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

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Abstract

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

**Methods:** We measured mtDNA by quantitative real-time PCR assay in peripheral leukocytes of 28 patients with non-atrophic gastritis (NAG), 74 patients with gastric cancer, and 48 matched asymptomatic controls. In parallel, the serologic level of IL8 was determined.

**Results:** Mean mtDNA level was higher in patients with gastric cancer ($P = 0.0095$) than in controls, with values $>8.46$ significantly associated with gastric cancer (OR, 3.93). Three ranges of mtDNA values were identified: interval I, $<2.0$; interval II, $2.0–20$; and interval III, $>20$. Interval I included mainly NAG cases, and few gastric cancer samples and interval III almost comprehensively with controls. Gastric All controls fell in interval II, together with some NAG and gastric cancer cases. IL8 levels were significantly higher in patients with gastric cancer ($P < 0.05$), with levels $>50$ pg/mL observed exclusively in patients with gastric cancer, allowing to distinguish them within interval II. We validated mtDNA results in a second cohort of patients, confirming that mtDNA was significantly higher in gastric cancer than in patients with preneoplasia.

**Conclusions:** Circulating levels of mtDNA and IL8 constitute a potential biomarker for the early detection of gastric cancer.

**Impact:** Our findings lead us to propose a new noninvasive method to detect patients with gastric cancer risk.

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Introduction

Gastric cancer represents a major health burden worldwide, affecting about 1 million people per year (1, 2). Gastric cancer 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); for example, in Japan, a country with the highest incidence of gastric cancer, nation-wide strategies based on improved tests for detection of early gastric cancer or precancerous lesions have decreased the incidence of gastric cancer and increased survival rate (5).

Although gastric cancer 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 gastric cancer cases (8). The prevalence of $H. pylori$ infection is high, with 80% to 95% of the population infected in developing countries and up to 30% to 40% of adults in industrialized countries (6). All infected individuals develop a gastritis that evolve to peptic ulcer diseases in about 10% of the cases, whereas gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma develop in <3% and 0.3% of infected subjects, respectively (9, 10). Two types of gastric cancer 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, dysplasia, and gastric cancer (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 gastric cancer (14) and confirmed that it was not enough to prevent all gastric cancer cases. These data raise the need for the development of biomarkers to detect precancerous lesions or early gastric cancer; the test should be a simple and noninvasive method, applicable in large-scale screening programs. In the case of intestinal-type gastric cancer, the measure of pepsinogen levels has been shown to be useful to detect gastric atrophy, although its use in non-Asian countries is controversial (15). Thus, despite several efforts to develop biomarkers to identify patients at risk for distal gastric cancer (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 to 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 patients with gastric cancer than in cancer-free patients (23). According to our previous studies, mtDNA mutations are induced \textit{in vitro} in \(H. pylori\)-infected gastric epithelial cells and in the mucosa of chronically infected mice (24, 25). A decrease in mtDNA content has also been described in most tumor tissues of advanced gastric cancer, compared with nearby nontumor control tissue (26–28). 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 cancers (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 gastric cancer and low mtDNA copy numbers in blood collected within the 2 years before cancer diagnosis. To our knowledge, there are no other reports on the variation of circulating mtDNA during the gastric carcinogenesis cascade, in particular comparing preneoplastic and neoplastic stages. To address this issue, in the present study, we measured levels of mtDNA in the peripheral leukocytes of patients with NAG, intestinal metaplasia, and gastric cancer. Our data showed significant variations in mtDNA levels during the progression from NAG to intestinal metaplasia and to gastric cancer, supporting the notion that circulating mtDNA levels can be useful as potential biomarkers for the identification of early steps of gastric carcinogenesis.

Materials and Methods

Study population

Two cohorts of Mexican adult patients were studied. Cohort 1 included 48 healthy asymptomatic \(H. pylori\)-negative blood donors, 28 patients with NAG, and 74 patients with gastric cancer, for a total of 150 adults recruited during the period 2009 to 2011. The blood donors were recruited at the blood bank of the Instituto Mexicano del Seguro Social (IMSS), Medical Center SXI in Mexico City. Cohort 2 was meant to validate results in cohort 1 and included 46 patients with NAG, 31 patients with intestinal metaplasia, and 49 patients with gastric cancer, for a total of 126 patients recruited during the period 1999 to 2002. Patients from both cohorts were adults who were attended for gastroduodenal diseases at the 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 NSAIDs for at least 2 preceding weeks. Diagnosis was based on endoscopic examination and histopathologic 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 histologic analysis

For each patient, 10 mL of blood was collected, and gastric tissue specimens were isolated. For patients with NAG or intestinal metaplasia, gastric biopsies collected from both the antrum and the corpus were taken. For patients with gastric cancer, one fraction of tumoral and adjacent tissues were collected during surgery. Biopsies were immersed in formalin and processed for hematoxylin and eosin (H&E) staining for histologic analysis and diagnosis of gastric lesions. The presence of \(H. pylori\) was confirmed by Giemsa staining and serology.

