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Estimating the Copy Number Variation of Mitochondrial DNA Between Two Age Groups of Iraqi Tobacco Smokers

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Abstract

Smoking exposes individuals to a range of harmful chemicals linked to various diseases and remains one of the most common unhealthy behaviours. The objectives of this research are to investigate the correlation between smoking and Mitochondrial DNA Copy Number (mtDNA-CN) by measuring the levels of Interleukin 6 (IL-6) and High-Sensitivity C-Reactive Protein (hs-CRP) as an inflammatory response. The current study included 80 smokers and 60 nonsmokers. The levels of IL-6 and hs-CRP were measured using the ELISA method, and mtDNA-CN was measured using quantitative real-time PCR. The results showed that the relative mtDNA/nDNA ratio was lower in smokers than nonsmokers (0.8186 vs. 1.740; $p = 0.0001$). The AUC value was 0.8442 ($p < 0.0001$). We also found that mtDNA-CN was inversely correlated with IL6 ($r = -0.5867$, $p < 0.0001$), and hs-CRP ($r= -0.4668$, $p < 0.0001$). The mtDNA-CN could potentially serve as a predictive biomarker for the impact of smoking on human health and diseases.
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Keywords: mtDNA copy number, hs-CRP, IL-6, qRT-PCR, Smoking.

تقدير التباين في عدد نسخ الحمض النووي للميتوكوندريا بين فئتين عمريتين من المدخنين العراقيين للتبغ

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الخلاصة

يعرض التدخين الأفراد لمجموعة من المواد الكيميائية الضارة المرتبطة بأمراض مختلفة ويظل أحد أكثر السلوكيات غير الصحية شيوعًا. هدفت هذه الدراسة الى التحقق من العلاقة بين التدخين و عدد نسخ الـ DNA المايتوكونديري mtDNA−CN ومستويات إنترلوكين 6 (IL−6) والبروتين التفاعلي سي عالي الحساسية (hs−CRP) كاستجابة للالتهابات. شملت الدراسة الحالية 80 مدخنًا و 60 من غير المدخنين. تم قياس مستويات IL−6 و hs−CRP باستخدام طريقة ELISA، وتم قياس mtDNA−CN باستخدام تفاعل

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البوليميراز المتسلسل الكمي في الوقت الحقيقي. أظهرت دراستنا أن نسبة mtDNA / nDNA كانت أقل لدى المدخنين مقارنة بغير المدخنين (0.8186 مقابل 1.740؛ p = 0.0001). كانت قيمة AUC=0.8422 (p < 0.0001). وجدنا أيضًا أن عدد نسخ الـ DNA المايتكوندريي مرتبطًا عكسيًا بمستوى IL6 (r = −0.5867, p < 0.0001)، وhs–CRP (r = −0.4668, p < 0.0001) . يمكن أن يعمل عدد نسخ الـ DNA المايتكوندريي كعلامة حيوية تنبؤية لتأثير التدخين على صحة الإنسان والأمراض.

1. Introduction

Smoking is an extremely significant public health concern worldwide because it is a chronic, unhealthy behaviour that is driven by nicotine dependence and is one of the major risk factors for lower life expectancy and diseases [1,2]. Most negative consequences of tobacco use are well documented and can have an impact on practically every organ in the body [3]. A World Health Organization (WHO) assessment estimated that there are approximately 11 billion tobacco smokers worldwide. Tobacco causes 8.7 million deaths every year, of which 1.3 million are attributable to secondhand smoke [4]. In recent years, the WHO report has also indicated that cigarette smoking is reduced in developed countries compared to developing countries [5]. The frequency of smoking remains high in many Arab nations and is typically lower among women than among men [6].

Each cigarette produces over 7,000 chemical compounds, 69 of which can cause cancer [7]. Inhaling these dangerous substances can cause oxidative stress in the body, resulting in inflammation, apoptosis, and aging [8]. Cigarette smoke damages DNA, lipids, and proteins by inducing oxidative stress via reactive oxygen species (ROS) [9]. Smoking impairs autophagy; however, the consequences of this defect, particularly in macrophages, are not fully understood [10]. CRP is a crucial inflammatory marker that responds to pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β [11]. Although CRP levels have been correlated with cigarette smoking, some studies have reported inconsistent correlations between smoking status and CRP levels [12].

