



# Chromosomal and oxidative DNA damage in non-functioning pituitary adenomas

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## Abstract

**Introduction:** Clinically non-functioning pituitary adenomas (NFPA) are common tumours of the pituitary gland and are mainly considered as benign. The primary aim of this study was to research the effects of NFPA on genome instability in patients with non-functioning pituitary adenoma by using the cytokinesis-block micronucleus cytome (CBMN-cyt) assay and 8-hydroxy-2'-deoxyguanosine (8-OHdG) assay. The second objective of this study was to assess whether there is a relationship between age, pituitary adenoma diameters, 8-OHdG levels, CBMN site assay parameters, and tumour aggressiveness.

**Material and methods:** The study was performed on 30 patients who had been diagnosed with NFPA and were admitted to the Department of Endocrinology and Metabolism, and 20 healthy subjects of similar age and sex.

**Results:** Micronucleus (MN), nucleoplasmic bridges (NPBs), nuclear bud (NBUD) frequencies, and apoptotic and necrotic cell frequencies in patients with NFPA were found to be significantly higher than in control subjects, and plasma 8-OHdG levels in patients with NFPA were statistically significantly lower than control subjects in this study.

**Conclusions:** It is believed that this is the first study to evaluate the aggressiveness of tumour with chromosome/oxidative DNA damage in patients with NFPA. However, further studies are needed in order to understand the cause of NFPA aggression and to evaluate these patients in terms of risk of cancer. (*Endokrynol Pol* 2021; 72 (2): 97–103)

**Key words:** 8-OHdG levels; DNA damage; micronucleus; non-functioning pituitary adenoma

## Introduction

Pituitary adenoma is a common disease that occurs in the pituitary gland and is clinically categorised as functioning and non-functioning pituitary adenomas (FPA and NFPA) [1–5]. Clinically compared to FPA, NFPA does not secrete active hormones and is not associated with clinical syndromes, for example acromegalic features, amenorrhoea-galactorrhoea, or hyperthyroidism. The vast majority of clinical NFPAs secrete gonadotropins, and it is generally known that they are gonadotroph pituitary adenomas [6]. The plurihormonality of pituitary adenomas can be defined as the ability to express more than one pituitary hormone [7]. The prevalence of NFPA is 15–30% of pituitary adenomas, and the annual incidence is 1 new case per 100,000 of the population [6, 8–11]. Small non-functioning tumours are generally asymptomatic, but most of them are macroadenomas (measuring more than 1 cm in size at the time of diag-

nosis). Most of non-functioning adenomas may be clinically more rapid in terms of invasiveness, recurrence, and aggressiveness, although it is usually histopathologically benign. When these tumours enlarge, they may compress surrounding structures and commonly cause a variety of symptoms, such as hypopituitarism, headache, and visual field defects [12, 13]. NFPAs are usually diagnosed by magnetic resonance imaging (MRI), hormone test, and vision test [14, 15]. NFPAs are more common in men and postmenopausal women [16].

The cytokinesis-block micronucleus cytome (CBMN-cyt) assay is comprehensive technique for measuring DNA damage, cytostasis, and cytotoxicity in cultured human lymphocytes. The events of DNA damage are scored specially in once-divided binucleated cells. These events include the following: micronucleus (MN), as a biomarker of chromosome breakage and/or loss; nucleoplasmic bridges (NPBs), as a biomarker of DNA misrepair and/or telomere end-fusions; and



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nuclear buds (NBUDs), as a biomarker of elimination of amplified DNA and/or DNA repair complexes. In addition, cytostatic effects are measured via the proportion of mono-, bi-, and multinucleated cells — this method also allows the measurement of cytotoxicity via necrotic and/or apoptotic cell ratios [17–20].

Oxidative stress plays an important role in the development of various diseases and is known to cause DNA damage involving point mutations because of base oxidation, single and double strand breaks, and/or genomic instability. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the predominant forms of damaged DNA products due to free radical-induced oxidative lesions, and it can be detected in various biological samples such as urine, cell culture, serum, and plasma. An increased level of 8-OHdG is widely used as an established marker of oxidative stress and cancer [21–23]. There is some literature on oxidative and chromosomal DNA damage in other pituitary adenomas, especially in GH-secreting and prolactin-secreting adenomas [24–26].

