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## CXCL-8 as a signature of severe *Helicobacter pylori* infection and a stimulator of stomach region-dependent immune response

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### ABSTRACT

*Helicobacter pylori* infection is involved in development of diverse gastro-pathologies. Our aim is to investigate potential signature of cytokines-chemokine levels (IL-17A, IL-1 $\beta$ , and CXCL-8) in *H. pylori*-infected patients and their impact on immune response in both corpus and antrum. Multivariate level analysis with machine learning model were carried out using cytokines/chemokine levels of infected Moroccan patients. In addition, Geo dataset was used to run enrichment analysis following CXCL-8 upregulation. Our analysis showed that combination of cytokines-chemokine levels allowed prediction of positive *H. pylori* density score with <5% of miss-classification error, with fundus CXCL-8 being the most important variable for this discrimination. Furthermore, CXCL-8 dependent expression profile was mainly associated to IL6/JAK/STAT3 signaling in the antrum, interferons alpha and gamma responses in the corpus and commonly induced transcriptional /proliferative activities. To conclude, CXCL-8 level might be a signature of Moroccan *H. pylori*-infected patients and an inducer of regional-dependent immune response at the gastric level. Larger trials must be carried out to validate the relevance of these results for diverse populations.

### 1. Introduction

*Helicobacter pylori* is a gram-negative spiral bacillus with flagella. This microaerophilic bacterium was first isolated by Warren and Marshall in 1982 [1]. Mostly acquired in early childhood, *H. pylori* persists in the gastric mucosa and causes several disorders such as gastritis, peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric carcinoma. According to the World Health Organization (WHO), this bacterium is estimated to be colonizing more than two thirds of the population in developing countries [2]. In Morocco, the prevalence of *H. pylori* infection in asymptomatic

individuals is over 90%. Moreover, gastric cancer prevalence is in constant increase in Moroccan infected patients [3]. *H. pylori* is classified as a class I carcinogen [2]. The progression towards gastric cancer is attributed to chronic inflammation, implicating immune cells recruitment and cytokines secretion in response to *H. pylori* infection. As a result, precancerous lesions (atrophy, intestinal metaplasia (IM) and dysplasia) evolve over time and could possibly reach gastric cancer stage [4,5].

C-X-C Motif Chemokine Ligand (CXCL)-8, (also known as interleukin 8 (IL-8)) is a pro-inflammatory chemokine, secreted by different cell types such as monocytes, macrophages, fibroblasts, endothelial cells,

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and epithelial cells. Its transcription is essentially mediated through NF- $\kappa$ B and activator protein-1 (AP-1), and its action is mediated through binding to two receptors namely CXCR-1 and CXCR-2 [6]. In response to *H. pylori* infection, gastric epithelial cells (GECs) secrete CXCL-8, which plays the role of chemoattracting neutrophils which are the first cells to be recruited in response to *H. pylori* and ensure recruitment of other cells, such as macrophages [6,7]. In different populations, several studies have shown heightened gastric mucosal CXCL-8's levels of *H. pylori*-infected patients when compared to noninfected ones [8,9]. Similarly, this chemokine upregulation is positively correlated to gastric inflammation degree through neutrophils recruitment as well as in gastric cancer [6]. In fact, CXCL-8 does also activate NF- $\kappa$ B signaling, which induce several pro-inflammatory mediators making it one of the implicated factors in inflammation and progression towards carcinogenesis, angiogenesis and metastasis [10]. As for Moroccan *H. pylori*-infected patients, we previously studied IL-1 $\beta$ , IL-17A and CXCL-8 expression in gastritis and precancerous lesions. Similarly to previous studies, showed that CXCL-8 is upregulated upon *H. pylori* infection [11].

Through bioinformatic and machine learning analysis application on our previous data [11], we aimed to evaluate the relevance of CXCL-8 level in both antrum and corpus, and to further support the idea of differential response to inflammatory stimuli upon *H. pylori* infection, we ran an enrichment analysis on data collected from Geo dataset platform to evaluate how these two gastric locations respond to CXCL-8 upregulation.

## 2. Materials & methods

### 2.1. Ethics statement

This research was conducted according to the principles set out in the Declaration of Helsinki and in local ethical guidelines. The study was approved by the Ethics Committee for Biomedical Research, Ibn Rochd Hospital, Casablanca, Morocco. Written informed consent was obtained from all patients.

### 2.2. Patients from bi-centric Morocco cohort

Quantification of CXCL-8, IL-17A and IL1- $\beta$ , and histopathological analyses were performed in stomach parts (antrum and fundus) from 70 Moroccan patients recruited in two distinct Moroccan hospitals: Ibn Rochd University Hospital of Casablanca (Hospital 1) and the Mohamed V<sup>th</sup> Military Training Hospital in Rabat (Hospital 2) [11].

The majority of the patients were recruited from hospital 1 ( $n = 59$ ) versus hospital 2 ( $n = 11$ ). There were few missing data but did not exceed 6%. For most parameters except gastritis evaluation, no bias was found between the recruitment between the two hospitals. All the other parameters were found equilibrated between the two hospitals.

### 2.3. Transcriptome dataset GSE27411 of stomach samples during atrophy and *H. pylori* infection

Transcriptome dataset normalized matrix (quantile normalization) from dataset GSE27411 was downloaded at the following web address: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27411> (accessed on 2021, nov 30th). The resulting uncompressed matrix contained quantification of transcript signal processed by Illumina humanRef-8 v2.0 expression beadchip technology. Annotation was performed with corresponding GEO platform GPL6255 available at the following web address: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL6255> (accessed on 2021, nov 30th). This transcriptome dataset investigated stomach mucosa biopsy from antrum and corpus parts in patients with or without *H. pylori* infection as well as atrophy stage [12].

### 2.4. Iterative chi.square test dependency between qualitative variables

In order to test dependency between qualitative variables of the study “chi2loop” R-package was developed to perform iteration chi. square test between character variables from the cohort dataset. This package is available at the following address: <https://github.com/cdesterke/chi2loop> (accessed on 2021, nov 30th). This package takes as input a dataset of qualitative variables. Dataset need to be imported with parameter stringsAsFactors = FALSE because qualitative variable needs to be in character format and not factor. On dataset cltest function could be applied to perform chi.square iteration between character columns and output result could be graphically evaluate with function nlplot. Subsequently “chinet” function could be applied also on results to detect variable community detection with Louvain classification algorithm. Univariate association plot on chi.square test residues were drawn with vcd R-package version 1.4–9. Factorial multiple analysis was processed with FactoMineR R-package version 2.4.

### 2.5. Machine learning

With quantile five discretization data of cytokines-chemokine IL-1 $\beta$ , IL-17A and IL-8 in antrum and corpus parts, a dataset was built to evaluate the prediction of *H. pylori* density score through the cohort. Random forest machine learning algorithm implemented in random-Forest R-package version 4.6–14 was trained with five hundred trees and default mtry parameter in order to evaluate the error of bagging during aggregation of the trees. To analyze the resulting confusion matrix of random forest model, a machine learning confusion matrix evaluation web site was programmed in javascript at the following address: <https://objective-easley-68bfc2.netlify.app/> (accessed on 2021, nov 30th) based on respective formula of precision, specificity, sensibility and F1-score defined at the address: <https://github.com/cdesterke/confusion> (accessed on 2021, nov 30th).

### 2.6. Transcriptome bioinformatics analyses

Bioinformatics analysis were performed in R software environment version 4.1.0. Gene correlated to CXCL-8 expression in stomach samples from dataset GSE27411 were detected with Pavlidis Template Matching algorithm [13]. Expression heatmap were drawn with pheatmap R-package version 1.0.12. Principal component analysis performed on CXCL-8 correlated genes was graphically output with autoplot function of ggfortify R-package version 0.4.13. Fisher ANOVA boxplot on expressed markers were drawn with ggplot2 R-package version 3.3.5. Functional enrichment performed on genes expression list was performed with enrichr web tools [14] on MSigDB Hallmark 2020 database [15]. Functional immune enriched network were drawn with Cytoscape standalone software version 3.6.0 [16].