Mitochondria are vital for respiration, oxidative energy production, steroid synthesis, calcium balance, cell growth, production of ROS, inflammation, and apoptosis [13]. The mtDNA-CN is a biomarker of mitochondrial dysfunction. Smoking is correlated with decreased mtDNA-CN in leukocytes [14]. A higher risk of several aging-related diseases has been linked to decreased mtDNA-CN levels in peripheral blood [15]. Changes in the mtDNA-CN can increase oxidative stress and contribute to inflammation responses.
Smoking has a wide range of harmful effects on human health, and several studies have linked smoking to an increased risk of developing diseases, particularly those related to aging. The current study aimed to evaluate the level of CRP and IL6 among male smokers and assess whether smoking affects the number of mtDNA copies. It also aimed to assess the correlation between CRP, IL6, and the number of mtDNA copy number.

2. Material and Methods

2.1 Study population

This study was carried out between November 2023 and January 2024. The study group included a population sample from Tikrit town, Salahuddin, Iraq. Blood samples and data were obtained from 156 participants aged 21–50 years who provided informed consent. Volunteers who were healthy and free of immunological, metabolic, or cardiopulmonary abnormalities, or any other diseases or conditions linked to systemic inflammatory responses were selected using the questionnaire. Additionally, 16 individuals with unreasonable mtDNA-CNs and immunological markers were excluded. Finally, 140 participants were included: 80 smokers (40 aged 18–30 years and 40 aged 30–50 years) and 60 nonsmokers.

The age distribution of the nonsmokers was matched to that of the smokers. The study protocol was approved by the Ethics Committee of Tikrit University, Iraq (NO. 285). All volunteers provided written informed consent, and all research methods were carried out in accordance with the Helsinki Declaration of 1975, as revised in 2000.

2.2 CRP and IL-6 measurement

A vacuum-sealed collection tube was used to collect anticoagulant-free blood samples. The samples were maintained at room temperature for 20 min to allow them to coagulate. The samples were then centrifuged at 1500 × g for 10 min to remove clots. After centrifugation, serum was promptly transferred to a sterile and labelled polypropylene tube. To prevent the freeze-thaw cycle, which could damage some serum components, the samples were handled at 2–8°C or frozen at −10 to −20 °C for short-term storage. Finally, a Human High-Sensitivity ELISA Kit was used to detect the concentrations of serum CRP and IL-6.

2.3. DNA isolation and qPCR amplification

Genomic DNA was extracted from the whole blood. The concentration and purity of DNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The primers used in this study are listed in Table 1. SYBR qPCR mix was used to measure the mtDNA-CN by a 7500 Real-Time PCR System, desktop (Applied Biosystems, USA). The real-time cycling conditions included an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 45 sec. Relative quantification of mtDNA-CN was performed using the ratio of mitochondrial genomic DNA [mitochondrial NADH dehydrogenase subunit 1 (mt-ND1)] to nuclear genomic DNA [actin beta (ACTB)]. The relative mtDNA-CN level was calculated using the $2^{-\text{Delta-Delta}}$ method ($2^{-\Delta\Delta Ct}$) where ΔCt is Ct mt-ND1 –Ct ACTB.

Table 1: The primer sequences were chosen for mt-ND1 and ACTB.

Genes		Nucleotides sequence	Reference
mt-ND1	F	CACCCAAGAACAGGGTTTGT	[16]
	R	TGGCCATGGGTATGTTGTTAA	
ACTB	F	ACCCACACTGTGCCCATCTAC	
	R	TCGGTGAGGATCTTCATGAGGTA	

2.4 Statistical Methods

GraphPad Prism Version 10.4.0 (621) was used for all statistical analyses. Statistical information is presented as mean ± standard deviation and compared using Student's T-test or Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant. To predict the potential of mtDNA-CN as the potential of mtDNA-CN as a marker for smoking-induced damage, we performed a receiver operating characteristic (ROC) analysis. Regions with acceptable cut-off values were compared using ROC curves, which were defined by the Youden index. The sensitivity, specificity, negative predictive value, and positive predictive value were also computed. The relative mtDNA-CN level was then calculated using the $2^{-\wedge\Delta\Delta Ct}$ method by using Excel. The degree of association between variables was investigated using Pearson correlation analysis. Pearson correlation produces a correlation coefficient (r) that measures the relationships between the parameters studied [17].

