Circulating Mitochondrial DNA Level, a Noninvasive Biomarker for the Early Detection of Gastric Cancer

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Running Title: Circulating mitochondrial DNA as a gastric cancer biomarker

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Abstract

Background: Gastric cancer (GC) represents a major health burden worldwide often diagnosed at an advanced-stage. Biomarkers for screening and prevention of GC are missing. Changes in peripheral blood mitochondrial DNA (mtDNA) have emerged as potential preventive/diagnosis biomarker for cancer risk. We aimed to determine whether peripheral leucocytes mtDNA levels are associated with stages of the gastric carcinogenesis cascade**.**

Methods: We measured mtDNA by quantitative real-time PCR assay in peripheral leucocytes of 28 non-atrophic gastritis patients (NAG), 74 GC patients and 48 matched-asymptomatic controls. In parallel, serological level of IL-8 was determined.

Results: Mean mtDNA level was higher in GC patients (p-value=0.0095), compared to controls, with values >8.46 significantly associated with GC (OR=3.93). Three ranges of mtDNA values were identified, interval I: <2.0, interval II: 2.0 to 20 and interval III: >20. Interval I included mainly NAG cases and few GC samples and interval III corresponded almost exclusively to GC patients. All controls felt in interval II, together with some NAG and GC cases; IL-8 levels were significantly higher in GC patients (p-value <0.05) with levels >50 pg/ml observed exclusively in GC patients allowing to distinguish them within interval II. We validated mtDNA results in a second cohort of patients, confirming that mtDNA was significantly higher in GC than in patients with preneoplasia.

Conclusions: Circulating levels of mtDNA and IL-8 constitute a potential biomarker for the early detection of GC.

Impact: Our findings lead us to propose a new non-invasive method to detect patients with GC risk.

Introduction

Gastric cancer (GC) represents a major health burden worldwide, affecting about one-million people per year (1),(2). GC is often diagnosed at an advanced stage and consequently carries a poor prognosis (3). Importantly, if it is detected at an early asymptomatic stage, it can be curable (4); e.g. in Japan, a country with the highest incidence of GC, nation-wide strategies based on improved tests for detection of early GC or precancerous lesions have decreased the incidence of GC and increased survival rate (5).

Although GC arises from the complex interplay of environmental and host genetic factors (6, 7), the major risk factor is *Helicobacter pylori* infection, which is associated with more than 80% of all distal GC cases (8). The prevalence of *H. pylori* infection is high, with 80-95% of the population infected in developing countries and up to 30%-40% of adults in industrialized countries (6). All infected individuals develop a gastritis which evolve to peptic ulcer diseases in about 10% of the cases, while gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma develop in <3% and 0.3% of infected subjects, respectively (9, 10). Two types of GC can be distinguished, the intestinal and diffuse types. The intestinal type develops through progressive changes in the gastric mucosa from non-atrophic gastritis (NAG), atrophic gastritis, intestinal metaplasia (IM), dysplasia and gastric cancer (GC) (11). It has been shown that eradication of the infection at an early stage can reverse gastric lesions and more importantly, prevent the development of preneoplasia ((7),(12),(13)). A clinical study conducted in Japan demonstrated the efficacy of *H. pylori* eradication to reduce the incidence of GC (14), and confirmed that it was not enough to prevent all GC cases. These data raise the need for the development of biomarkers to detect precancerous lesions or early GC; the test should be a simple and non-invasive method, applicable in large scale screening programs. In the case of intestinal type GC, the measure of

pepsinogens levels has been shown to be useful to detect gastric atrophy, although its utility in non-Asian countries is controversial (15). Thus, in spite of several efforts to develop biomarkers to identify patients at risk for distal GC (16), no efficacious screening test is yet available.

Mitochondria are essential organelles of eukaryotic cells that possess their own genome. Mitochondrial DNA (mtDNA) is a circular molecule present at 2-10 copies per organelle (17). Both, mutations and alterations of mtDNA content have been described in many different cancer types (18-20). MtDNA mutations have been detected at early stages of gastric carcinogenesis (21, 22). In *H. pylori*-infected patients, mtDNA mutations are significantly more frequent in GC patients than in cancer free patients (23). According to our previous studies, mtDNA mutations are induced *in vitro* in *H. pylori*-infected gastric epithelial cells and in the gastric mucosa of chronically infected mice (24),(25). A decrease in mtDNA content has also been described in most tumor tissues of advanced GC, compared with nearby non-tumor control tissue (26),(27),(28). Changes in peripheral blood mtDNA levels have recently emerged as a potential preventive/diagnosis biomarker associated with cancer risk (18). Circulating mtDNA levels were significantly higher in patients with urologic malignancies (29), breast (30) colorectal (31) and lung cancer (32, 33). These studies indicated that increased mtDNA content in peripheral blood is associated with elevated cancer risk. However, contrasting reports have found mtDNA depletion in the blood of patients with stage I breast tumors (34). A recent large prospective cohort study in women from Shanghai, found no association between the leukocyte mtDNA copy number and the presence of gastric tumor (35). However, they observed a positive association between a risk of developing GC and low mtDNA copy numbers in blood collected within the two years prior to cancer diagnosis. To our knowledge, there are no other reports on the variation of circulating mtDNA during the gastric carcinogenesis cascade, in particular comparing pre-neoplastic and

neoplastic stages. To address this issue, in the present study we measured levels of mtDNA in the peripheral leucocytes of NAG, IM and GC patients. Our data showed significant variations in mtDNA levels during the progression from NAG to IM and to GC, supporting the notion that circulating mtDNA levels can be useful as potential biomarkers for the identification of early steps of gastric carcinogenesis.

