VAG-F	CAATCTGTCCAATCAAGCGAG	570	m1	[8]
		VAG-R	GCGTCAAAATAATTCCAAGG	645	m2	
	Intermediate	VacF1	GTTGGGATTGGGGGAATGCCG			
		C1R	TTAATTTAACGCTGTTTGAAG	426	i1	[9]
		C2R	GATCAACGCTCTGATTTGA	426	i2	
cagA	**cagA constant region**	Con-F Con-R	GTGCCTGCTAGTTTGTCAGCG TTGGAAACCACCTTTTGTATTAGC	402	*cagA*+	[10]

Supplementary Table S2. Primers used for RT-qPCR

Gene	*HGCN Gene symbol*	*Sequence 5'-3'*	*Product size (bp)*	*PCR efficiency*[a]	*Ref.*
Interleukins					
Interleukin-8	IL8	CTCTTGGCAGCCTTCCTGA AGTTCTTTAGCACTCCTTGGCA	71	1.742	[11]
Interleukin-23 subunit alpha precursor p19 (IL23p19)	IL23A	AGTGTGGAGATGGCTGTGACC GCTGGGACTGAGGCTTGGAATCTG	245	1.775	[12]
Interleukin-12 subunit alpha precursor p35 (IL12p35)	IL12A	TGCCGCGGCCACAGGTCT AGGCCAGGCAACTCCCATTAG	398	1.767	This study
Interleukin-12 subunit beta precursor p40 (IL12p40)	IL12B	CATTCGCTCCTGCTGCTTCAC TACTCCTTGTTGTCCCCTCTG	267	1.754	[13]
Signaling					
Transcription factor GATA-6	GATA6	CCCATGACTCCAACTTCCA GCCCATCTTGACCCGAATAC	166	1.791	[14]
Myeloid differentiation primary response protein MyD88	MYD88	CAGAGGAGGATTGCCAAAAG GGGGTCATCAAGTGTGGTG	134	1.809	[15]
Suppressor of cytokine signaling 2	SOCS2	GGATGGTACTGGGGAAGTATGACTG AGTCGATCAGATGAACCACACTGTC	244	1.793	[16]
Signal transducer and activator of transcription 6	STAT6	ATGCCAAAGCCACTATCCTG ATCAAACCACTGCCAAAAGG	296	1.746	[17]
Housekeeping					
beta-2-microglobulin	B2M	ACTGAATTCACCCCCACTGA CCTCCATGATGCTGCTTACA	114	1.801	[18]

HGCN: HUGO Gene Nomenclature Committee database
[a] calculated by LinregPCR v12.0

Supplementary Table S3. miRNAs analyzed by RT-qPCR

Gene	*Accession Number*[a]	*PCR efficiency*[b]	*%CV$_{intra}$*	*Cq range (min-max)*
hsa-miR-9	MI0000466	1.855	6.98%	32.6-40.0
hsa-miR-96	MI0000098	1.909	7.94%	31.0-35.6
hsa-miR-98	MI0000100	1.837	10.0%	29.2-34.6
hsa-miR-146a	MI0000477	1.761	9.93%	29.5-38.9
hsa-miR-155	MI0000681	1.900	7.30%	28.5-35.8
hsa-miR-204	MI0000284	1.871	22.7%	31.4-38.1
hsa-miR-650	MI0003665	1.901	23.3%	30.7-40.0
hsa-miR-137	MI0000454	Very low expression levels (Cq>35). Data not suitable for LinRegPCR analysis		
hsa-miR-196a	MI0000238	Very low expression levels (Cq>35). Data not suitable for LinRegPCR analysis		
hsa-miR-190	MI0000486	Not available as validated Exiqon LNA primer set		
hsa-miR-618	MI0003632	Not available as validated Exiqon LNA primer set		
hsa-miR-9*	MIMAT0000441	Not done		
hsa-miR-146b-5p	MI0003129	Not done		
hsa-miR-493	MI0003132	Not done		
hsa-miR-498	MI0003142	Not done		
hsa-miR-502-5p	MI0003186	Not done		
hsa-miR-766	MI0003836	Not done		
hsa-miR-519e	MI0003145	Primer set not worked		

[a]miRBase accession number
[b] calculated by LinregPCR v12.0

Supplementary Table S4. Results of globaltest analysis which tested the association between miRNA expression profiles and demographic or clinical diagnosis variables.