Circulating mtDNA

Peripheral blood (10 mL) 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 microtechnique and frozen at −70°C until tested for mtDNA quantification. The serum fraction was separated from cells and frozen at −20°C until tested for serology to \(H. pylori\) antigens and IL8 levels.

Determination of IgG against \(H. pylori\) antigens and of IL8 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 ELISA previously validated by us (37). Serological levels of IL8 were measured by ELISA (BD Biosciences).

Quantification of mtDNA

mtDNA levels were measured on DNA isolated from circulating leukocytes by qPCR using the StepOne 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: 12S rRNA (forward): 5′-GCTGGCG-GAAACATCGAG; (reverse): 5′-CAGGGTTTCTGAGAACAG-GATGCC; 18S rRNA (forward): 5′-GAGAAACGG-CTACCACATCC; (reverse): 5′-GCCTCGAAAGACTGCCT-GTAT (39). The qPCR reaction was carried out in 20 μL of total volume containing 5 μL of DNA (200 pg), 10 μL of primers (10 μmol/L) using an initial denaturation step at 95°C for 10 minutes and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Samples were analyzed in triplicate. The average threshold cycle number values for nDNA (18S rRNA) and mtDNA (12S rRNA) were obtained. The relative mtDNA level was calculated using the ∆Ct of average Ct of nDNA and mtDNA (ΔCt = Ct nDNA − Ct mtDNA) as 2−ΔΔCt, as previously described (40).

Statistical analysis
The Student t test and Pearson χ2 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 < 0.05. For OR determination, the 95% confidence intervals (CI) were determined according to the Woolf method (41). The sensitivity and specificity of mtDNA and IL8 tests were calculated using ROC analyses. Statistical tests were performed using GraphPad Prism4 for Macintosh (GraphPad Inc.).

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 patients with NAG were H. pylori–positive compared with 71% in the gastric cancer group. In the control group, we only selected H. pylori–negative blood donors (n = 48). Patients in the NAG and gastric cancer groups were older (mean ages, 56 and 62 years, respectively), compared with individuals from the asymptomatic group (mean age, 32 years; P < 0.0001). Twenty-eight patients with gastric cancer 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 gastric cancer cases were of intestinal type, 7 presented hyperplasia, 7 metastasis, and 1 case unresectable. In cohort 2, 78% of the patients with NAG were H. pylori–positive, compared with 84% in intestinal metaplasia and 59% in gastric cancer. Overall, patients with intestinal metaplasia and gastric cancer were older than patients with NAG.

Levels of mtDNA in peripheral leukocytes from patients with NAG and gastric cancer
In cohort 1, mtDNA was quantified in patients with NAG and gastric cancer as described in Materials 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, the minimum and maximum mtDNA levels were 0.05 and 29.3, respectively. Even though in patients with gastric cancer, the minimum mtDNA level was 1.74, the maximum value measured was 60 (Fig. 1A). Mean mtDNA levels were significantly higher in patients with gastric cancer than in asymptomatic controls (2-fold; P = 0.0013) and patients with NAG (2.2-fold; P = 0.0095). As reported in Fig. 1A, 3 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 patients with NAG (46% of the cases) and gastric cancer (14% of the cases) 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 gastric cancer cases. We performed an ROC analyses (42) to determine a cutoff value of mtDNA that will differentiate gastric cancer 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 gastric cancer (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 gastric cancer subgroups according to gastric cancer types showed that the mean mtDNA values were similar among diffuse (n = 28) and intestinal types (n = 20) of patients with gastric cancer and significantly higher than healthy and NAG samples (Fig. 1B). The severity of gastric cancer did not affect mtDNA, as no significant differences were identified between cases with hyperplasia, metastasis, or late stage (unresectable; Fig. 1B). The presence of H. pylori infection had no effect on mtDNA content neither in patients with NAG nor in patients with gastric cancer (Supplementary Fig. S1). In addition, age had no influence on mtDNA levels among asymptomatic controls, NAG and gastric cancer groups, although a weak correlation was observed when considering the whole cohort (Supplementary Fig. S2A). However, no effect of age was detected on mtDNA on both healthy and NAG samples, despite the presence of old individuals (Supplementary Fig. S2A; insert graph, cohort 1). Furthermore, a slight mtDNA decrease in smokers compared with non-smokers was noticed (Supplementary Fig. S3A).