Material and methods

Study population

Two cohorts of Mexican adult patients were studied. Cohort 1 included 48 healthy asymptomatic *H. pylori* negative blood-donors, 28 NAG and 74 GC patients for a total of 150 adults recruited during the period 2009-2011. The blood donors were recruited at the blood bank of the Instituto Mexicano del Seguro Social (IMSS), Medical Center SXXI in Mexico City. Cohort 2 was meant to validate results in cohort 1, and included 46 NAG patients, 31 patients with IM and 49 with GC for a total of 126 patients recruited during the period 1999-2002. Patients from both cohorts were adults who were attended for gastroduodenal diseases at IMSS. We selected patients who were not under treatment for cancer and who had not been treated with antibiotics, bismuth compounds, proton pump inhibitors and non-steroidal anti-inflammatory drugs for at least two preceding weeks. Diagnosis was based on endoscopic examination and histopathology analysis (36). All patients and asymptomatic controls were informed and asked to sign a consent letter. The study was approved by the ethical committee from the National Council for Research on Health, IMSS.

Collection of clinical samples and histological analysis

For each patient, 10 ml of blood were collected and gastric tissue specimens isolated. For patients with NAG or IM, gastric biopsies collected from both the antrum and the corpus were

taken. For patients with GC, one fraction of tumoral and adjacent tissues were collected during surgery. Biopsies were immersed in formalin and processed for haematoxilin-eosin (H&E) staining for histology analysis and diagnosis of gastric lesions. The presence of *H. pylori* was confirmed by Giemsa staining and serology.

Circulating mtDNA

Peripheral blood (10ml) was taken from each patient, and mononuclear cells were purified by centrifugation through a Ficoll-Hypaque density gradient. DNA was isolated from these cells using the salting-out micro-technique and frozen at -70°C until tested for mtDNA quantification. The serum fraction was separated from cells and frozen at -20ºC until tested for serology to *H. pylori* antigens and IL-8 levels.

Determination of IgG against H. pylori antigens and of IL-8 in the serum fractions

H. pylori serology was determined with IgG antibodies against *H. pylori* whole-cell antigens and against CagA *H. pylori* protein using an enzyme-linked immune-absorbent assays (ELISA) previously validated by us (37). Serological levels of IL-8 were measured by ELISA (BD Biosciences).

Quantification of mtDNA

MtDNA levels were measured on DNA isolated from circulating leukocytes by quantitative Polymerase Chain Reaction (q-PCR) using the StepOne^{TM} Plus Real-Time PCR system and FastStart Universal SYBR Green Master (Applied Biosystems) as previously described (38). MtDNA was quantified using a region in the *12S ribosomal RNA* gene, and the nuclear encoded *18S ribosomal RNA* gene as an endogenous reference. Primers used were: *12SrRNA* (forward): 5'-GCTCGCCAGAACACTACGAG; (reverse): 5'—CAGGGTTTGCTGAAGATGGCG; *18SrRNA* (forward): 5'-

GAGAAACGGCTACCACATCC; (reverse): 5'-GCCTCGAAAGAGTCCTGTAT (39). The qPCR reaction was carried out in 20µl of total volume containing 5µl of DNA (200 pg), 10µl of FastStart Universal SYBR Green Master, 0.2µl of primers (10µM) using an initial denaturation step at 95°C for 10 minutes and 40 cycles of 15 secondes at 95°C and 1 minute at 60°C. Samples were analyzed in triplicate. The average threshold cycle number values for nDNA (*18SrRNA*) and mtDNA (*12SrRNA*) were obtained. The relative mtDNA level was calculated using the delta Ct (ΔCt) of average Ct of nDNA and mtDNA ($\Delta Ct = Ct_{nDNA} - Ct_{mtDNA}$) as $2^{\Delta Ct}$ as previously described (40).

Data analysis.

The Student's t-test and Pearson's chi^2 test were used to compare mtDNA level in peripheral blood between healthy subjects and patients at various stages of the gastric pathologies. Differences were considered significant for p-values<0.05. For Odd Ratio (OR) determination the 95% confidence intervals were determined according to the Woolf's method (41). The sensitivity and specificity of mtDNA and IL-8 tests were calculated using receiver operator characteristic (ROC) analyses. Statistical tests performed using GraphPad Prism4 for Macintosh (GraphPad Inc., La Jolla, CA).

Results

Characteristics of the studied cohorts

The general characteristics of the patients included in this study are described in Table 1. In Cohort 1, 50% of the NAG patients were *H. pylori*-positive compared to 71% in the GC group. In the control group, we only selected *H. pylori* negative blood donors (n=48). Patients in the NAG and GC groups were older (mean age 56 and 62 respectively), compared to individuals from the asymptomatic group (mean age 32) (p-value<0.0001). Twenty-eight GC

patients were diagnosed as diffuse-type, of which 6 had metastasis and 9 considered unresectable by surgeons because of a very advanced stage (late-stage). Twenty GC cases were of intestinal type, 7 presented hyperplasia, 7 metastasis and 1 case unresectable. In Cohort 2, 78% of the NAG patients were *H. pylori* positive, compared to 84% in IM and 59% in GC. Overall, IM and GC patients were older compared to NAG patients.