### 2.7. Statistical analysis

Statistical analysis was performed in software environment version 4.1.0. Factor evaluation for difference between groups based on cytokine-chemokine and their receptors quantification was evaluated firstly by two way – Analysis of variance (ANOVA) Fisher test. Factor significance was assessed if corresponding  $p$ -value was estimate for  $p < 0.05$  after validation of no significant interaction between factors. Subsequently, one way ANOVA were done based on cytokine-chemokine quantifications in each part of stomach between groups of samples followed by Tukey post Hoc test. Significance was also assessed for  $p$ -value  $< 0.05$  during one way ANOVA processing. For testing data normality of the cytokine-chemokine quantifications, Shapiro-Wilk normality test was employed: normality exclusion was done for a  $p$ -value  $> 0.05$ .

### 3. Results

#### 3.1. Fundus and antrum cytokine-chemokine patterns in gastric *H. pylori*-infected biopsies

Cytokines-chemokine quantifications (IL-1 $\beta$ , IL-17A, CXCL-8) were performed on stomach regional parts (antrum and fundus) of the 70 patients recruited from 2 distinct Moroccan hospitals (supplementary table 1). Statistical distribution analysis of the 6 cytokines-chemokine quantifications in these samples revealed their heterogeneity. 3 of them were found to have a Gaussian distribution like: antrum CXCL-8 (Shapiro test  $p = 0.14$ , Supplementary Fig. 1), antrum IL-17A (Shapiro test,  $p = 0.25$ , Supplementary Fig. 1) and Fundus IL-17A (Shapiro test,  $p = 0.11$ , Supplementary Fig. 1). The 3 other cytokines-chemokine quantifications were found with a distribution far from Gaussian distribution: antrum IL-1 $\beta$  (Shapiro test,  $p = 2.19\text{e-}7$ , Supplementary Fig. 1), fundus IL-1 $\beta$  (Shapiro test,  $p = 8.46\text{e-}5$ , Supplementary Fig. 1) and fundus CXCL-8 (Shapiro test,  $p = 7.89\text{e-}3$ , Supplementary Fig. 1). Taking in account this great heterogeneity of the variable distribution, we decided to perform a data discretization of the cytokines-chemokine levels in quantile 5 transformation. In order to test the variable dependency for the parameters of the cohort, iterative chi.square test was performed with chi2loop R-package. This loop analysis revealed some significant associations between cytokines-chemokine levels but also between cytokines-chemokine levels as well as clinical and biological parameters from the cohort (supplementary table 2).

Cytokines-chemokine levels were shown to have a significant association with *H. pylori* density score (Fig. 1A and supplementary table 2). These results suggest that cytokines-chemokine levels, especially fundus CXCL-8, could be considered as important parameters to predict *H. pylori* detection.

Starting from parameter associations found by iterative chi.square tests, a detection of parameter communities was applied with Louvain classification algorithm (Fig. 1B). With this classification analysis, four main clusters were observed during integrative analysis of clinical and biological parameters with cytokines-chemokine levels. The three cytokines-chemokine levels performed in fundus were aggregated in the light green cluster while the other three performed in antrum were aggregated in the pink cluster. In the distinct purple cluster, the different clinical parameters were associated, with stronger association between smoking and gender. Also, metaplasia was found to be strongly associated to atrophy and in a weaker manner to smoke. In a last dark green cluster, some clinical and epidemiological parameters like gastritis and hospital were found mixed with biological parameters such as gastritis activity and *H. pylori* density score. Inside this mixed cluster, a strong association was found between *H. pylori* density score and gastritis activity (Fig. 1B). On this community graph, great association was observed between *H. pylori* density score (dark green cluster) and both with cytokines-chemokine from antrum (pink cluster) and from fundus (light green cluster) (Fig. 1B). In dark green cluster, these associations revealed that there is a regional pattern of cytokines-chemokine levels in patients' stomach and these quantifications are mainly linked to *H. pylori* density score. A multivariate factorial model was built on cytokines-chemokine quantifications in antrum and fundus taking in account illustrative parameters which have been linked such as: *H. pylori* density score and gastritis activity (Fig. 1C). This multivariate model based on cytokines-chemokine levels showed that these quantifications mainly have the power to discriminate non-infected patients (H0) from infected ones (H1, H2 and H3) (Fig. 1D). These results were confirmed by univariate analyses of the chi.square residues of each cytokine-chemokine in fundus part of the stomach (Supplementary Fig. 2) and in antrum part of the stomach (Supplementary Fig. 3).

In order to evaluate the efficiency to predict the positivity of *H. pylori* density score with the cytokines-chemokine quantifications, a random forest machine learning model was trained on 500 trees of classification with the 6 distinct cytokines-chemokine levels and supervised on the 2

distinct classes: negative *H. pylori* density score (H0) versus positive *H. pylori* density scores (H1, H2 and H3). During learning of the model, progressive reduction of error rate by class was observed (Fig. 2A). After bagging of the trees, at the end of the learning, the model harbored a small global error of bagging (4.55%), meaning <5% of misclassification during prediction of the classes with this model. Indeed, matrix confusion analysis revealed that this model based on antrum and fundus cytokines/chemokine levels harbored a precision of 0.95, a specificity of 0.75, a sensibility of 1 and a F1-recall score of 0.97 (Fig. 2B). As confirmed by multidimensional scaling only 3 negative patients were miss-classified as false positive (Fig. 2C). Analysis of the variable importance (VIMP) in the model showed that cytokines-chemokine levels in the fundus are more efficient than cytokines-chemokine levels in the antrum to predict the positivity of the *H. pylori* density score especially for fundus CXCL-8 (Fig. 2D). This important role of the fundus cytokine-chemokine was confirmed by performing iterative association studies by chi.square test (Fig. 2E) and cluster community detection by Louvain algorithm which classified by *H. pylori* density score positivity in the blue cluster of fundus cytokines-chemokine (Fig. 2F).

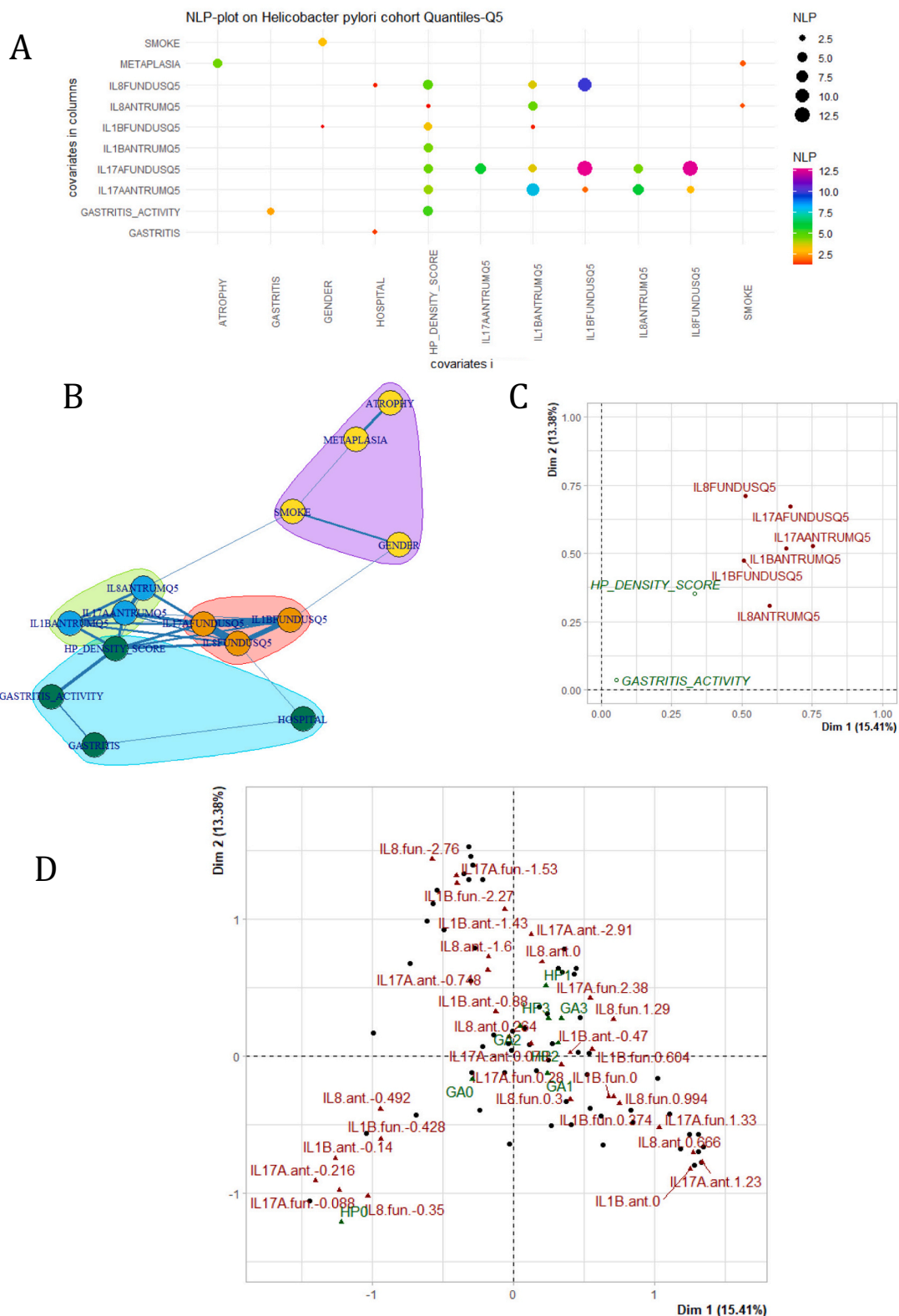
#### 3.2. Co-regulation of CXCL-8 and its receptor CXCR2 in the antrum mucosa during *H. pylori* infection