3. Results

The most important characteristics observed in the smoker group, which included 80 volunteers over the age of 30 years and 40 volunteers under the age of 30 years, were that 32% of the volunteers had been smoking for five to ten years, while 48% had been smoking

for ten to thirty years. By family history, 30% of the participants had fathers who smoked, 8.75% had mothers who smoked, and 4% had both parents who smoked. Additional demographic data is presented in Table 2.

Table 2: Demographics characterization of the smokers group.

Variable	Numbers
Age, years	40 under 30 years 40 above 30 years
BMI, kg/m2	22.78 ± 4.5 (under 30 years) 29.32 ± 5.6 (above 30 years)
Physical activity, n (%)	2-4 hours/week 18 (80) Below 1 hour/week 62 (80)
Smoking period	5 to 10 years in 32 (40%) 10 to 30 years in 48 (60%)
Father smokers	24 (30%)
Mather smokers	7 (8.75%)
Father and mother are smokers	4 (5%)

The values of IL-6 were compared between 2 groups: smokers and nonsmokers. The mean ± SD IL-6 values showed a significant difference between the smokers and the nonsmokers (51.39 ± 6.899 vs. 45.51 ± 7.809 pg/mL, $p < 0.0001$) (Table 3). The ROC analysis was performed to evaluate the diagnostic efficiency of IL-6. With an AUC value of 0.7019 (95% CI: 0.6122-0.7915) $P < 0.0001$), a specificity of 72.5%, and a sensitivity of 65% (figure 1). In general, an AUC of 0.7 to 0.8 is considered acceptable but not excellent and, therefore, not ideal for considering as a biomarker diagnosis in smokers.

Table 3: Statistics parameters of IL-6 and hs CRP in the population study.

	IL 6		hs CRP	
Volunteer	Smokers	NonSmokers	Smokers	NonSmokers
Median	49.96	46.21	2.050	1.300
Range	37.27	28.92	2.570	1.670
Mean	51.39	45.51	2.148	1.281
Std. Deviation	6.899	7.809	0.7640	0.4313
Lower 95% CI of mean	49.85	43.49	1.978	1.169
Upper 95% CI of mean	52.92	47.52	2.318	1.392
Coefficient of variation	13.43%	17.16%	35.56%	33.68%
P value	0.0001		<0.0001	

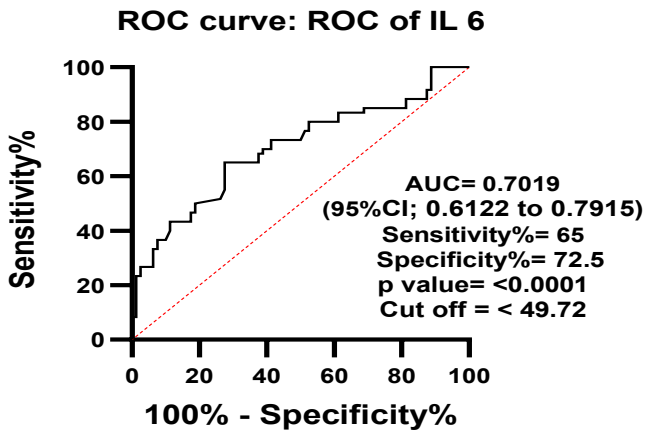

Figure 1: ROC curve analysis of IL6 for smoking males. ROC receiver operating characteristic, AUC area under ROC curve, 95% CI= 95% confidence interval, Sensitivity, Specificity, P value, Cut off.

The current study's data indicates a significant difference in hs-CRP levels between smokers and nonsmokers. The concentration of hs-CRP was lower in the smokers' group, measuring 2.148 ± 0.7640 pg/mL, compared to the nonsmokers' group, which had a concentration of 1.281 ± 0.4313 pg/mL. This difference was statistically significant (p = 0.0001) (Table 3). Additionally, ROC analysis was conducted to assess the diagnostic efficiency of hs-CRP levels. The results revealed an AUC value of 0.8196 (95% CI: 0.7528-0.8864) with a p-value less than 0.0001. ROC analysis revealed a specificity of 73.75% and a sensitivity of 76.67% (Figure 2). A positive AUC score of 0.8196 suggested that the diagnostic effect of the model was strong in the smoker group.