Levels of mtDNA in peripheral leukocytes from patients with NAG and GC

In Cohort 1, mtDNA was quantified in NAG and GC patients as described in material and methods, and compared with asymptomatic *H. pylori* negative subjects. In asymptomatic controls, the relative mtDNA values ranged from 2 to 17.09, whereas in NAG group minimum and maximum mtDNA levels were 0.05 and 29.3 respectively. Even though in GC patients minimum mtDNA level was 1.74, the maximum value measured was 60 (Figure 1A). Mean mtDNA levels were significantly higher in GC patients compared to asymptomatic controls (2-fold) (p-value=0.0013) and to NAG patients (2.2-fold) (p-value=0.0095). As reported in Figure 1A, three intervals of mtDNA values can be distinguished: whereas for all healthy individuals, mtDNA values grouped between 2 and 20 (interval II); in the mtDNA <2.0 group (interval I) only NAG (46% of the cases) and GC (14% of the cases) patients were observed. Importantly, samples with mtDNA<0.5 corresponded exclusively to NAG cases. In contrast, mtDNA values >20 (interval III) were not observed in healthy individuals, and only in 7% of NAG, but in 28% of GC cases. We performed a ROC analyses (42) to determine a cut-off value of mtDNA that will differentiate GC cases from the asymptomatic *H. pylori*–negative controls and found a value of 8.46, with a specificity of 80%, but a sensitivity of 47%. However, using this value we found that mtDNA>8.46 was significantly associated with GC (OR=3.93; 95% CI=1.75-8.81) but not with NAG (OR=1.24; 95% CI=0.42-3.67) (Table

2), in agreement with an association between the presence of gastric malignancy and increased peripheral leukocytes mtDNA content.

An analysis of GC subgroups according to GC types, showed that the mean mtDNA values were similar among diffuse (n=28) and intestinal types (n=20) of GC patients, and significantly higher compared to healthy and NAG samples (Figure 1B). The severity of GC did not affect mtDNA as no significant differences were identified between cases with hyperplasia, metastasis or late-stage (unresectable) (Figure 1B). The presence of *H. pylori* infection had no effect on mtDNA content neither in NAG or in GC patients (Supplementary Figure S1). In addition, age had no influence on mtDNA levels among asymptomatic controls, NAG and GC groups, although a weak correlation was observed when considering the whole cohort (Supplementary Figure S2A). However, no effect of age was detected on mtDNA on both healthy and NAG samples, despite the presence of old individuals (Supplementary Figure S2A, insert graph Cohort 1). Furthermore, a slight mtDNA decrease in smokers compared to non-smokers was noticed (Supplementary Figure S3A).

In order to validate results in Cohort 1, and to further test whether mtDNA levels differentiate patients with pre-neoplasia from NAG patients, mtDNA was quantified in Cohort 2 which included patients with NAG (n=46), intestinal metaplasia (IM) (n=31) and GC (n=49) (Table 1). Samples from Cohort 2 were collected 10 years earlier (1999-2002) than Cohort 1 (2009-2011). This time lapse might be the reason why mtDNA levels in all patients of Cohort 2 were lower than in Cohort 1. Still, behavior between groups was similar and mean mtDNA levels were significantly higher in GC than in both NAG (p-value=0.0012*)* and IM patients (p-value=0.0022) (Supplementary Figure S4). Whereas no significant differences were observed between NAG and IM patients. In this cohort, higher mtDNA values (>2.0) were significantly associated with GC, as compared with NAG (OR=8.48; 95% CI 2.22-32.46) and with IM patients (OR=5.87; 95% CI 1.51-22.84). Thus, in agreement with data from Cohort 1,

peripheral leucocytes mtDNA levels shifted to higher values in GC patients as compared to pre-neoplastic NAG and IM patients. Age had no influence on mtDNA levels, in NAG, IM and GC groups, although a weak association was found for the whole cohort (p-value=0.04) (Supplementary Figure S2C). As for Cohort 1, the same analysis including only non cancer groups did not show any effect of age on mtDNA levels, despite the advanced age of some patients in these groups, specially among IM patients for which mean age was similar to GC patients (Supplementary Figure S2C, insert graph Cohort 2).

Comparison of serological levels of IL-8 and mtDNA in NAG and GC patients

Chronic gastric inflammation is associated with the promotion of carcinogenesis and inflammation can be correlated with plasma mtDNA levels (43). We wondered whether there was any correlation between plasma IL-8 and mtDNA in patients with NAG and GC using samples from Cohort 1. IL-8 levels are not affected by parameters as age (Supplementary Figure S2B). Compared to NAG patients and controls, IL-8 levels were significantly higher in GC patients (4- and 2.3-fold respectively) (p-value=0.016) (Figure 2A) either with diffuse (p-value=0.019) or intestinal type (p-value=0.036) of cancer and with metastasis (p-value=0.003) or unresectable (p-value=0.0015) (Figure 2B). However, no correlation was observed between IL-8 and mtDNA levels in GC patients. It should be noticed that among GC patients, IL-8 levels were higher in smokers than in non-smokers (Supplementary Figure S3B). Eighty percent of samples with IL-8 >50 pg/ml corresponded to GC cases, among which 67% were in the mtDNA interval II. Moreover, 89% of GC samples with the highest mtDNA level (interval III), showed IL-8<50 pg/ml. For NAG patients, a weak but significant correlation was observed between IL-8 and mtDNA (r=0.586*; P*=0.0041), although the analysis included only 25 samples. Thus, in most of the cases higher concentration of IL-8 differentiated GC

patients with “normal” mtDNA levels (interval II), in spite of the lack of correlation between mtDNA and IL-8 levels (Figure 3).