On the other hand, we have not found a report in the literature on oxidative and chromosomal DNA damage in patients with NFPA. For this reason, we assessed the frequencies of MN, NPBs, NBUDs, necrotic and apoptotic cells, and nuclear division index (NDI) using the CBMN-cyt assay and oxidative DNA damage using the 8-OHdG assay, in patients with non-functioning pituitary adenoma. The relations among age, pituitary adenoma diameters, 8-OHdG levels, and CBMN-Cyt assay parameters were also examined. Thus, 8-OHdG levels and CBMN-Cyt assay parameters may be used as novel biomarkers for early detection of aggressive or invasive pituitary tumours.

## Material and methods

### *Patients and controls*

The study was performed in 30 patients who had been diagnosed with NFPA and were admitted to the Department of Endocrinology and Metabolism at Erciyes University Medical Faculty from December 2011 to August 2013. Twenty age- and sex-matched healthy controls were also included in the study.

All patients included in the study were recorded with name, surname, file number, age, sex, place of residence, telephone number, complaints on arrival, basal pituitary hormone values before surgery, magnetic resonance (MR) imaging results, and postoperative pathology reports.

The diagnosis of NFPA was made based on clinical and biochemical findings. At diagnosis, the majority of these patients with NFPA had macroadenoma, visual field defects, and at least growth deficiency and hypogonadism. Additionally, a magnetic resonance imaging (MRI) scan of the pituitary gland was used to detect, locate, and determine the size of adenomas. Patients with NFPA were diagnosed with complaints, and underwent a physical examination and pituitary MRI. A non-functioning adenoma was diagnosed in patients with a pituitary adenoma and no hormonal excess. All patients with NFPA were assessed by the same endocrinologist (FB). The patients in the study included 13 females and 17 males, 22–72 years old. All patients included in the study had been newly di-

agnosed and had not received any medical or surgical therapy for NFPA. Twenty-nine of the patients had macroadenoma (96.67%) and only one had microadenoma (3.33%). The control group was selected from healthy subjects matched for age, gender, and socioeconomic status. None of the participants were taking medications for medical or other reasons, or had diseases such as hypertension, diabetes mellitus, heart disease, or cancer. A standardised questionnaire was designed to obtain relevant details of their current health status, history, and lifestyle, and to collect information on past medical history, drug, and smoking habits. The study excluded patients and controls subjects who reported consumption of alcohol or more than three cups/day of tea and/or coffee, and subjects who had a history of occupational and environmental exposure to known genotoxic chemicals.

The local Ethics Committee approved the study protocol, and all patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and local laws.

### *Measurement of basal pituitary hormones*

The basal pituitary hormones and necessary peripheral hormones were examined in pre-operative follow-ups of the patients.

Blood samples were obtained from patients for the measurement of growth hormone (GH), insulin-like growth factor 1 (IGF-1), follicle-stimulating hormone (FSH), luteinising hormone (LH), total testosterone (tT), free testosterone (fT), free thyroxine (fT<sub>4</sub>), free tri-iodothyronine (fT<sub>3</sub>), prolactin (PRL), oestradiol (e<sub>2</sub>), adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), and cortisol. All serum samples were collected early in the morning after fasting for 8–10 h.

Basal hormone levels were measured using radioimmunoassay (RIA), immunoradiometric assay (IRMA), or chemiluminescence assay at the Erciyes University Medical Faculty Biochemistry Laboratory.

### *Immunohistochemical evaluation*

The operation material was sent to the Erciyes University Medical Faculty pathology laboratory for those who underwent surgery among the patients included in the study. The tissues were kept in 10% formaldehyde for 24 hours, and then tissues were taken for follow-up. Cross-sections of 0.4 microns were made in the paraffin-embedded tissues. These sections were stained with haematoxylin and eosin and evaluated on a microscope. PRL, TSH, GH, ACTH, FSH, and LH paints were applied to each case. All of the cases were evaluated as NFPA with pathologic result and radiographic changes. According to immunohistochemical results, LH- and FSH-positive staining appeared in 17 (5 LH, 1 FSH, 11 FSH and LH positive) patients with NFPA, while in 13 patients with NFPA null-cell adenomas were seen. All immunohistochemical examinations and pathologic evaluations were performed by the same pathologist.