To validate results in cohort 1 and to further test whether mtDNA levels differentiate patients with preneoplasia from patients with NAG, mtDNA was quantified in cohort 2 which included patients with NAG (n = 46), intestinal metaplasia (n = 31), and gastric cancer (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 gastric cancer than in both patients with NAG \((P = 0.0012)\) and patients with intestinal metaplasia \((P = 0.0022;\) Supplementary Fig. S4). No significant differences were observed between patients with NAG and intestinal metaplasia. In this cohort, higher mtDNA values \((>2.0)\) were significantly associated with gastric cancer than with NAG \((\text{OR, 8.48; 95\% CI, 2.22–32.46})\) and with patients with intestinal metaplasia \((\text{OR, 5.87; 95\% CI, 1.51–22.84})\). Thus, in agreement with data from cohort 1, peripheral leukocytes mtDNA levels shifted to higher values in patients with gastric cancer than in patients with pre-neoplastic NAG and intestinal metaplasia. Age had no influence on mtDNA levels, in NAG, intestinal metaplasia, and gastric cancer groups, although a weak association was found for the whole cohort \((P = 0.04;\) Supplementary Fig. 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, especially among patients with intestinal metaplasia for which mean age was similar to patients with gastric cancer \((\text{Supplementary Fig. S2C; insert graph, cohort 2})\).

### Comparison of serological levels of IL8 and mtDNA in patients with NAG and gastric cancer

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 IL8 and mtDNA in patients with NAG and gastric cancer using samples from cohort 1. IL8 levels are not affected by parameters such as age \((\text{Supplementary Fig. S2B})\). Compared with patients with NAG and controls, IL8 levels were significantly higher in patients with gastric cancer \((4- and 2.3-fold, respectively; \text{P = 0.016; Fig. 2A})\) either with diffuse \((P = 0.019)\) or intestinal type \((P = 0.036)\) of cancer and with metastasis \((P = 0.003)\) or unresectable \((P = 0.0015;\) Fig. 2B). However, no correlation was observed between IL8 and mtDNA levels in patients with gastric cancer. It should be noticed that among patients with gastric cancer, IL8 levels were higher in smokers than in nonsmokers \((\text{Supplementary Fig. S3B})\). Eighty percent of samples with IL8 \(> 50 \text{pg/mL}\) corresponded to gastric cancer cases, among which 67\% were in the mtDNA interval II. Moreover, 89\% of gastric cancer samples with the highest mtDNA level (interval III) showed IL8 < 50 pg/mL. For patients with NAG, a weak but significant correlation was observed between IL8 and mtDNA \((r = 0.586; P = 0.0041)\), although the analysis included only 25 samples. Thus, in most of the cases, higher concentration of IL8 differentiated patients with gastric cancer with “normal” mtDNA levels (interval II), despite of the lack of correlation between mtDNA and IL8 levels (Fig. 3).
a real need to develop appropriate screening strategies for its early detection. In Japan, one of the countries with the highest incidence of gastric cancer, strategies that include the eradication of *H. pylori* among young people and periodic endoscopic examination have been proposed to reduce gastric cancer deaths (5). However, endoscopy is

![Table 2](image.png)

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

<table>
<thead>
<tr>
<th>Group</th>
<th>mtDNAa</th>
<th>OR (95% CI)</th>
<th>(\chi^2)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>40</td>
<td>8</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>NAG</td>
<td>21</td>
<td>7</td>
<td>1.24 (0.42–3.67)</td>
<td>0.15</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>36</td>
<td>38</td>
<td>3.93 (1.75–8.81)</td>
<td>11.7</td>
</tr>
</tbody>
</table>

aCutoff value for mtDNA was >8.462 based on ROC analyses.
an invasive and a costly method that requires special equipment and well-trained personnel to offer reliable diagnosis. Despite all recent developments in molecular diagnostics (16), identification of biomarkers with reliable predictive value is 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 types of tumors (29–32, 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 gastric cancer than in healthy subjects. We identify a distribution of samples according to 3 intervals of mtDNA levels measured as previously described (38, 40): interval I, mtDNA < 2.0; II with mtDNA between 2.0 and 20.0; and III corresponding to mtDNA values >20.0. All asymptomatic individuals fell in the interval II, together with