Discussion

Gastric cancer remains the second cause of death by cancer, often detected at an advanced stage. Thus, there is a real need to develop appropriate screening strategies for its early detection. In Japan, one of the countries with the highest incidence of GC, strategies that include the eradication of *H. pylori* among young people, and periodic endoscopic examination have been proposed to reduce GC deaths (5). However, endoscopy is an invasive and costly method that requires special equipment and well-trained personnel to offer reliable diagnosis. Despite all recent developments in molecular diagnostic (16), identification of biomarkers with reliable predictive value are still unavailable. Peripheral blood mtDNA copy number has recently emerged as a potential preventive/diagnosis biomarker associated with the risk of various cancers (18),(19). Levels of circulating mtDNA were found significantly higher in patients with various type of tumors (29),(30),(31),(32),(44). Peripheral mtDNA content has also been proposed as a novel molecular marker for tracing tumor progression (34). In the present study, we found more variation of circulating leukocytes mtDNA level in patients with NAG or GC as compared with healthy subjects. We identify a distribution of samples according to three intervals of mtDNA levels measured as previously described (38),(40), intervals I: mtDNA<2.0; II with mtDNA comprised between 2.0 and 20.0 and III corresponding to mtDNA values >20.0. All asymptomatic individuals felt in the interval II, together with some NAG and GC patients. Importantly, in the interval I near half of NAG and very few GC patients but no healthy individuals were observed, whereas in the interval III almost only GC patients were found. Interestingly, low mtDNA values (<0.5) corresponded only to NAG patients whereas high mtDNA levels (>30) were exclusively observed in GC

patients. According to these data, by a simple blood sampling, the classification of an individual in intervals I or III would be an indication of the presence of gastric inflammation and probable GC, respectively. This detection would constitute a first indication for the patients, upstream endoscopic investigation and specific follow-up.

In a recent large prospective study, no association between mtDNA copy number and GC was found (35). However a positive association between low mtDNA copy number and risk of GC was observed in blood collected within the two years prior to cancer diagnosis, suggesting that a decrease in mtDNA level is an early indicator of the malignant transformation of gastritis lesions. In agreement, in our study the lowest mtDNA levels correspond to NAG patients. Gastritis is characterized by a chronic inflammation of the gastric mucosa, associated with high production of oxidative species known to damage DNA, rendering mtDNA highly susceptible to damage and depletion. This is probably the reason for the lower mtDNA levels observed in some NAG cases. Although the role of mtDNA depletion in the cancer process remains unknown, it might decrease mitochondrial genes expression and impair the oxidative phosphorylation chain activity, leading to an increased production of ATP by glycolysis, a mechanism occurring during tumorigenesis, known as the Warburg effect (45). Low mtDNA copy number has also been reported associated to an increased risk in various cancers (46),(47). A lower blood mtDNA level has been described in stage I breast cancer as compared to advanced stages II to IV, leading to suggest that cancer cells deplete a larger amount of mtDNA in circulating cells at an early stage of cancer development. The initial decline in mtDNA content should be compensated at later stage of the cancer process to restore respiratory functions (34), which is in agreement with the increase we observed in many of the CG cases studied. This kinetic of mtDNA levels during the carcinogenic process would also be in agreement with results from Cohort 2 patients showing significant lower mtDNA levels in NAG and in precancerous IM patients, compared

to most GC patients. This tendency was observed in Cohort 2 in spite of lower levels of mtDNA in all patients studied, which was probably due to the long time lapse of storage samples before testing. This observation would argue to the importance of using only fresh specimens to have reliable and clinically useful values. It also suggest that mtDNA levels cannot distinguish NAG patients and patients with early pre-neoplastic lesions.

Summarizing results from both cohorts, in healthy *H. pylori* negative individuals we observed a limited range of mtDNA levels (interval II) and values below this range were found mostly in patients with precancerous stages NAG and IM lesions (interval I); whereas values above this range were observed mostly in patients with GC (interval III). Based on these results and in agreement with a previous study (35), patients with decreased mtDNA levels potentially represent individuals with increased risk for GC, which should be monitored periodically to effectively prevent GC development. Whereas in patients with increased mtDNA, the presence of GC lesions should be suspected and further tested by endoscopy. However, in both, precancerous and GC groups we observed patients with mtDNA levels in the same range as healthy individuals. These patients need to be further studied to identify if they represent a group with a different carcinogenesis process or cancer stage. Among GC cases, we noticed a tendency, although not statistically significant, for mtDNA to increase according to the severity of the disease, from patients with hyperplasia or metastasis to later-stage (unresectable). In any case, it should be studied in a larger number of patients, to confirm if mtDNA may help differentiate GC stages and subtypes.