In order to study regional influence of chemokines-cytokine quantifications, we investigated the analysis of Gene Expression Omnibus (GEO) transcriptome dataset GSE27411 [12]. This dataset is composed of transcriptome results performed on biopsies of both stomach parts, antrum and corpus, in context of *H. pylori* infection including the atrophy stage. Two-way ANOVA performed on CXCL-8 quantification showed a significant variation during *H. pylori* infection and atrophy stage (Fig. 3A,  $p\text{-value} = 1.64\text{e-}4$ ) without any influence of the stomach part ( $p\text{-value} = 0.77$ , Fig. 3A) and without interaction between the two factors ( $p\text{-value} = 0.91$ , Fig. 3A). One way ANOVA of CXCL-8 quantification performed on antrum part showed a significant regulation between *H. pylori* infected and no infected samples ( $p\text{-value} = 7.18\text{e-}3$ , Fig. 3B), and the same observation was done in corpus part of the stomach ( $p\text{-value} = 0.0165$ , Fig. 3B). In addition, evaluation of IL-1 $\beta$  and IL1-7A quantification by two-way ANOVA and downstream one-way ANOVA on stomach parts did not show any significance (Supplementary Fig. 4). These results confirmed the CXCL-8 regulation in distinct region of stomach during *H. pylori* infection. In a second time, it could be possible to perform the same transcriptome investigation for CXCL-8 chemokine receptors: CXCR1 and CXCR2. Two-way ANOVA and downstream one-way ANOVA on both stomach parts performed for CXCR1 regulation study did not show any significance (Fig. 3C and D) while Two-way ANOVA performed on CXCR2 quantification showed a significant regulation during *H. pylori* infection and atrophy stage ( $p\text{-value} = 0.012$ , Fig. 3E) without interaction of stomach part factor (interaction  $p\text{-value} = 0.13$ , Fig. 3E). Downstream one way ANOVA performed on each stomach part highlighted a significant regulation of CXCR2 during *H. pylori* infection in antrum part ( $p\text{-value} = 0.0131$ , Fig. 3F) but not in the corpus stomach part (Fig. 3F).

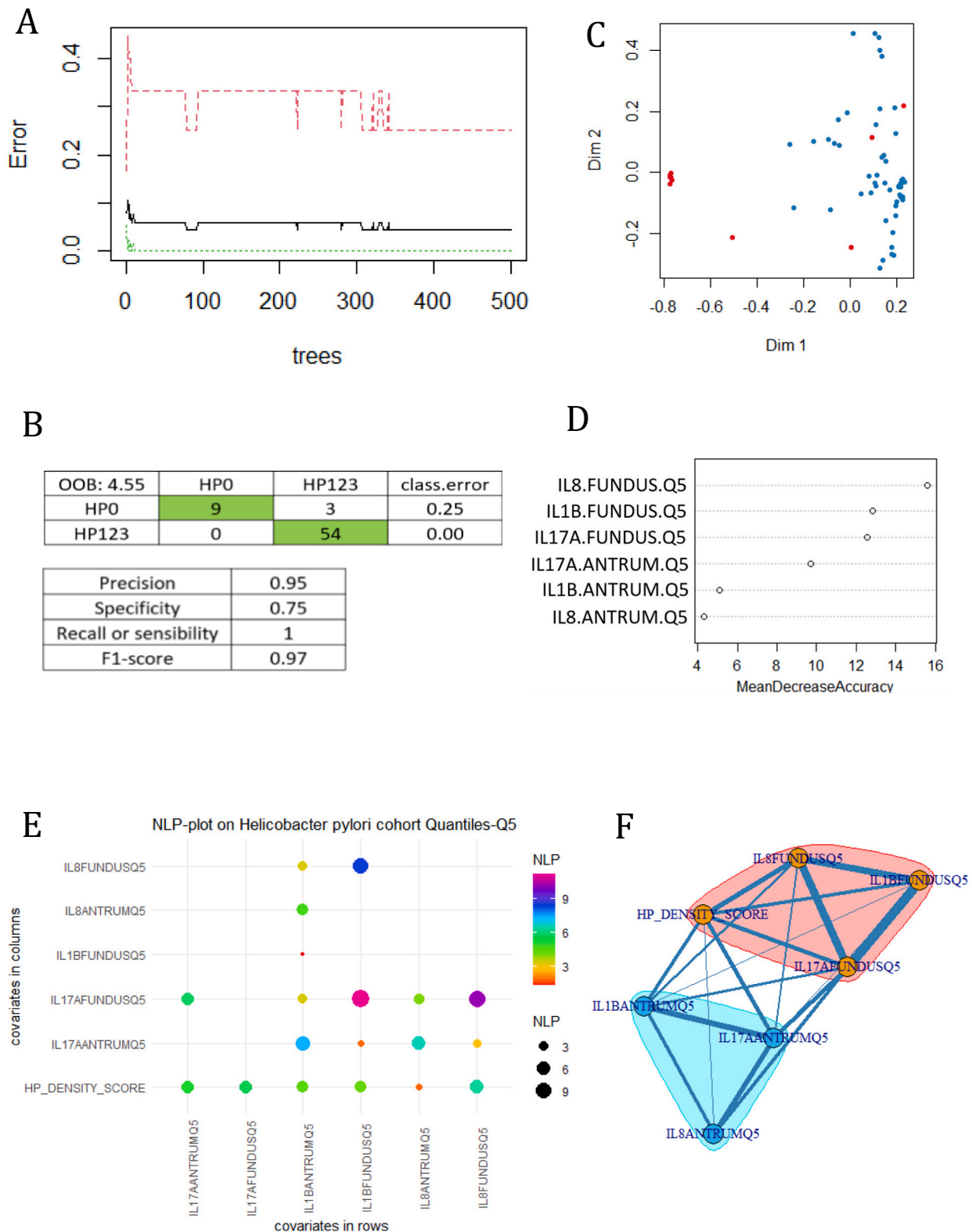
#### 3.3. Main implication of IL6/JAK/STAT3 signaling in expression profile of CXCL-8 dependent antrum mucosa during *H. pylori* infection

As we previously observed, significant regulation of CXCL-8 chemokine during *H. pylori* infection in both antrum and corpus. Expression profile CXCL-8 dependent in each stomach part was investigated with Pavlidis Template Matching algorithm [13]. Overlapping between CXCL-8 expression profiles found in antrum and corpus mucosa revealed a specific signature of the antrum of the stomach during *H. pylori* infection composed of 418 genes (Fig. 4A and B). This antrum specific CXCL-8 dependent expression profile allowed to discriminate experimental groups on first axis of principal component (PCA1 weight:

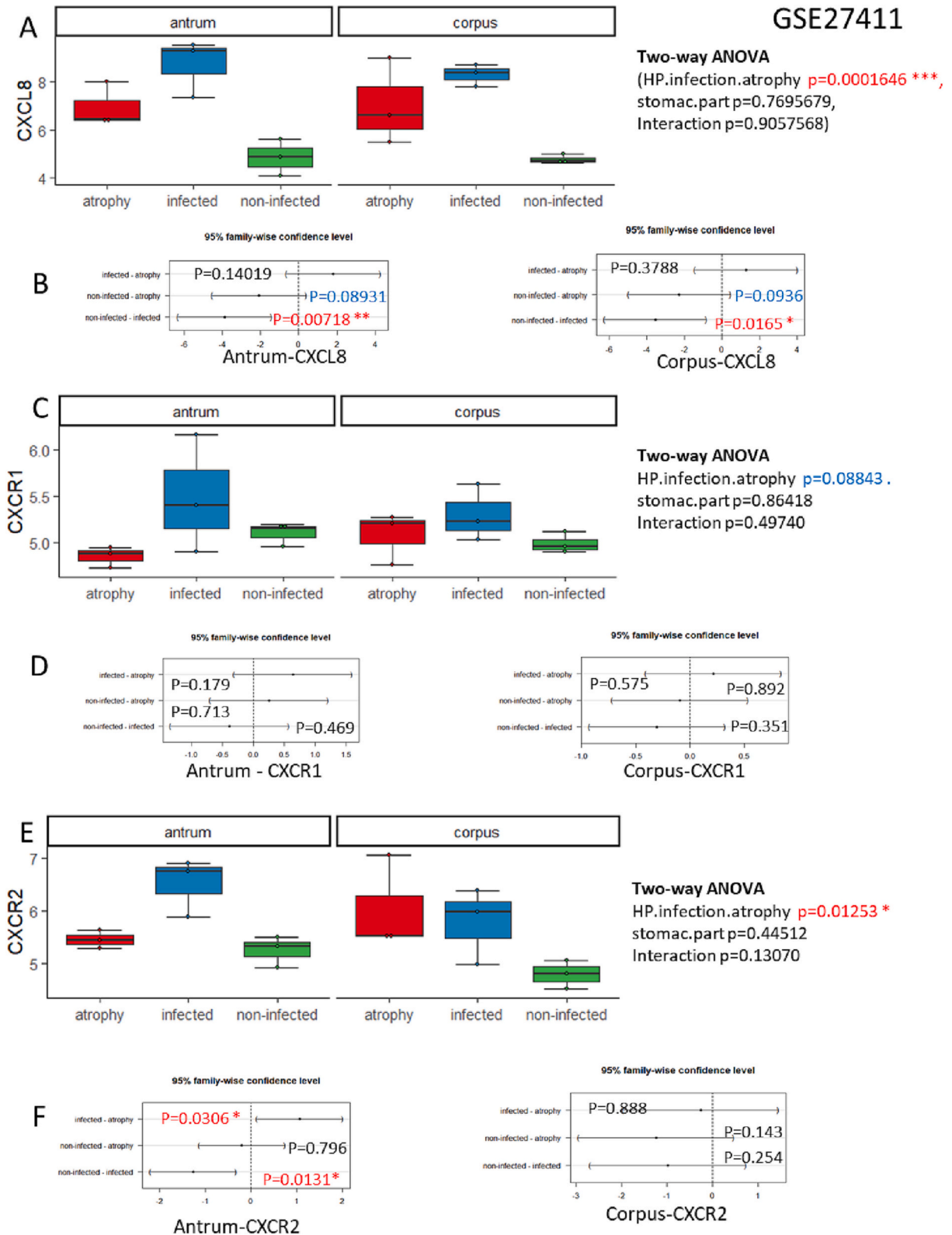




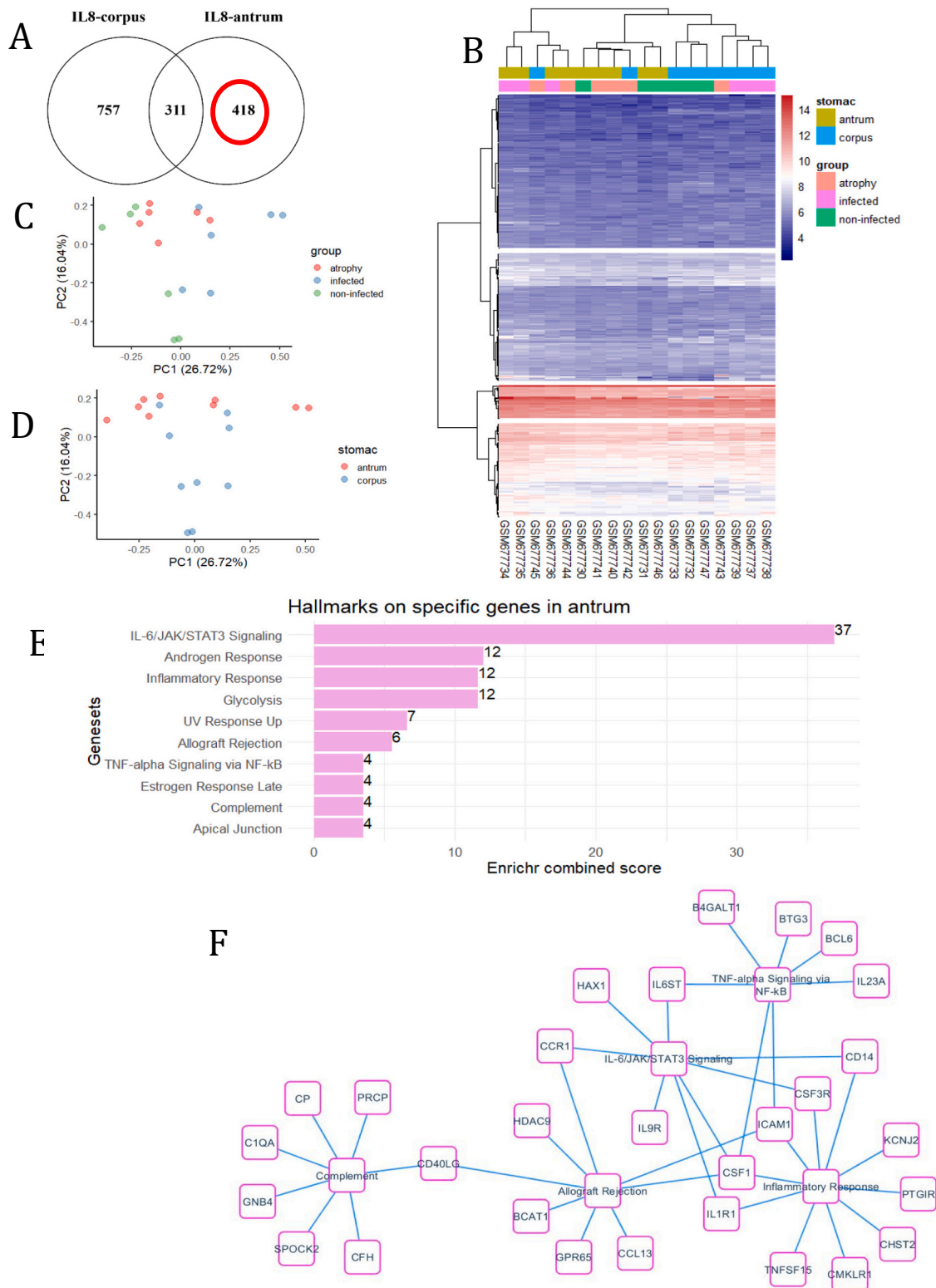
**Fig. 1.** Fundus and antrum cytokine-chemokine patterns in *H. pylori*-infected patients. A/ NLPplot (NLP: negative log<sub>10</sub> *p*-values) testing dependency between clinical variables and cytokine-chemokine quantifications by iterative chi-square test: size and color of the dots are relative to the significance between the x and y axes tested variables, for cytokine-chemokine quantifications in antrum and fundus Q5 meaning quantile five discretization; B/ Variable community detection by Louvain classification algorithm performed on variable association: size of the edges is relative to significance between the two linked variables; C/ Variable map of unsupervised factorial analysis performed on cytokine-chemokine quantifications (in red) and linked significant clinical covariates as illustrative (in green); D/ Group map of unsupervised factorial analysis performed on cytokine-chemokine quantifications (in red) and linked significant clinical covariates as illustrative (in green). GA0, GA 1, GA 2 and GA3: gastritis activity scores. H0, H1, H2 and H3: *H. pylori* density scores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Prediction of HP (*H. pylori*) density score through antrum and fundus cytokine-chemokine patterns. A/ random forest machine learning error rate plot testing discrimination of positive versus negative *H. pylori* density score as outcome based quantification of IL-8, IL-17A and IL-1 $\beta$  in fundus and antrum; B/ machine learning estimation of parameters defining the model; C/ Multidimensional scaling (MDS) plot of random forest model with negative samples in red and positive samples in blue for *H. pylori* density score status; D/ Variable importance plot for variables included in random forest model with *H. pylori* density score as outcome; E/ NLP plot testing independence of the cytokine-chemokine quantifications versus HP density scores, the size and color of the bubbles are relative to the significance between the x and y axes tested variables. F/ Variable community detection by Louvain classification algorithm performed on variable association, the size of the edges is relative to significance between the two linked variables. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Regulation of CXCL-8 and its receptors in antrum and corpus of the stomach during *H. pylori* infection. A/ two-way ANOVA barplot performed on IL-8 transcriptome quantification in GSE27411 dataset: stomach parts (antrum versus corpus) and sample conditions (non-infected, HP infected and atrophy) were used as factors during Fisher test. B/ One way ANOVA was performed on IL-8 quantification between groups with Fisher test and Tukey post hoc tests for Antrum and Corpus parts. C/ two-way ANOVA barplot performed on CXCR1 transcriptome quantification in GSE27411 dataset: stomach parts (antrum versus corpus) and sample conditions (non- infected, HP infected and atrophy) were used as factors during Fisher test; D/ one way ANOVA was performed on CXCR1 quantification between groups with Fisher test and Tukey post hoc tests for Antrum and Corpus parts. E/two-way ANOVA barplot performed on CXCR2 transcriptome quantification in GSE27411 dataset: stomach parts (antrum versus corpus) and sample conditions (non-infected, HP infected and atrophy) were used as factors during Fisher test; F/ one way ANOVA was performed on CXCR2 quantification between groups with Fisher test and Tukey post hoc tests for Antrum and Corpus parts.