### *Radiological evaluation*

Three-dimensional volumetric pituitary MR imaging was performed in pre-operative follow-up to patients at the Department of Radiology at Erciyes University (Philips Gyroscan Intera 1.5 Tesla; 35 Best, Netherlands). The evaluation and reporting of the MRs was done by the Radiology Department of the Medical Faculty of Erciyes University.

### *Whole-blood cultures of human lymphocytes*

After written informed consent had been obtained, the heparinised blood samples (3 mL) were obtained from all patients and controls. Approximately 0.4 mL of heparinised whole blood samples were cultured in 5 mL of culture medium (peripheral blood karyotyping medium) with 1.5% phytohaemagglutinin-M (PHA-M) to stimulate T-lymphocytes in a 37°C incubator for 72 h (all materials from Biological Industries, Kibbutz Beit Haemek, Israel). Duplicate cultures were made for each patient and control subject, to determine intra-individual differences [27].

### CBMN-Cyt assay

CBMN-Cyt assay was performed according to some modifications of the protocol described by Fenech in 2000 and 2007 [18, 28]. Cells were blocked with cytochalasin-B (Sigma-Aldrich, St. Louis, Mo.) at a final concentration of 3 mcg/mL to each culture tube, after 44 hours of incubation to block cytokinesis. The cultures were stopped at 72 h of incubation, treated with hypotonic solution (0.1 M KCl) for 4 min and fixed with two changes of methanol:acetic acid (3:1) [18, 29]. To prepare the slides, the fixed cells were dropped (7–8 drops) onto glass slides and air-dried. The slides were stained for 10 min with 5% Giemsa (Merck KGaA, Darmstadt, Germany) in Sorensen buffer. To determine the intra-individual differences, the different slides of two parallel cultures for each patient and control subject were prepared and evaluated. All slides were scored under a light microscope (Nikon Alphaphot-2 YS2-H, Inc., Tokyo, Japan) with a 40 × 10 magnification, and CBMN-cyt assay parameters such as MN, NBUDs, and NPBs were additionally verified under 1000 × magnification. For each slide obtained from each duplicate culture, the score was obtained with two different scores with an identical microscope.

The number of mono-, bi-, tri-, and tetra-nucleated cells per 1000 viable mono-nucleated cells was scored in peripheral blood lymphocytes of all individuals to determine cytostatic effects. NDI was calculated using the following formula:

$$NDI = (M1 + 2 M2 + 3 M3 + 4 M4) / N,$$

where M1-M4 are the numbers of cells with 1-4 nuclei and N is the total number of viable cells scored, excluding necrotic and apoptotic cells [18, 30].

### Determination of 8-OHdG levels

Two-millilitre heparinised blood samples taken from the patients were immediately centrifuged at room temperature for 15 minutes at 1512 × g for analysis of 8-OHdG. The plasma was stored in microtubes at –80°C until it was analysed. Plasma 8-OHdG levels were measured using an ELISA kit (NWK-8-OHdG02; Northwest Life Science Specialties, LLC, Vancouver, WA), and the intra-assay coefficient of 8-OHdG assay was calculated to be 5.9%. Plasma 8-OHdG levels were expressed in ng/mL. Calibration, curve fitting, and data analysis were performed according to the manufacturer's instructions.

### Statistical analyses

The data were analysed using the SPSS for Windows statistical package, version 15.0. The non-parametric Mann-Whitney U test

was used to test the differences in CBMN-Cyt assay parameters and 8-OHdG levels between patients with NFPA and control subjects. Spearman's rho correlation analysis was used to assess the relation between age, pituitary adenoma diameter, 8-OHdG level, and CBMN-Cyt assay parameters. Differences were considered statistically significant when p values were less than 0.05.

## Results

Table 1 shows the results for age, adenoma diameter, CBMN-cyt assay parameters, and 8-OHdG level for 30 patients with NFPA and 20 control subjects.

Table 2 shows Spearman's rho correlation coefficients and significance for age and pituitary adenoma diameters for patients with NFPA and control subjects.

### DNA damage in peripheral blood lymphocytes

Micronucleus, NPB, and NBUD frequencies in patients with NFPA were found to be significantly higher than those in control subjects ( $p < 0.001$ ,  $p < 0.001$ , and  $p < 0.001$ , respectively) (Tab. 1 and Fig. 1). No correlation was found between age and pituitary adenoma size and DNA damage parameters (MN, NPB, and NBUD frequencies) in patients with NFPA and control subjects ( $p > 0.05$ , Tab. 2).