Chronic inflammation contributes to the promotion of cancer. Recently, a positive association has been reported between plasma level of pro-inflammatory cytokines, IL-6 and IL-8, and high GC incidence, suggesting them as potential biomarkers for GC (48). We did not find any correlation between IL-8 and mtDNA levels, indicating that the inflammatory process does not always drive the observed increased mtDNA levels in GC. In fact, we found

mostly low IL-8 levels in GC cases with high mtDNA level (interval III). In addition, higher values of IL-8 were detected mostly in GC cases with "normal" mtDNA level (interval II), which supports that determination of both, mtDNA and IL-8, improves the identification of patients with GC in interval II. It is important to notice that most of these GC patients (mtDNA-interval II and IL-8>50 pg/ml) were smokers. MtDNA levels can be also affected by other parameters as infections, that should be taken into account (49).

In conclusion, we identified three different ranges of peripheral leucocytes mtDNA levels, one present in all healthy *H. pylori* uninfected subjects and considered as "normal", one with lower mtDNA observed mainly in patients with gastric precancerous lesions and a third increased range corresponding almost exclusively to GC patients. According to our data, quantification of mtDNA by a simple blood sampling would permit an early detection of the presence of lesions and to design a personalized clinical follow-up of patients. Patients with low mtDNA level are likely to present precancerous or GC lesions and should be periodically monitored by endoscopy. Patients with high mtDNA level are more likely to already present GC and should be thoroughly studied by endoscopy. We should notice that there were patients with precancerous and with GC lesions with mtDNA levels in the same range as the asymptomatic group, which deserve more studies. Detection can be improved by testing in addition IL-8 levels, particularly in GC patients that present "normal" mtDNA (interval II). These data should be further validated in larger patient groups, also including patients from different geographical origin. Our findings indicate that testing for circulating mtDNA and IL-8 might offer reliable minimaly invasive biomarkers, to screen populations at risk for GC. They pave the way to the development of circulating mtDNA measure as a predictive/early-diagnostic biomarkers that are particularly needed in developing countries in Asia and Latin America, regions with the highest GC mortality rates.

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Figure legends

Figure 1. Circulating mtDNA content in leukocytes of NAG and GC patients compared to healthy blood donors in Cohort 1. A) MtDNA was quantified by quantitative real-time PCR (40), from DNA isolated from peripheral leukocytes isolated from healthy blood donors *H. pylori*-negative (open triangles), NAG (open circles) and GC (closed circles) patients. (40)Intervals I, II and III correspond to range of mtDNA level: <2, 2-20 and >20, respectively. **B)** Peripheral leukocytes mtDNA level in NAG and GC patients according to the type of cancer either diffuse (n=31) or intestinal (n=20) (left part) or the presence of hyperplasia (n=19), metastasis (n=19) or late-stage cancer judged unresectable by surgeons (n=17) (right part). As in control group, mean mtDNA levels are lower in NAG patients compared to GC of diffuse (*) intestinal type (**) with hyperplasia (*), metastasis (**) and late-stage cancer (*). Each symbol corresponds to a single patient. Bars indicate mean ± SEM. Samples were tested in triplicate in three independent experiments.

*p-value<0.05; ** <0.01: ***<0.001**.**

Figure 2. IL-8 serological levels in NAG and GC patients. A) IL-8 was measured by ELISA in sera from controls, NAG and GC patients from cohort 1. **B)** IL-8 levels were compared between diffuse and intestinal type of GC, as well as among GC with hyperplasia, metastasis or at late-stage. Bars correspond to mean ± SEM. *p-value<0.05; **<0.01

Figure 3. Distribution of mtDNA levels according to IL-8 in healthy subjects, NAG and GC patients related to interval II. No correlation was observed in all cases between mtDNA and IL-8 levels. However, 75% of samples with IL-8>50pg/ml correspond to GC cases. Each symbol corresponds to a single patient.