**Fig. 4.** Immune regulation antrum specific associated to IL-8 expression. Transcriptome data from GSE2741 were used during these analyses; A/ Venn diagram highlighting common genes found correlated to IL-8 and specific to the antrum part of stomach during *H. pylori* infection; B/ Expression heatmap drawn with IL-8 antrum correlated genes; C/ Principal component analysis performed on IL-8 antrum correlated genes stratified groups (non-infected, infected and atrophy). D/ Principal component analysis performed on IL-8 antrum correlated genes stratified on stomach regional parts. E/ Enrichr Functional enrichment performed on MsigDB\_Hallmark\_2020 database with IL-8 antrum correlated genes. F/ functional enrichment network performed with IL-8 antrum correlated genes implicated in immune functions.



26.72%, Fig. 4C) and also stomach regions on second axis of principal component analysis (PCA2 weight: 16.04%, Fig. 4D). Functional enrichment performed with CXCL-8 antrum specific gene expression profile on Hallmarks gene sets of MSigDb database revealed main enrichment of IL6/JAK/STAT3 signaling in this signature (Enrichr combined score = 37, Fig. 4E). Some other immune gene sets were also found enriched in this antrum signature such as: inflammatory response (Enrichr combined score = 12, Fig. 4E), allograft rejection (Enrichr combined score = 6, Fig. 4E), TNF- $\alpha$  signaling via NF- $\kappa$ B (Enrichr combined score = 4, Fig. 4E), complement (Enrichr combined score = 4, Fig. 4E). Main enriched IL6/JAK/STAT3 signaling gene set involved regulation of some myeloid markers such as CD14, CSF3R receptor and CSF1 growth factor (Fig. 4F). This enrichment analysis in antrum also highlighted the specific regulation of several cytokine and chemokine receptors such as IL6ST, IL9R, IL-1R1 and CCR1 (Fig. 4F).

### 3.4. Major induction of interferons alpha and gamma responses are CXCL-8 dependent in stomach corpus mucosa during *H. pylori* infection

Overlapping between CXCL-8 expression profiles found in antrum and corpus mucosa revealed a specific signature of the corpus during *H. pylori* infection composed of 757 genes (Fig. 5A and B). This corpus specific CXCL-8 dependent expression profile allowed to discriminate experimental groups on first axis of principal component (PCA1 weight: 38.97%, Fig. 5C) and stomach regions on second axis of principal component analysis (PCA2 weight: 9.47%, Fig. 5D). Functional enrichment performed with CXCL-8 corpus specific gene expression profile on Hallmarks gene sets of MSigDb database revealed main enrichment of interferon gamma (IFN- $\gamma$ ) response in this signature (Enrichr combined score = 175, Fig. 5E), but also interferon alpha (IFN- $\alpha$ ) response (Enrichr combined score = 145, Fig. 5E). These IFN- $\gamma$  and  $\alpha$  induced response in corpus shared some Interferon Gamma Inducible proteins such as IFI30 and IFI35 but also PML transcription factor, some components of the proteasome and immune proteasome such as: PSMB9, PSMB2, PSMB8, PSMA2, PSMB10. Simultaneously to the interferon response a large network of immune molecules was found regulated in the corpus and involved in allograft rejection (Enrichr combined score = 175, Fig. 5E). In this corpus signature, it could be observed a minor implication of IL6/JAK/STAT3 signaling (Enrichr combined score = 15, Fig. 5E) through regulation of STAT3 (Fig. 5F) and also TNF- $\alpha$  via NF- $\kappa$ B signaling (Enrichr combined score = 12, Fig. 5E) where both were associated to IL-1 $\beta$  (Fig. 5F).

### 3.5. Common CXCL-8 dependent expression profile between antrum and corpus mainly reflects mucosal transcription and proliferation activities associated to an inflammatory response

Overlapping between genes co-regulated to CXCL-8 in antrum and corpus of the stomach during *H. pylori* infection and atrophy revealed 311 common co-regulated genes (Fig. 6A and B). Unsupervised principal component analysis performed with these 311 co-regulated genes to CXCL-8 allowed to well discriminate group samples on first principal axis (PC1 weight: 62%, Fig. 6C) and stomach parts on second principal axis (PCA2 weight: 11.68%, Fig. 6D). Functional enrichment performed on Hallmarks gene sets of MSigDb database revealed main implication of proliferation activity in this common expression profile through regulations of the G2/M checkpoint gene set (Enrichr combined score = 974, Fig. 6E) and the Mitotic spindle gene set (Enrichr combined score = 93, Fig. 6E). Also, a transcription activity was detected in this common expression profile through E2F targets gene set (Enrichr combined score = 755, Fig. 6E). Among this enrichment analysis, it could be observed some enriched immune gene sets such as TNF- $\alpha$  signaling via NF- $\kappa$ B (Enrichr combined score = 63, Fig. 6E) and inflammatory response (Enrichr combined score = 63, Fig. 6E). These two immune gene sets involved some shared molecules such as: CCL20, CXCL6 chemokines, TNFAIP6, and IL-1 $\alpha$  cytokine (Fig. 6F). All together, these results suggest

that independently to the stomach part (corpus and antrum), CXCL-8 upregulation is mainly associated to mucosal proliferation and transcription activities and less to immune activities including inflammatory response and TNF-signaling via NF- $\kappa$ B.