		Statistic.Q	Expected.Q	sd.of.Q	P.value	num.mirna.with.Z>3
Sex	***Female vs Male***	7,904	10	4,033	0,660	12
Age	***<45 vs ≥45***	11,65	10	4,033	0,267	27
H.pylori status	***Reference Positive vs Negative***	37,07	10	3,972	**0.0002**	153
Endoscopic diagnosis	***Duodenal Ulcus vs Normal***	16,19	10	3,972	0,073	70
Chronic Gastritis	***Present vs Absent***	18,38	10	3,995	**0,039**	93
H.pylori virulence	***High (cagA+ VacA s1m1i1) vs Low (cagA- VacA s2m2i2)***	16,51	10	4,648	0,087	79

Supplementary Table S5. Spearman's correlation coefficients of miRNA expression values in relation to *H.pylori* status and gastritis activity.

***H.pylori* negative without gastritis (Spearman r_s)**

	miR-650	*miR-204*	*miR-146a*	*miR-98*	*miR-155*	*miR-96*	*miR-9*
miR-650	-	0.6778	**0.8787**	0.5774	**0.8285**	0.6946	0.4268
miR-204		-	0.4667	**0.8167**	0.4667	**0.8167**	0.1500
miR-146a			-	0.6000	**0.9000**	0.6167	0.4667
miR-98				-	0.5500	**0.9167**	0.2500
miR-155					-	0.6833	0.4333
miR-96						-	0.2500
miR-9							-

***H.pylori* negative with non-active gastritis (Spearman r_s)**

	miR-650	*miR-204*	*miR-146a*	*miR-98*	*miR-155*	*miR-96*	*miR-9*
miR-650	-	0.2873	0.1381	0.2320	0.3591	0.2983	-0.0055
miR-204		-	0.3901	0.6484	0.0275	0.2692	**0.7363**
miR-146a			-	**0.7418**	**0.7033**	**0.7198**	-0.0055
miR-98				-	0.6209	**0.7967**	0.2967
miR-155					-	0.6264	-0.1154
miR-96						-	-0.1703
miR-9							-

***H.pylori* positive with active gastritis (Spearman r_s)**

	miR-650	*miR-204*	*miR-146a*	*miR-98*	*miR-155*	*miR-96*	*miR-9*
miR-650	-	0.2755	0.1517	0.2982	0.5294	0.3725	0.3643
miR-204		-	0.4530	0.5872	**0.6544**	**0.5955**	-0.0072
miR-146a			-	0.5769	**0.7108**	0.2549	0.3375
miR-98				-	**0.6985**	**0.6285**	0.1373
miR-155					-	0.3358	0.5343
miR-96						-	-0.2198
miR-9							-

Correlations with p<0.01 are indicated in bold.

Supplementary Table S6. Correlation between miRNAs and interleukins or signaling molecules in relation to *H.pylori* status and gastritis activity.

***H.pylori* negative without gastritis**							
(Spearman rs)							
	***miR*-650**	***miR*-204**	***miR*-146a**	***miR*-98**	***miR*-155**	***miR*-96**	***miR*-9**
IL12p40							
IL12p35	0,158997	-0,083333	0,316667	0,116667	0,366667	0,116667	**0,883333**
IL23p19	-0,242680	-0,150000	-0,200000	-0,250000	-0,566667	-0,533333	-0,083333
IL8	0,150629	-0,133333	0,466667	0,166667	0,300000	0,000000	0,666667
GATA6	0,083683	0,316667	0,166667	0,366667	0,300000	0,316667	0,316667
SOCS2	0,351468	0,233333	0,416667	0,433333	0,500000	0,416667	**0,866667**
STAT6	0,510465	0,250000	0,550000	0,216667	0,616667	0,216667	0,766667
MYD88	0,393309	-0,016667	0,566667	0,150000	0,533333	0,083333	0,666667