Table 1. Characteristics of the study population

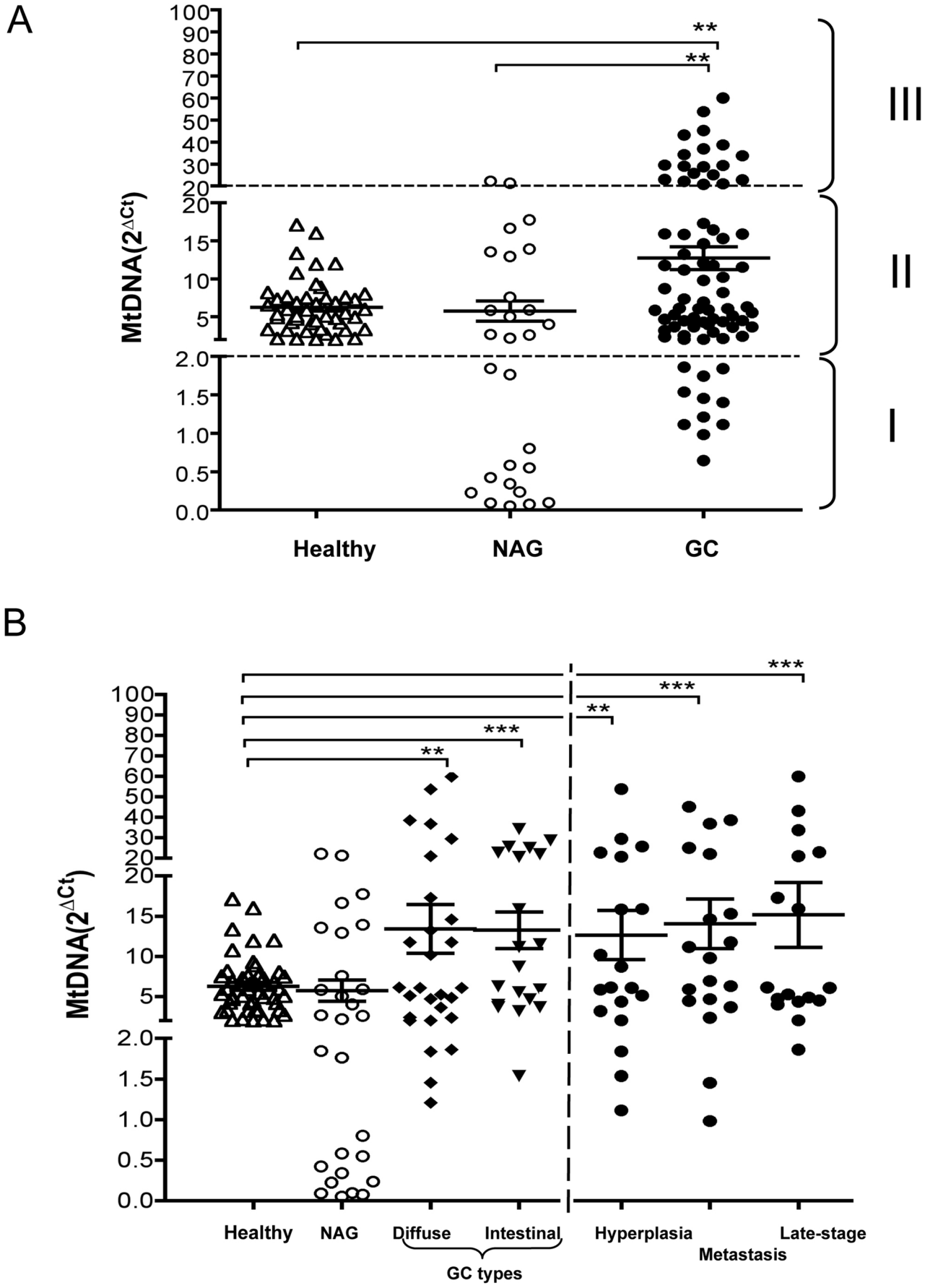
		Number of patients	Mean age (range)	Sex ratio (M/F)	*H. pylori* positive %
Cohort 1	Healthy[a]	48	32 (18-62)	0.92	-
	Non-atrophic gastritis (NAG)	28	56 (21-84) $p<0.0001$[b]	2.25	50
	Gastric cancer (GC)	74	62 (31-87) $p<0.0001$[b] $p=0.019$[c]	1.23	71
	Total number of patients (NAG+GC)	102			
Cohort 2	Non-atrophic gastritis (NAG)	46	51 (30-78)	0.45	78
	Intestinal Metaplasia (IM)	31	60 (33-80) $p=0.007$[d]	0.55	84
	Gastric cancer (GC)	49	62 (31-86) $p=0.0003$[d]	1.88	59
	Total number of patients (NAG+IM+GC)	126			

Two different groups of patients are analysed in the study. In each groups, samples were collected around the same period of time, from 2009-2011 for Cohort 1 and 1999-2002 for Cohort 2. [a] Healthy subjects are *H. pylori* negative blood donors. P-values in the « Mean age » column represent statistical analyses for age distribution in, [b]NAG and GC patients compared to healthy individuals; [c]GC patients compared to the NAG group (Cohort 1); [d]IM and GC patients compared to the NAG group (Cohort 2).

Table 2. Increased mtDNA is associated with significant risk for gastric cancer

	mtDNA*				
Group	<8.462	>8.462	OR (CI 95%)	X^2	p-value
Healthy	40	8	1.00		
NAG	21	7	1.24 (0.42-3.67)	0.15	0.69
GC	36	38	3.93 (1.75-8.81)	11.7	0.0006