## 4. Discussion

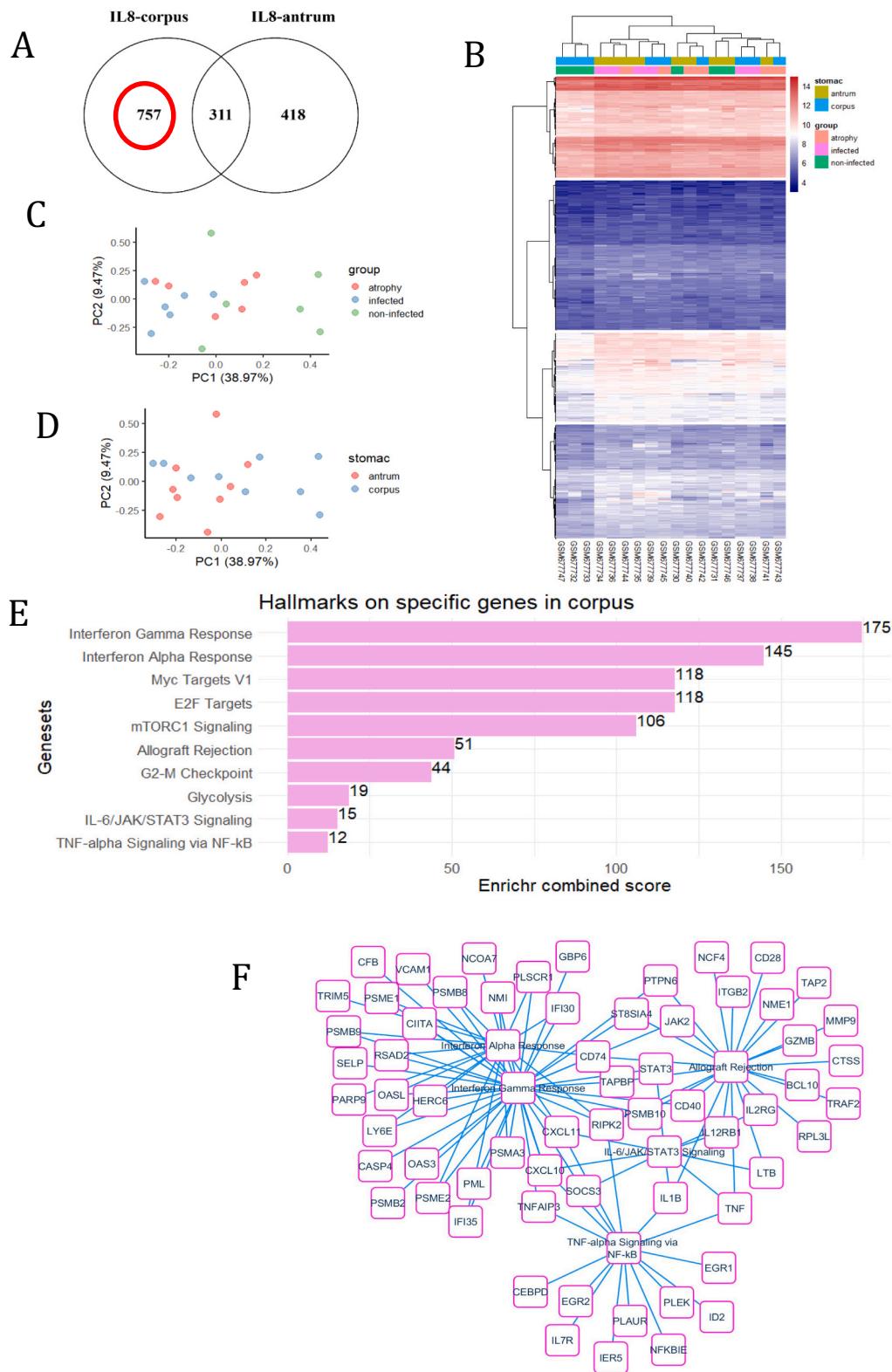
In this paper, we first investigated the correlation within clinical and biological parameters as well as cytokines-chemokine expression levels previously carried out [11]. *H. pylori* density score showed significant correlation to most of the clinical and biological parameters, especially to CXCL-8, IL-1 $\beta$  and IL-17A in both regions of the stomach, namely the antrum and the fundus. Only CXCL-8 and IL-1 $\beta$  levels positively correlated to the *H. pylori* density according to literature [17,18]. Furthermore, our results showed that patients exhibiting different *H. pylori* density scores were differentiated through the cytokines-chemokine profile, suggesting that this triad (IL-1 $\beta$ , IL-17 and CXCL-8) can undeniably differentiate infected from non-infected patients, especially through corpus CXCL-8 expression.

To further explore CXCL-8 role in *H. pylori* infection, we used Geo dataset to investigate coregulated genes to CXCL-8 upregulation. As we previously demonstrated that each gastric region has a specific cytokine-chemokines pattern [11], we investigated the potential pathways involved in response to *H. pylori* induced CXCL-8 in both antrum and corpus. According to the retrieved data, CXCL-8 was upregulated in infected patients in both parts of the stomach. Similarly, we previously showed similar CXCL-8 expression pattern in the corpus, unlike the antrum where CXCL-8 showed no significant variation [11]. As for this chemokine receptors, and in concordance with GEO dataset findings, *H. pylori*-infected patients exhibited an increased level of CXCR1 and CXCR2 in the antrum [7].

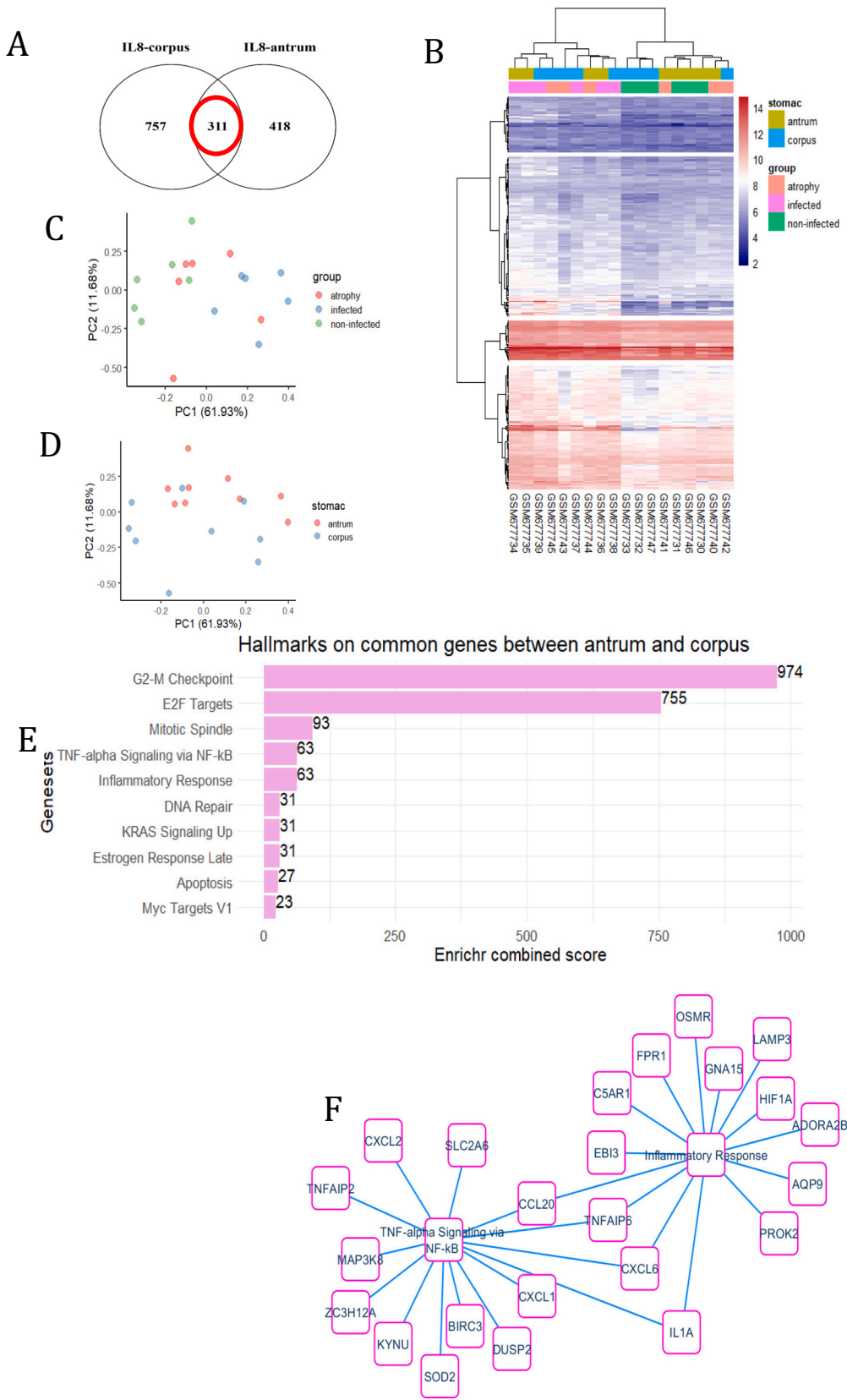
In response to *H. pylori* infection, GECs secrete CXCL-8 which plays the role of chemoattracting neutrophils [7]. These cells are the first to be recruited in response to *H. pylori* and ensure recruitment of other cells, such as macrophages [6]. Strong association between CXCL-8 and the inflammation severity was established, resulting in higher risk of *H. pylori*-associated pathologies [19]. CXCL-8 is also involved in carcinogenesis through angiogenesis, epithelial-mesenchymal transition and cancer cells survival [9].