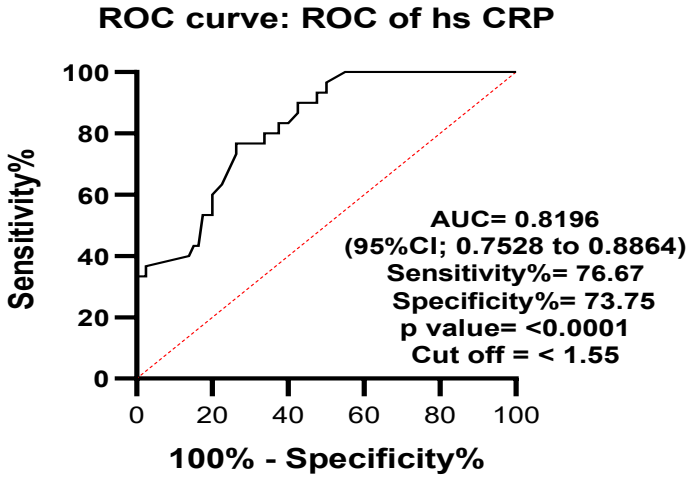

Figure 2: ROC curve analysis of hs CRP for males who smoke. ROC stands for receiver operating characteristic, AUC is the area under the ROC curve, and 95% CI represents the 95% confidence interval, sensitivity, specificity, P value, and cut-off.

In the current study, the relative mtDNA-CN in leukocytes from 80 smokers and 60 nonsmokers was estimated. The volunteers were further categorized into two age groups: > 30 years and < 30 years. qPCR was performed to obtain accurate results. Our findings reveal a noteworthy relationship between smoking status and mtDNA CNs. We observed a significant difference in mtDNA-CN in the leukocytes of nonsmokers and smokers. The mean ± SD of

mtDNA-CN in the nonsmoker group was 1.740 ± 0.8940, while the mean ± SD of the smoker group was 0.7521 ± 0.2990. This difference was statistically significant (p = 0.0001), indicating that smoking clearly impacts mtDNA-CN levels, as shown in Table 4 and Figure 3 (A).

In addition to this comparative analysis, as detailed in Figure 3 (B), ROC analysis was performed to evaluate the diagnostic potential of mtDNA-CN as an indicator of smoking status. The AUC value was 0.8442, with a 95% confidence interval (CI) ranging from 0.7798 to 0.9086 and a p-value of less than 0.0001. These findings showed a sensitivity of 81.67% and a specificity of 66.25%. The ROC AUC score of 0.8442 suggested a strong diagnostic capability, indicating the potential use of mtDNA-CN as a valuable biomarker for assessing the risks associated with smoking.

Table 4: Statistics parameters of mtDNA\ nDNA ratio in the population study.

Relative mtDNA \ nDNA ratio	Smokers	Nonsmokers
Median	0.7524	1.454
Range	1.116	2.970
Mean	0.8186	1.740
Std. Deviation	0.2990	0.8940
Lower 95% confidence interval of mean	0.7521	1.509
Upper 95% confidence interval of mean	0.8852	1.970
P value	0.0001	

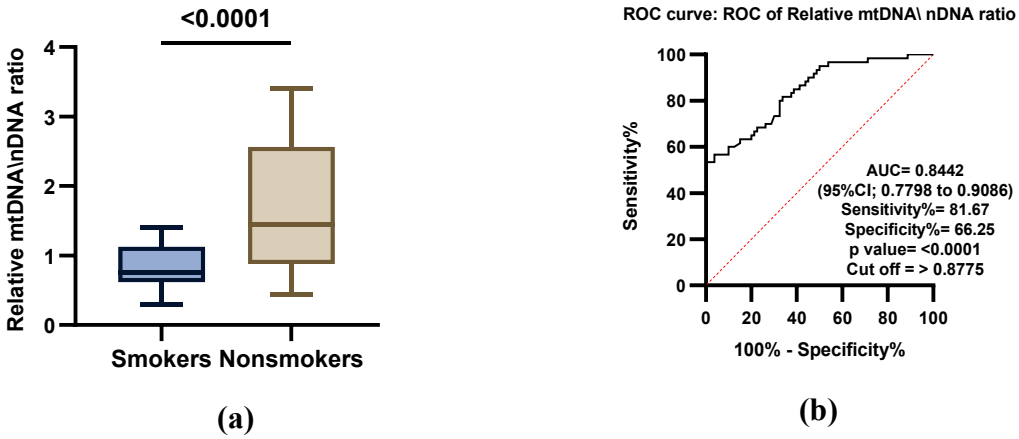

Figure 3: (a) Box plot of the mtDNA\ nDNA ratio in the population study. (b) ROC curve analysis of Relative mtDNA\ nDNA ratio for smoking males. ROC receiver operating characteristic, AUC area under ROC curve, 95% CI= 95% confidence interval, sensitivity, specificity, P value, cut-off.