*cutoff value for mtDNA was >8.462 based on a ROC analyses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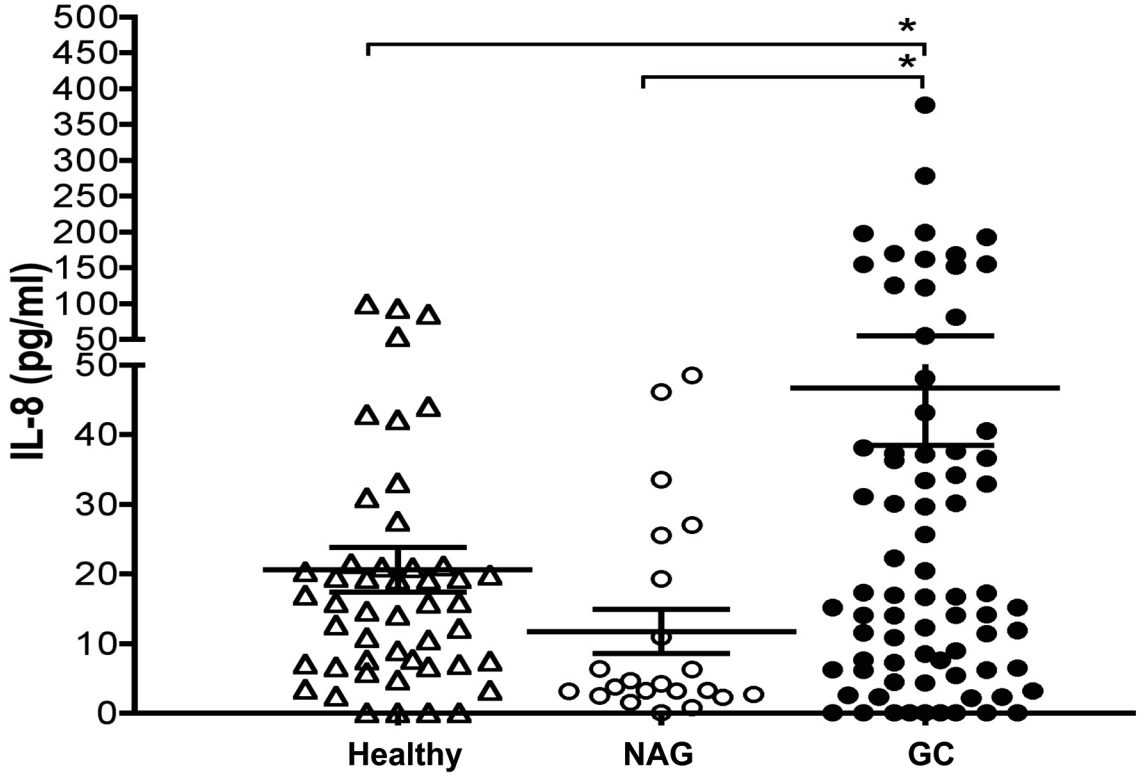
MtDNA(2^ΔCt)
100
90
80
70
60
50
40
30
20
15
10
5
2.0
1.5
1.0
0.5
0.0
**
**
III
II
I
Healthy
NAG
GC
B
MtDNA(2^ΔCt)
***
***
**
**
***
Healthy
NAG
Diffuse
Intestinal
GC types
Hyperplasia
Late-stage
Metastasis

Figure 1

A


IL-8 (pg/ml)
500
450
400
350
300
250
200
150
100
50
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40
30
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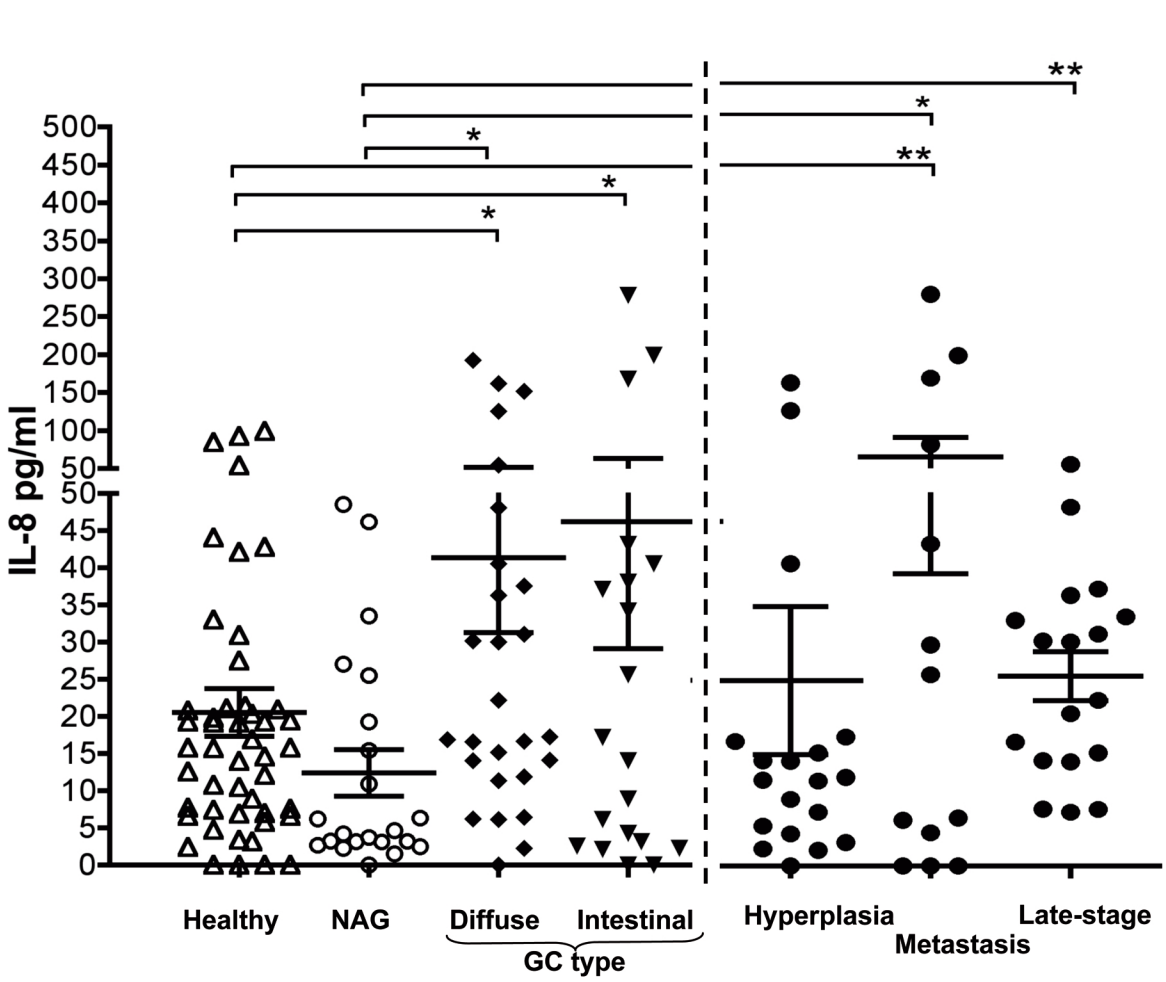
10
0
*
*
Healthy
NAG
GC