Enrichment analysis showed that a predominant IL-6/JAK/STAT3 pathway as well as inflammatory response in the antrum were coregulated to CXCL-8 upregulation. Interestingly, IL-6 upregulated in the antrum of patients with gastritis [20], intestinal metaplasia [21] and antrum-localized gastric cancer [22]. In physiological conditions, IL-6 is a critical factor for maturation and differentiation of multiple immune cells, and is shown to activate STAT3 through JAK recruitment and phosphorylation [23]. *H. pylori*-infected patients with precancerous lesions showed heightened STAT3 phosphorylation [24]. In addition, and along with other signaling pathway, IL-6/JAK/STAT3 was implicated in inducing CDX2 expression, which is implicated in intestinal metaplasia development [25]. IL-6-JAK-STAT3 axis is also involved in gastric cancer promotion and progression through enhanced cell proliferation and angiogenesis [24]. This axis was associated to 8 genes which can be classified as cytokine receptors (IL6ST, IL1R1, IL9R and CCR1), myeloid markers (CSF3R, CSF1 and CD14) and pro-oncogenes factors (HAX1), with CD14, CSF3R, CSF1 and IL-1R being also associated to inflammatory response. Moreover, IL-6 mediated-signaling involves binding to a secreted form of the IL-6R (sIL-6R), followed by complexation with Interleukin 6 Cytokine Family Signal Transducer (IL6ST), also known as gp130, resulting in JAKs/STAT3 or SHP-2 recruitment, which is associated to PI3K and ERK/MAPK pathway activation [26,27]. Mice lacking IL6ST SHP-2 binding site leads to constitutive activation of STAT3, resulting in the development of intestinal metaplasia as well as intestinal-type gastric adenocarcinoma [28]. In vitro, unphosphorylated CagA favors the activating JAK/STAT3 axis, and thereby, contributing to carcinogenesis through c-Myc expression and enhancing cell





**Fig. 5.** Immune regulation corpus specific associated to IL-8 expression. Transcriptome data from GSE2741 were used during these analyses. A/ Venn diagram highlighting common genes found correlated to IL-8 and specific to the corpus part of stomach during *H. pylori* infection. B/ Expression heatmap drawn with IL-8 corpus correlated genes. C/ Principal component analysis performed on IL-8 corpus correlated genes stratified on groups (non-infected, infected and atrophy). D/ Principal component analysis performed on IL-8 corpus correlated genes stratified on stomach regional parts; E/ Enrichr Functional enrichment performed on MsigDB\_Hallmark\_2020 database with IL-8 corpus correlated genes. F/ functional enrichment network performed with IL-8 corpus correlated genes implicated in immune functions.



**Fig. 6.** Immune regulation common between antrum and corpus stomach parts and associated to IL-8 modulation of expression. Transcriptome data from GSE2741 were used during these analyses; A/ Venn diagram highlighting common genes found correlated to IL-8 in corpus and in antrum parts of stomach during *H. pylori* infection. B/ Expression heatmap drawn with antrum-corpus common genes correlated to IL-8. C/ Principal component analysis performed on antrum-corpus common genes stratified on groups (non-infected, infected and atrophy). D/ Principal component analysis performed on antrum-corpus common genes stratified on stomach regional parts. E/ Enrichr Functional enrichment performed on MSigDB\_Hallmark\_2020 database with antrum-corpus common genes correlated to IL-8. F/ functional enrichment network performed with antrum-corpus common genes implicated in immune functions.

migration [29]. Likewise, ex vivo studies showed an increase in STAT3 activation in *H. pylori*-infected patients with gastritis and overall poor survival gastric cancer [21]. Furthermore, IL-6 was shown to induce Colony-Stimulating Factor 1 (CSF-1 or macrophage-CSF) through IL-6R signaling pathway [30]. CSF-1 might play an important role in gastric carcinogenesis by upregulating c-Jun transcription factor [31]. Moreover, granulocyte-colony stimulating factor (G-CSF) receptor, CSF3R, leads to activation of JAK/STAT signaling pathways [32], and insure proliferation and differentiation of neutrophils [33]. Indeed, CSF3R-null mice exhibited impaired neutrophil production and mobilization with higher susceptibility to apoptosis [34]. To our knowledge, no studies were carried out regarding CSF3R role in *H. pylori* infection, but neutrophil's role was largely studied in this infection. Acute *H. pylori* infection was marked by neutrophils recruitment which contribute to oxidative burst through ROS production and heighten the risk of cancer development through impairing DNA repair mechanisms [35]. As for gastric cancer, CSF3R was upregulated in patients' biopsies, and its expression was positively correlated to angiogenesis through VEGF expression. On the other hand, HAX1 protein plays a role in the neutrophil's maturation process [36]. Although no study showed HAX1 implication in *H. pylori* infection, ex vivo and in vitro studies showed that HAX1 overexpression is associated to malignant progression and poor prognosis of colorectal cancer and hepatocellular carcinoma [36,37].

Unlike the two other myeloid markers related to IL6/JAK/STAT3 axis, CD14 involvement in *H. pylori* infection has been investigated. *H. pylori*-positive subjects tended to have higher serum CD14 levels compared with *H. pylori*-negative subjects [38]. Gastric cancer cells exhibited higher CD14 expression, which was positively correlated to EMT and thereby metastasis [39]. Progression towards this can also be insured by CCR1, leading to recruitment of myeloid-derived suppressor cells, mesenchymal stem cells, tumor-associated macrophages and neutrophils [40]. IL-1 receptor 1 (IL-1R1) binds to IL-1 $\alpha$ , IL-1 $\beta$ , and IL-1Ra and induce NF- $\kappa$ B activation, which is associated to proinflammatory mediators expression [41]. IL-1 $\beta$  was shown to act on committed and polarized Th17 cells through IL-1R1; indeed, IL-1R deficient mice showed defective IL-17 production [42]. In *H. pylori* infection, IL-1 $\beta$  was associated with severe gastric inflammation and cancer, while certain genotypes of IL-1Ra were associated with decreased inflammation and thus lower risk to develop gastric cancer [43,44]. Regarding IL-9R, its signaling activates the JAK1/STAT(1, 3 and 5) pathway as well as NF- $\kappa$ B pathway [45]. In *H. pylori* infection, chronic gastritis exhibited higher Th9 cells numbers compared to non-infected/healthy individuals [46], but their role was not further clarified. Nevertheless, Th9 response promotes immune cells recruitment through chemokines secretion, and thereby aggravating inflammation [47]. However, IL9 and IL9R overexpression was involved in cervical cancer regression [48]. These overall data suggest that IL-6/JAK/STAT3 as well as its associated genes may enhance inflammation mainly through neutrophils and macrophages activation, thereby contributing to atrophy, IM and gastric cancer.

Next, we carried out a similar enrichment analysis at the corpus level and showed a predominant interferon gamma and alpha responses upon CXCL-8 upregulation. *H. pylori* infection induced Th1 immune response and up-regulation of IFN- $\gamma$  [49]. In addition, the development of precancerous lesions is highly associated to a strong infiltration of Th1-polarized response characterized by CD4<sup>+</sup> IFN- $\gamma$  - producing T cells in the gastric mucosa [50]. However, Tu et al reported that IFN- $\gamma$  overexpression inhibits gastric inflammation, metaplasia and carcinogenesis in mice [51]. IFN- $\gamma$  response was associated to several genes including Caspase-4, SELP and VCAM-1. Caspase-4 is involved in the upregulation of pro-inflammatory cytokines such as CXCL-8 and IL-1 $\beta$  through TNFR-associated factor 6 (TRAF6) activation [52]. In severe gastritis patients, this protease was upregulated, suggesting that its higher expression might be correlated to inflammation severity [53]. Both SELP (Selectin P) and VCAM-1 ensured cell recruitment towards inflammatory sites and

were upregulated in the sera of patients with gastritis compared to non-infected ones [54,55].