Another study investigated the effect of smoking on the quantity of mtDNA-CNs across different age groups. Our analysis revealed no significant difference in mtDNA-CN within the same group between smokers and nonsmokers when comparing individuals younger than 30 years to those older than 30 years. The statistical results showed a mean difference of 0.1469 with a p-value of 0.998 for the smokers' group and a mean difference of 0.02607 with a p-value of 0.803 for the nonsmokers group, indicating a lack of notable variation between these age categories. However, a striking disparity was found in mtDNA-CN when contrasting smokers with nonsmokers within the same age group. Specifically, we found that mtDNA-CN

was significantly decreased in smokers under 30 years of age compared to non-smoking volunteers in the same age range. The mean difference was calculated at -0.9813, with a p-value of 0.001. In addition, for those aged > 30 years, mtDNA-CN exhibited a significant decrease in smokers compared to that in nonsmokers, with a mean difference of -0.8344 and a p-value of 0.001. These findings indicated that smoking affects mtDNA-CN in both age groups. For detailed statistical comparisons across different age groups, refer to Table 5 and Figure 4.

Table 5: Tukey's multiple comparisons test of mtDNA\nDNA ratio according to age group.

Test details	Mean 1	Mean 2	Mean Diff.	95.00% CI of diff.	P value	q	DF
S1 vs. S2	0.8317	0.8056	0.02607	-0.34 to 0.3922	.998	0.262	136
S1 vs. NonS1	0.8317	1.813	-0.9813	-1.377 to -0.5859	<.001	9.129	136
S1 vs. NonS2	0.8317	1.666	-0.8344	-1.230 to -0.4390	<.001	7.762	136
S2 vs. NonS1	0.8056	1.813	-1.007	-1.403 to -0.6120	<.001	9.372	136
S2 vs. NonS2	0.8056	1.666	-0.8605	-1.256 to -0.4650	<.001	8.005	136
NonS1 vs. NonS2	1.813	1.666	0.1469	-0.2758 to 0.5697	.803	1.279	136

S1= smokers under 30 years, S2= Smokers above 30 years, NonS1= Nonsmokers under 30 years, NonS2= Nonsmokers above 30 years.

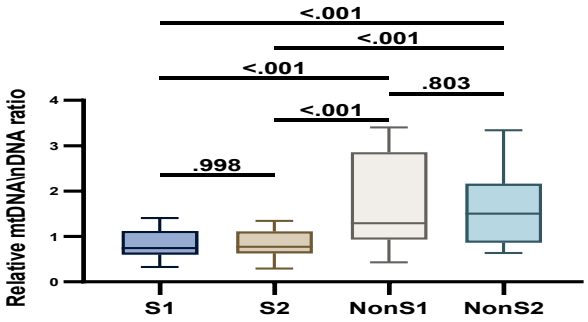

Figure 4: Box plot of the mtDNA\ nDNA ratio according to age group.

The data in Table 6 indicate a significant inverse correlation between IL-6 levels and mtDNA copy number in smokers. This correlation was quantified by Spearman's correlation coefficient of r = -0.5867, and the statistical significance was strong, with a p-value less than 0.0001. Furthermore, an inverse correlation was found between CRP levels and smoking status, reflected in Spearman's correlation coefficient of r = -0.4668, with a p-value= < 0.0001. These findings imply that higher concentrations of IL-6 and CRP are associated with decreased mtDNA copy numbers, highlighting their potential implications for understanding inflammation and mitochondrial activity in smokers.

Table 6: Pearson Correlation Coefficient of IL 6 and hs CRP serum concentration with mtDNA copy number in smokers (n=80).

Parameters	r	95% confidence interval	R squared	P value
IL 6	-0.5867	-0.7143 to -0.4213	0.3442	<0.0001
hs CRP	-0.4668	-0.6226 to -0.2753	0.2179	<0.0001

4. Discussion

Smoking remains a widely recognized unhealthy behavior in several countries, with Iraq being a notable example. The detrimental impact of smoking on human health has been well documented and tends to intensify with age. Therefore, the main aim of this study was to investigate the effects of smoking on mtDNA-CN. Additionally, we will consider the role of aging in this relationship in young and middle-aged groups.