B


IL-8 pg/ml
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450
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10
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0
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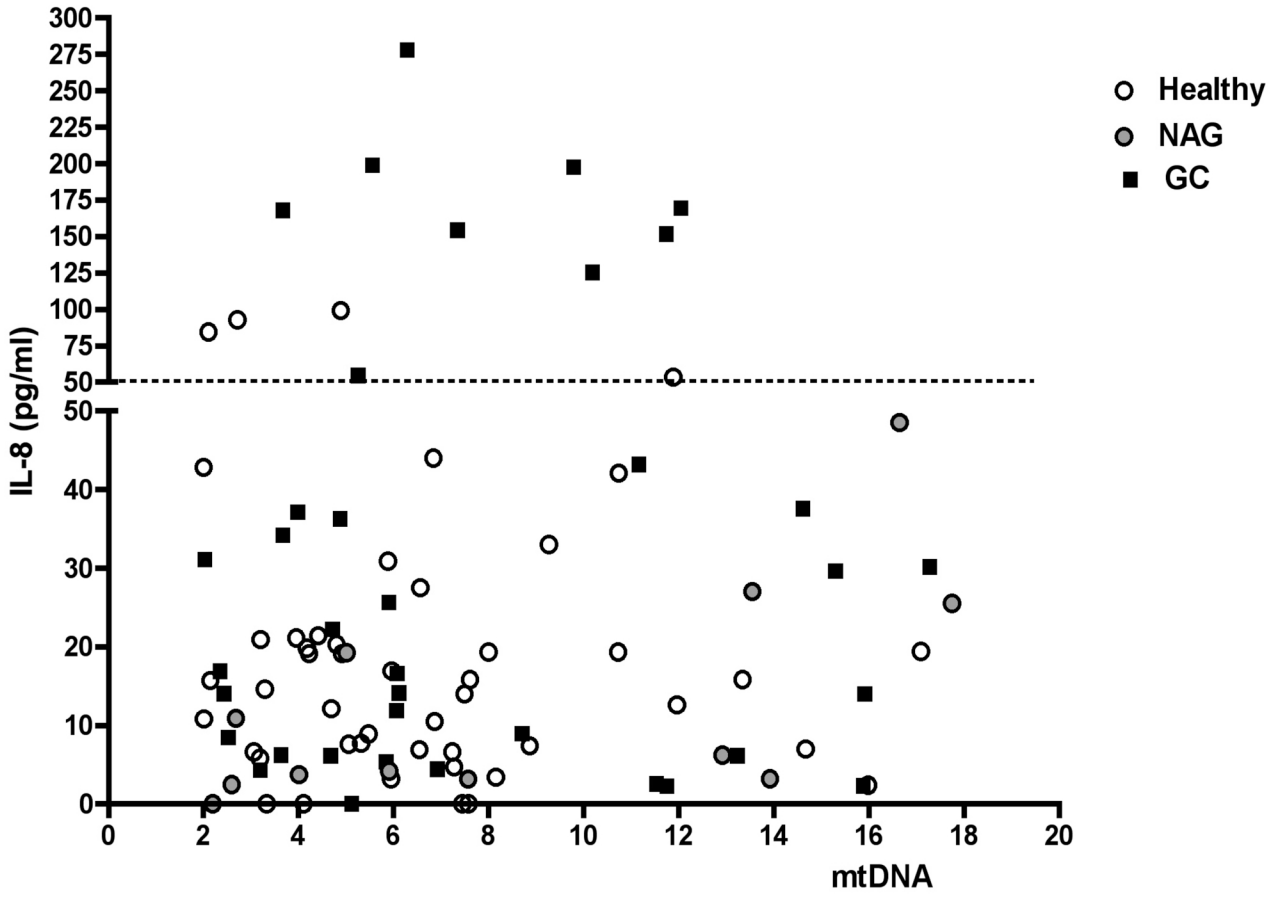
Healthy
NAG
Diffuse
Intestinal
GC type
Hyperplasia
Late-stage
Metastasis

Figure 2


300
275
250
225
200
175
150
125
100
75
50
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10
0
IL-8 (pg/ml)
0
2
4
6
8
10
12
14
16
18
20
mtDNA
Healthy
NAG
GC

Figure 3