### Cytotoxicity in peripheral blood lymphocytes

Apoptotic and necrotic cell frequencies in patients with NFPA were found to be significantly higher than in control subjects ( $p < 0.001$  and  $p < 0.005$ , respectively, Tab. 1 and Fig. 2). There was a negative correlation between pituitary adenoma size and apoptotic and necrotic cell frequency in patients with NFPA ( $p < 0.05$ ,  $r: -0.387$ ,  $r: -0.408$ , respectively, Tab. 2).

### Cytostasis in peripheral blood lymphocytes

The NDI values of patients with NFPA were found to be significantly higher than the control subjects ( $p < 0.001$ ,

**Table 1. Results of CBMN-Cyt assay parameters and 8-OHdG levels in patients with non-functioning pituitary adenomas (NFPA) and control subjects (mean ± SD)**

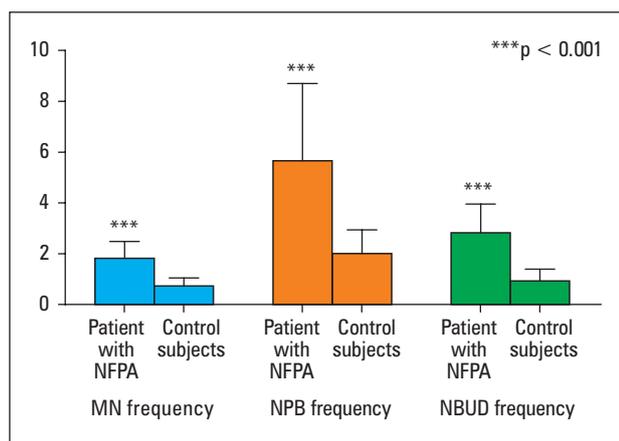
	Patients with NFPA (n = 30)	Control subjects (n = 20)	p value
Age [yrs]	53.00 ± 13.39	48.95 ± 13.84	0.29
Adenoma diameters [mm]	26.83 ± 10.32	–	–
MN frequency (%)	1.82 ± 0.66	0.73 ± 0.32	< 0.001
NPB frequency (%)	5.66 ± 3.04	2.01 ± 0.93	< 0.001
NBUD frequency (%)	2.83 ± 1.13	0.93 ± 0.46	< 0.001
Frequency of apoptotic cells (%)	5.70 ± 3.52	1.30 ± 0.81	< 0.001
Frequency of necrotic cells (%)	5.09 ± 3.05	2.86 ± 1.08	< 0.005
NDI	1.29 ± 0.79	1.20 ± 0.87	< 0.001
8-OHdG levels (ng/mL)	0.43 ± 0.32	0.64 ± 0.18	< 0.001

MN — micronucleus; NBUD — nuclear bud; NPB — nucleoplasmic bridge; NDI — nuclear division index; 8-OHdG — 8-hydroxy-2'-deoxyguanosine

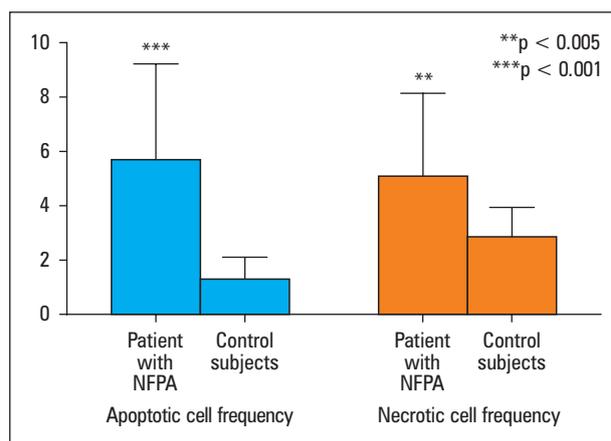
**Table 2.** Spearman's rho correlation coefficients and significance values for age and pituitary adenoma diameters with CBMN-Cyt assay parameters and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in patients with non-functioning pituitary adenoma and control subjects