This study revealed that smokers had a significant increase in IL-6 and hS-CRP levels compared to the levels in nonsmokers. Chemical compounds in tobacco smoke alter several systemic immune characteristics, including an increase in the number of macrophages, neutrophils, eosinophils, and mast cells, as well as the functionality of various immune cells [18]. Evidence from previous observations that have demonstrated that concentrations of IL-6, IL-10, and TNF-α are higher in smokers [19]. Activation of the phosphatidylinositol 3-kinase (PI3K), serine/threonine kinase (Akt), and nuclear factor-Kb (NF-κB) signaling pathways plays a crucial role in the enhanced migration of cells dependent on IL-6 when exposed to cigarette smoke [20]. Nicotine also increases the expression of IL-6 via the p-38 MAPK/AP-1 and ROS/STAT-3 signaling pathways, leading to endothelial injury [21]. Cigarette smoke causes respiratory system inflammation. This can increase levels of cytokines like IL-6 and Tumor necrosis factor alpha (TNFα), stimulating the production of hs-CRP in the liver [22]. This finding broadly supports the findings of other studies that link smoking status with increased levels of IL-6 [23] and hs-CRP [24].

In this study, the mtDNA-CN was found to be lower in smokers than in nonsmokers. Long-term exposure to cigarette smoke increases ROS levels, decreases total antioxidant capacity, and disrupts DNA repair processes, ultimately leading to oxidative DNA damage [25]. ROS trigger autophagy, which removes damaged proteins and organelles and contributes to cellular homeostasis [26]. Autophagy is an evolutionarily conserved lysosomal degradation process that protects cells from oxidative stress by eliminating damaged components such as mitochondria [27]. Autophagy is the only known process responsible for mitochondrial turnover [28]. Long-term exposure to cigarette smoke has been reported to lead to a decline in mtDNA_CN and has been observed in nerve cells [29], the cardiac mitochondrial [30], and the peripheral blood of healthy smokers compared to healthy nonsmokers [14]. The correlation between cigarette smoking and lower mtDNA-CN varied significantly by sex and race/ethnicity, and this association was more pronounced among men and black participants [29].

A decrease in mtDNA-CN in the 30–50-year age group was found compared to the group of individuals under 30 years. However, these differences were not statistically significant in either group. The upper age limit was set at 50 years to directly assess the impact of smoking in this age range, particularly because mtDNA-CN is known to decline with aging. Previous studies have indicated that a decrease in mtDNA-CN begins at approximately 50 years of age, with a half-life of approximately 60 years in healthy humans [31]. On average, the harmful effects of smoking generally appear at least 20–30 years after smoking onset [32].

When cells experience damage or stress, mtDNA is released from mitochondria, leading to the activation of immune and inflammatory responses [33]. During inflammation, mitochondrial ROS stimulate inflammatory cells and produce IL-6, the primary activator of protein synthesis, including that of CRP [33]. Therefore, a low mtDNA-CN is associated with inflammation, as shown by elevated levels of hs-CRP and IL-6. Consistent with the present results, previous studies have demonstrated that individuals with higher levels of hs-CRP, hs-IL-6, and neutrophil-to-lymphocyte ratios have lower blood mtDNA content [35].

The limitations and merits of our study should be considered when interpreting the results. All study participants were Iraqi. Consequently, these correlations could not be extrapolated to other racial or ethnic groups. In addition, the sample size was small. These findings are reassuring. Further studies that consider the correlation between smoking and mtDNA-CN are needed.

5. Conclusions

Given the large number of toxins found in smokers that are directly related to numerous diseases, it is crucial to regularly and accurately assess the negative effects of smoking. Compared to nonsmokers, the smokers' group's mtDNA-CN data showed a decrease. Nevertheless, additional research is required to elucidate the molecular mechanisms underlying this connection and validate their predictive diseases and potential clinical usefulness.

6. Financial support and sponsorship

Nil.

7. Conflict of interest

no conflicts

8.Author Contributions

M. H. S.: conceptualization, data curation, formal analysis, Visualization, Writing the original draft, and writing the review and editing.

SH. J. A.: conceptualization, data curation, Methodology, Writing—original draft, and writing —review and editing.

DH. S. N: Data curation, formal analysis, methodology, writing—original draft and writing—review and editing.

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