![Figure 2. IL8 serological levels in patients with NAG and gastric cancer (GC). A, IL8 was measured by ELISA in sera from controls, patients with NAG, and gastric cancer from cohort 1. B, IL8 levels were compared between diffuse and intestinal type of gastric cancer, as well as among gastric cancer with hyperplasia, metastasis, or at late stage. Bars correspond to mean ± SEM. *, P < 0.05; **, P < 0.01.](image)
some patients with NAG and gastric cancer. Importantly, in the interval I, nearly half of NAG and very few patients with gastric cancer but no healthy individuals were observed, whereas in interval III, almost only patients with gastric cancer were found. Interestingly, low mtDNA values (<0.5) corresponded only to patients with NAG, whereas high mtDNA levels (>30) were exclusively observed in patients with gastric cancer. According to these data, by a simple blood sampling, the classification of an individual in interval I or III would be an indication of the presence of gastric inflammation and probable gastric cancer, 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 gastric cancer was found (35). However, a positive association between low mtDNA copy number and risk of gastric cancer was observed in blood collected within the 2 years before cancer diagnosis, suggesting that a decrease in mtDNA level is an early indicator of the presence of gastric inflammation and probable gastric cancer, respectively. This detection would constitute a first indication for the patients, upstream endoscopic investigation, and specific follow-up.

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Circulating Mitochondrial DNA as a Gastric Cancer Biomarker

in patients with gastric cancer (interval III). On the basis of these results and in agreement with a previous study (35), patients with decreased mtDNA levels potentially represent individuals with increased risk for gastric cancer, which should be monitored periodically to effectively prevent gastric cancer development. Whereas in patients with increased mtDNA, the presence of gastric cancer lesions should be suspected and further tested by endoscopy. However, in both precancerous and gastric cancer groups, we observed mtDNA levels to be 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 gastric cancer 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 gastric cancer stages and subtypes.

Chronic inflammation contributes to the promotion of cancer. Recently, a positive association has been reported between plasma level of proinflammatory cytokines, IL6 and IL8, and high gastric cancer incidence, suggesting them as potential biomarkers for gastric cancer (48). We did not find any correlation between IL8 and mtDNA levels, indicating that the inflammatory process does not always drive the observed increased mtDNA levels in gastric cancer. In fact, we found mostly low IL8 levels in gastric cancer cases with high mtDNA level (interval III). In addition, higher values of IL8 were detected mostly in gastric cancer cases with "normal" mtDNA level (interval II), which supports that determination of both, mtDNA and IL8, improves the identification of patients with gastric cancer in interval II. It is important to notice that most of these patients with gastric cancer (mtDNA-interval II and IL8 > 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 3 different ranges of peripheral leukocyte 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 one increased range corresponding almost exclusively to patients with gastric cancer. 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 levels are likely to present precancerous or gastric cancer lesions and should be periodically monitored by endoscopy. Patients with high mtDNA levels are more likely to already present gastric cancer and should be thoroughly studied by endoscopy. We should notice that there were patients with precancerous and gastric cancer lesions with mtDNA levels in the same range as the asymptomatic group, which deserves more studies. Detection can be improved by testing additional IL8 levels, particularly in patients with gastric cancer who 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 IL8 might offer reliable minimally invasive biomarkers to screen populations at risk for gastric cancer. They pave the way for the development of circulating mtDNA measure as a predictive/early diagnostic biomarker that is particularly needed in developing countries in Asia and Latin America, regions with the highest gastric cancer mortality rates.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Fernandes, J. Torres, E. Touati Development of methodology: M. Camorlinga-Ponce, A. Gomez, C. Maldonado, E. Touati Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Fernandes, V. Michel, C. Maldonado, J. Torres Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Fernandes, V. Michel, A. Gomez, C. Maldonado, J. Torres, E. Touati Writing, review, and/or revision of the manuscript: A. Gomez, H. De Reuse, J. Torres, E. Touati Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Michel, C. Maldonado Study supervision: J. Torres, E. Touati

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