	MN frequency (%)	NPB frequency (%)	NBUD frequency (%)	Frequency of apoptotic cells (%)	Frequency of necrotic cells (%)	NDI	8-OHdG levels [ng/mL]
<b>Patients with non-functioning pituitary adenoma (n = 30)</b>							
Age [yrs]							
r	-0.133	0.103	0.161	-0.054	0.011	-0.176	0.065
p	0.482	0.590	0.396	0.776	0.954	0.352	0.732
Pituitary adenoma diameters [mm]							
r	-0.202	-0.280	-0.236	-0.387*	-0.408*	-0.110	-0.168
p	0.284	0.134	0.209	0.034	0.025	0.562	0.375
<b>Control subjects (n = 20)</b>							
Age [yrs]							
r	0.160	0.134	0.406	0.020	-0.272	-0.296	0.002
p	0.501	0.575	0.075	0.935	0.246	0.205	0.995

Correlation is significant ( $p < 0.05$ ); MN — micronucleus; NBUDs — nuclear bud; NPBs — nucleoplasmic bridge; NDI — nuclear division index; 8-OHdG — 8-hydroxy-2'-deoxyguanosine



**Figure 1.** The frequencies of micronucleus (MN), nuclear bud (NBUD), and nucleoplasmic bridge (NPB) in patients with non-functioning pituitary adenomas (NFPA) and control subjects



**Figure 2.** Apoptotic and necrotic cell frequencies in patients with non-functioning pituitary adenomas (NFPA) and control subjects

Tab. 1 and Fig. 3). No correlation was found between age and pituitary adenoma size and NDI values in patients with NFPA and control subjects ( $p > 0.05$ , Tab. 2).

### Plasma 8-OHdG levels

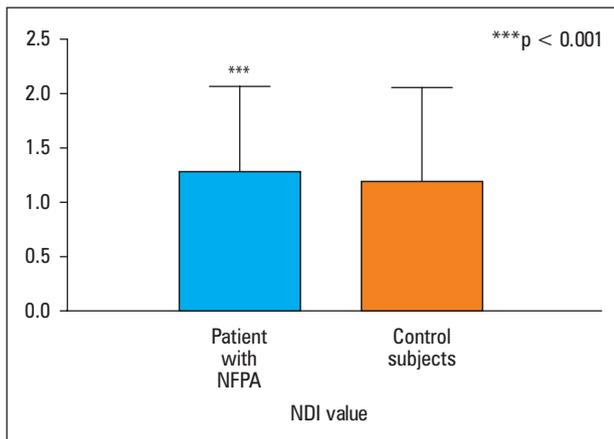
Plasma 8-OHdG levels in patients with NFPA were statistically significantly lower than in control subjects ( $p < 0.001$ , Tab. 1 and Fig. 4). There was no significant correlation between plasma 8-OHdG levels and age and pituitary adenoma size in patients with NFPA and control subjects ( $p > 0.05$ , Tab. 2).

In addition, the MN frequency in patients with non-secreted hormone NFPA was found to be

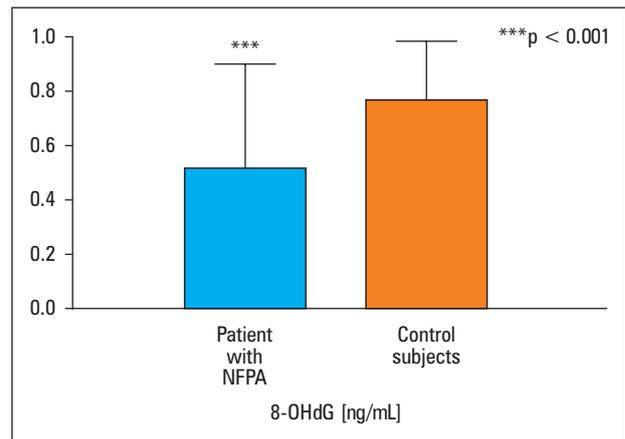
higher than in patients with secreted hormone NFPA ( $p < 0.05$ ), while plasma 8-OHdG levels were found to be lower ( $p < 0.05$ ), from CBMN-cyt assay parameters and plasma 8-OHdG levels in this study.

### Discussion

Clinically, NFPA are common benign tumours, but they are usually large at the time of diagnosis [31, 32]. The main clinical problems that arise in NFPA are hormonal insufficiency due to the effect of pressure on the optic chiasm or the influence of the pituitary hormone-releasing cells due to the mass effect [16].