***H.pylori* negative with non-active gastritis**							
(Spearman r_s)							
	***miR*-650**	***miR*-204**	***miR*-146a**	***miR*-98**	***miR*-155**	***miR*-96**	***miR*-9**
IL12p40	-0,206305	0,101139	-0,156043	-0,280300	-0,419005	-0,372770	0,358322
IL12p35	0,502770	-0,109890	-0,219780	-0,181319	0,115385	-0,159341	0,071429
IL23p19	-0,318122	-0,662999	-0,134801	-0,162311	0,145805	0,107290	-0,530950
IL8	-0,005525	0,016484	0,065934	-0,148352	0,115385	-0,346154	0,313187
GATA6	0,337022	0,681319	0,170330	0,456044	0,082418	0,164835	**0,725275**
SOCS2	0,160223	0,291209	-0,104396	0,104396	-0,027473	0,027473	0,582418
STAT6	0,342547	0,225275	-0,263736	0,010989	-0,225275	0,016484	0,324176
MYD88	0,060774	-0,093407	-0,362637	-0,197802	-0,241758	-0,142857	0,142857

***H.pylori* positive with active gastritis**							
(Spearman r_s)							
	***miR*-650**	***miR*-204**	***miR*-146a**	***miR*-98**	***miR*-155**	***miR*-96**	***miR*-9**
IL12p40	0,236815	0,187177	0,050672	-0,147880	0,179362	0,116857	0,344365
IL12p35	0,446852	0,102167	0,046440	-0,269350	0,164216	-0,205366	0,494324
IL23p19	**0,596799**	-0,112545	-0,334538	-0,404750	0,047823	-0,196180	0,160041
IL8	0,329205	0,100103	0,120743	0,023736	0,139706	0,250774	0,275542
GATA6	-0,042312	0,023736	-0,133127	-0,207430	-0,274510	-0,254902	-0,052632
SOCS2	-0,157895	-0,042312	-0,137255	-0,141383	-0,264706	-0,126935	0,050568
STAT6	0,104231	-0,083591	-0,139319	-0,170279	-0,250000	-0,201238	0,149639
MYD88	0,055756	0,148684	-0,002065	0,065049	-0,114040	0,012390	0,042334

Supplementary Table S7. Expression of validated miRNAs in relation to *cagA* status and *vacA* genotypes.

		cagA				
		Absent		**Present**		
N (%)		**5 (29.4%)**		**12 (70.6%)**		
miRNA (N_0/µg RNA)					**Z –adjusted**	**2sided-exact p**
miR-650	1047	[882-2698]	1889	[787-5510]	-0,7379	0,5058
miR-204	923	[709-1267]	1464	[1052-2245]	-1,6866	0,1037
miR-146a	90400	[80400-146000]	146500	[73200-186000]	-0,9487	0,3827
miR-98	10100	[8610-15500]	13800	[9935-17950]	-0,7379	0,5058
miR-155	21950	[17200-25950]	36350	[16950-55450]	-1,2127	0,2615
miR-96	5289	[1940-7230]	7693	[6191-11160]	-1,7920	0,0818
miR-9	2115	[1919-2132]	2353	[1498-2868]	-0,8433	0,4421
		vacA				
		s1		**s2**		
N (%)		**11 (64.7%)**		**6 (35.3%)**		
miRNA (N_0/µg RNA)					**Z –adjusted**	**2sided - exact p**
miR-650	2040	[684-5993]	1235	[882-2698]	0,8040	0,4623
miR-204	1544	[1123-2781]	944	[709-1267]	2,0101	**0,0477**
miR-146a	145000	[69000-203000]	118200	[80400-152000]	0,4020	0,7325
miR-98	12700	[8770-17100]	12800	[8610-18800]	0,2010	0,8836
miR-155	36400	[15500-56200]	23000	[20900-28900]	0,9630	0,3773
miR-96	7424	[6170-13317]	6260	[1940-8431]	1,3065	0,2161
miR-9	2348	[1413-2761]	2123	[1919-2559]	0,1005	0,9612
		m1		**m2**		
N (%)		**8 (47.1%)**		**9 (52.9%)**		
miRNA (N_0/µg RNA)					**Z –adjusted**	**2sided - exact p**
miR-650	3123	[1210-8581]	1047	[882-2698]	1,2509	0,2359
miR-204	1627	[1189-2843]	982	[923,9-1384]	1,8283	0,0745
miR-146a	155000	[91700-244000]	90400	[69000-148000]	1,2509	0,2359
miR-98	13800	[9870-19050]	12700	[8770-15500]	0,4811	0,6730
miR-155	45550	[22600-63450]	21950	[15950-32600]	1,6803	0,1049
miR-96	8482	[6112-13761]	6212	[5289-7424]	1,6358	0,1139
miR-9	2487	[19005-2868]	2115	[1475-2358]	1,2509	0,2359
		i1		**i2**		
N (%)		**11 (68.8%)**		**5 (31.3%)**		
miRNA (N_0/µg RNA)					**Z –adjusted**	**2sided –exact p**
miR-650	1422	[556-5028]	2698	[1737-4285]	-0,6231	0,5833
miR-204	1256	[964-1710]	1267	[717-1563]	0,5098	0,6612
miR-146a	152000	[69000-203000]	106000	[90400-146000]	0,8497	0,4409
miR-98	11300	[8770-18800]	14900	[8640-15500]	-0,0566	1,0000
miR-155	23000	[13500-54700]	32650	[24900-36600]	-0,6528	0,5714
miR-96	7962	[5977-13317]	6212	[5289-7230]	1,0762	0,3196
miR-9	2216	[1413-2761]	2132	[2115-2358]	-0,6231	0,5833