Although no study investigated the IFN- $\alpha$  role in the context of *H. pylori* infection, its role was investigated in the gastric cancer stage, where it induced apoptosis by impairing Bcl-2, Bcl-X and BAK expression levels as well as through TRAIL activation [56,57]. IFN- $\alpha$  response displayed an association to Poly-ADP-ribose polymerase family member 9 (PARP9) which was shown to enhance inflammation through TNF $\alpha$  and IL-1 $\beta$  expression [58].

Both interferon responses were associated to other genes, majorly involved in antigen presentation, inflammatory mediators' production and immune cells recruitment. Gastric epithelial cells and APCs express class II MHC, which is regulated by both CIITA and CD74. MHC class II transactivator (CIITA) was shown to be downregulated in macrophages by *H. pylori* [59], implying that its modulation might be an escape immune mechanism. In gastric cancer, CIITA expression was subject to hypermethylation, suggesting that its suppression contribute to the absence of immune system activation [60]. As for CD74, its engagement with *H. pylori* leads to the production of CXCL-8 from GECs which in return enhance CD74, leading to a successful colonization [61]. In gastric cancer, CD74 expression was also upregulated and was associated to prevention of tumor antigen presentation to T cells, rendering tumors less immunogenic [61]. Furthermore, multiple proteasome subunits such as PSMB9, PSMB2, PSMB8, PSMB10 and PSMA2 were linked to the interferon responses. Some of these subunits belong to the immunoproteasome, which is induced by both IFN- $\gamma$  and IFN- $\alpha$  and is involved in the processing of class I MHC peptides driving a T cells dependent response [62]. Regarding inflammatory mediators' production, receptor-interacting serine/threonine kinase 2 (RIPK2) dependent pathways were involved in chemokines production such as CXCL-8, and thereby contributing to the gastric inflammation severity [63] as well as pro-tumorigenic effect in gastric cancer [64]. As for immune cells recruitment, CXCL-10 and CXCL-11 are upregulated in *H. pylori*-infected patients compared to non-infected ones and gastric CXCL-10 showed a positive association with gastritis severity [6,65]. In carcinogenesis, CXCL-10, CXCL-11/CXCR3 signaling in cancer cells increases their carcinogenic potential in an autocrine manner, and ensure immune suppression through T regulatory cells recruitment, explaining their link to poor prognosis in different cancers [66]. However, Gao et al. reported that upregulation of CXCL-11 following chemotherapy drug docetaxel exhibited an anti-tumor effect through increased CD8<sup>+</sup> T cells recruitment [67]. Interferon response was also associated to four genes implicated in antiviral response, but were not investigated in *H. pylori* infection such as TRIM, Lys6, OAS and IFI. Some of them were implicated in pro-inflammatory cytokines and Gram negative bacteria's survival [68,69]. Our results also showed that additionally to IFN response, corpus IL6/JAK/STAT3 signaling axis was also observed but in a minor manner. Robust pSTAT3 expression was seen in the corpus of mice overexpressing IFN- $\gamma$ , as well as upregulated corpus IL-6 [70], suggesting IFN- $\gamma$  association to the pro-tumorigenic IL6/STAT3 signaling axis. It is worth mentioning that this axis is associated to many genes including IL-1 $\beta$ , which is a major contributor the *H. pylori* infection progression towards advanced stages such as gastric cancer [71].

Since CXCL-8 is upregulated in gastritis, IM and gastric cancer, we suspect that IFN response might act at these three stages. Among the proinflammatory mediators induced by *H. pylori*, IFN- $\gamma$  was involved in gastritis development [72]. In this early stage, the IFN response might exacerbate inflammation severity through enhancing *H. pylori* attachment to the corpus mucosa, resulting in immune cells recruitment. Indeed, *H. felis* mice models showed that antigen-specific T cells are the cause of gastric pathology [74]. In addition, T cells as well as IFNs are a necessary components for the induction of atrophic gastritis through GECs apoptosis via Fas-FasL interaction and FADD/caspase-8 signaling, respectively [74]. Furthermore, parietal cell loss might be also induced through autoantigen (H<sup>+</sup>/K<sup>+</sup> ATPase)-specific CD4<sup>+</sup> T cells [74]. In chronic *H. pylori* gastritis with corpus glands atrophy, the gastric H<sup>+</sup>/K<sup>+</sup>-

ATPase has been identified as the major autoantigen [74]. Cell loss alone was not enough to induce IM but needed inflammatory stimuli such as IL-1 $\beta$  [75], which is a marker of this advanced stage in the corpus [11]. Overall, IFN responses-associated genes showed carcinogenic effect and have a potential role in *H. pylori* infection progression towards gastric cancer development. As for the corpus/ antrum common pathways, they were mainly implicated in cell cycle control, and thereby in carcinogenesis. In addition, associated genes to the major immunity-related pathways (TNF $\alpha$ -NF- $\kappa$ B axis and inflammatory response) were commonly associated to C—C Motif Chemokine Ligand 20 (CCL-20) and CXCL-6 as well as TNFAIP6 and IL-1 $\alpha$ . In *H. pylori* infection, the bacterium stimulates the CCL-20 secretion from GECs, T cells and DC and was characterized as a Th17 cell-attracting chemokine [6]. As for gastric cancer, CCL-20 was associated to progression of carcinogenesis as well as decreased overall survival [76]. Likewise, serum levels of CXCL-6 was significantly upregulated in *H. pylori* positive juveniles [65] and CXCL-6 expression promotes tumor angiogenesis, tumor invasion and metastasis through neutrophils recruitment [77]. Similarly, IL-1 $\alpha$  expression was also upregulated in earlier stages of *H. pylori* infection [78] and its upregulation in gastric cancer patients was positively correlated to lymph node metastasis and a decreased overall survival [79]. The TNF $\alpha$ -stimulated gene-6 (TNFAIP6) role was mostly described in the context of cancers. Indeed, its expression increased in gastric cancer tissues and showed positive correlation to tumor invasion, lymph node metastasis and poor overall survival [80].

These data show that these genes have a high inflammatory effect by contributing to promotion as well as progression of gastric carcinogenesis. Some of the molecules were not studied in the context of *H. pylori* infection nor in gastric carcinogenesis, and need to be investigated for more insights on their role in *H. pylori* related gastropathology. Etiologically, *H. pylori* infection is established in the antrum/antrum-corporum junction (incisura angularis), serving as critical niche due to its favorable pH [4,75]. In response to this aggression, inflammation rises and upon its aggravation that might be due to host genetics, resistance to/absence of treatment and/or immune escape of *H. pylori*, the infection spreads towards corpus along the lesser curvature [81–83]. In fact, clinical observations showed that gastric IM was mostly observed in the incisura angularis as well as in the antrum and progress towards corpus in the same manner [84].

On the other hand, and although previous studies showed that significant racial differences were seen in biomarkers for some diseases [85–87], our study aimed to depict the immunological response upon *H. pylori* infection and its associated disease's progression in Moroccan patients. Overall, our results suggested that corpus CXCL8 might be a severity signature within Moroccan patients.

## 5. Conclusions

Since CXCL-8 showed great importance to our data and differentiated between infected and non-infected patients, we explored its impact on each one of the gastric regions. Interestingly, they showed a differential response to CXCL-8 upregulation suggesting that gastric location greatly impact the immune response. The overall genes resulting from the enrichment analysis might contribute to severity of inflammation, which is correlated with progression of *H. pylori* infection. This infection exposes the gastric mucosa to multiple inflammatory stimuli such as CXCL-8. Future investigations must focus on studying CXCL-8 levels in patients with antrum-dominant vs corpus-dominant/pangastritis patients to determine the relevance of our results suggesting that corpus CXCL-8 might be a marker of corpus infection, which is a marker of *H. pylori* infection progression to more severe stage. At last, the differential response of each gastric location and the impact of the overall inflammatory stimuli might highlight the need for adapted therapeutic strategies as it will clarify the behavior of both gastric regions towards *H. pylori* infection, supporting the idea that these two are different not just morphologically, but also in many other aspects such as immune

response. Further and larger studies are needed to corroborate our results in regards to the other populations.

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## Author contribution

El Filaly, Desterke and Akarid: conception and design of the study; drafting the article. Desterke and El Filaly: analysis and interpretation of data. Outlioua: acquisition of data. Outlioua, Akarid, Badre, Rabhi, Karkouri, Riyad, Khalil and Arnoult: revising it critically for important intellectual content. All authors gave the final approval of the version to be submitted.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2023.109648>.

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