**Figure 3.** Nuclear division index (NDI) values in patients with non-functioning pituitary adenomas (NFPA) and control subjects



**Figure 4.** Plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in patients with non-functioning pituitary adenomas (NFPA) and control subjects

Since there is no overexpression of a specific hormone in non-functioning pituitary adenomas, it is difficult to correlate the increases in the CBMN-Cyt assay parameters (such as frequency of MN, NPBs, and NBUDs) of these patients with any hormone.

NFPA is a very complex all-body disease with multiple molecular dynamic changes in genomic, transcriptomic, proteomic, and metabolomic levels [1, 33–36]. The increase in DNA damage parameters (MN, NPB, and NBUD frequencies) in patients with NFPA in our study may be associated with multiple molecular dynamic alterations in these patients.

In our study, 8-OHdG levels in patients with NFPA were significantly lower than control subjects. Base damage that occurs in DNA is repaired by a base excision repair mechanism. Errors that occur during this repair mechanism cause an increase in DNA chain breaks. Therefore, we believe that oxidative DNA damage occurs in these patients and that some of these damages may be repaired. But faults during repair of chromosomal DNA damage may also contribute to the increase in MN, NPB, and NBUD frequencies. According to literature reviews, there has been no study of plasma 8-OHdG levels in these patients before, and these results need to be supported by further studies.

Apoptotic and necrotic cell frequencies were significantly higher in patients with NFPA than in control subjects. However, despite this increase in cytotoxicity in patients with NFPA, the NDI ratio was found to be increased compared to control subjects. An increase in cell death due to increased genomic instability is an expected condition. We can assume that these DNA damages seen in NFPA are repaired in accordance with the increase of NDI ratio and that the cells continue to multiply and not die.

Increased DNA damage and NDI values and reduced oxidative DNA damage in NFPA may be associated with late diagnosis, and the pituitary adenoma size of these patients is considerably larger than that of other adenomas. Furthermore, because there is no evidence of excessive secretion of a clinically important hormone in non-functioning adenomas, these results may be directly related to the disease, a stimulating growth factor that enlarges the adenoma. However, according to CBMN-Cyt assay parameters, high MN frequencies found in non-secretory patients may be predictable as biomarkers of cancer.

It is unknown why these tumours develop. They are thought to arise from a mutation or mutations in a single pituitary gland cell, but it is unknown why or how this happens. On the other hand, NFPA has a high degree of heterogeneity and difficulties in early diagnosis and treatment. It may help in the understanding of exploration of variations in molecular mechanisms and discoveries that are effective and reliable biomarkers and therapeutic targets in NFPA.

In the general population, MN frequency is considered to be a predictor of increased risk of cancer. On the other hand, the formation of nuclear anomalies and/or chromosomal DNA damage parameters including MN, NPBs, and NBUDs are also events that can be seen in the early stages of carcinogenesis [19, 37]. Therefore, increased chromosomal DNA damage parameters in NFPA may be associated with possible cancer risk in these patients. In addition, it has been reported that genomic instability frequently occurs in pituitary tumours [38–43]. However, limited information is available on genomic damage in pituitary adenomas. In our previous studies, we have shown that the MN, NPB, and NBUD frequencies increased in patients with acromegaly and prolactinoma [25–27]. In this study, the increased

genome damage in patients with NFPA that are not associated with clinical manifestations of pituitary hormone hypersecretion may have contributed to their progression from benign adenomas to malign tumours. Thus, CBMN-Cyt assay parameters including MN, NPB, and NBUD frequencies may be used as novel biomarkers for early detection of aggressiveness and invasion of pituitary tumourigenesis. However, carcinogenesis, such as the development of pituitary adenoma, is also a multi-step and multifactorial process. Further studies are needed in order to understand the cause of NFPA and to evaluate these patients in terms of risk of cancer.

### Acknowledgement

This work was supported by research Projects Units (Project Number: TSD-10-3327).

### Funding

There has been no significant financial support for this work that could have influenced its outcome.

### Conflict of interest

The authors declare that they have no conflict of interest.

### Ethics approval

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. The local Ethics Committee approved the study protocol, and all patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and local laws.

### Consent to participate

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

### Consent for publication

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

### Availability of data and material

Data statements for this study can be accessed from Table 1 and 2.

### Code availability

No software application or special code can be applied for this study.

### Authors' contributions

All authors contributed to the study. They read and approved the final version of the article.

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