Results are median [lower quartile-upper quartile].

Mann-Whitney U Test. Significant P values are in bold.

Supplementary Table S8. Diagnostic accuracy of IL-8 mRNA levels and selected miRNAs.

	ROC Curves				***Sensitivity and Specificity***			
Test	***AUC (95% CI)***		***P value***	***Cut-off***	***Sensitivity (95% CI)***		***Specificity (95% CI)***	
IL-8 mRNA	0.9886	(0.9659 to 1.011)	**< 0.0001**	> 1425	94.4	(72.7% to 99.8%)	95.5	(77.2% to 99.8%)
Ratio *miR*-96/ *miR*-155	0.9665	(0.9215 to 1.012)	**< 0.0001**	< 0.5700	84.2	(60.4% to 96.6%)	95.5	(77.2% to 99.8%)
Ratio *miR*-96/ *miR*-650	0.9618	(0.9078 to 1.016)	**< 0.0001**	< 13.92	85.0	(62.1% to 96.7%)	94.1	(71.3% to 99.8%)
Ratio *miR*-96/ *miR*-9	0.8857	(0.7794 to 0.9921)	**< 0.0001**	< 4.500	70.0	(45.7% to 88.1%)	95.2	(76.2% to 99.8%)
***miR*-650**	0.8659	(0.7580 to 0.9738)	**< 0.0001**	> 1150	60.0	(36.0% to 80.8%)	95.5	(77.2% to 99.8%)
Ratio *miR*-204/ *miR*-9	0.8655	(0.7378 to 0.9932)	**< 0.0001**	< 1.370	90.0	(68.3% to 98.7%)	85.7	(63.7% to 96.9%)
Ratio *miR*-204/ *miR*-650	0.8607	(0.7425 to 0.9789)	**0.0002**	< 2.360	79.0	(54.4% to 93.9%)	82.4	(56.6% to 96.2%)
***miR*-9**	0.8455	(0.7186 to 0.9724)	**0.0002**	> 1334	85.0	(62.1% to 96.7%)	72.7	(49.8% to 89.3%)
***miR*-155**	0.8373	(0.7177 to 0.9570)	**0.0002**	> 18200	79.0	(54.4% to 93.9%)	72.7	(49.8% to 89.3%)
***miR*-96**	0.8364	(0.7116 to 0.9612)	**0.0002**	< 10426	80.0	(56.3% to 94.2%)	81.8	(59.7% to 94.8%)
Ratio *miR*-204/ *miR*-55	0.8330	(0.6934 to 0.9726)	**0.0002**	< 0.1200	100.0	(83.2% to 100.0%))	77.3	(54.6% to 92.2%)
***miR*-146a**	0.8261	(0.6925 to 0.9597)	**0.0003**	> 76600	75.00	(50.9% to 91.3%)	90.91	(70.8% to 98.8%)
***miR*-204**	0.7330	(0.5757 to 0.8903)	**0.0098**	< 2082	75.0	(50.9% to 91.3%)	72.7	(49.8% to 89.3%)
***miR*-98**	0.6432	(0.4729 to 0.8134)	0.1127	-	-	-	-	-

Tests are ordered by decreasing values of AUC.

ROC: Receiver-Operating-Characteristic curves; AUC: Area under the curve.

Significant *P* values are in bold

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