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# Role of nesfatin in endometrial cancers

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**Summary**

*Aims:* Endometrial cancers (ECs) represent a heterogeneous group of cancers with different types of histology, etiology, and prognosis. The largest risk factors for EC are obesity and diabetes. Nesfatin regulates food intake and glucose homeostasis. The aim of this multicentre study was closer recognition of nesfatin and other molecular factors in ECs. *Materials and Methods:* Using sections of paraffin-embedded preparations and immunohistochemistry, the expression of nesfatin (NESF 1), estrogen receptors (ER α and β1), and progesterone receptor (PR) was examined in 146 cancer patients (115 EC type I and 33 type II). *Results:* In EC type I, a higher expression of PR, but lower ER β1 was found. No significant difference was detected in NESF 1 and ER α expression between the two types of ECs. ER α expression was higher in early stages of EC, and in more advanced cases ER β1 staining was weaker. In EC types I and II, a high expression of NESF 1 correlated with a high expression of ER α. Lower nesfatin expression correlated with low cells differentiation (grading) in EC type I. A tendency towards high expression of nesfatin was noted in patients with a lack of ER β1 expression. No correlation was found between the expression of NESF 1 and PR in any of the groups. *Conclusions:* The role of nesfatin in EC is still poorly understood. In EC type I, a high expression of NESF 1 seems to represent a favourable prognostic factor, while in type II its role is still unknown.

*Key words:* Enodmetrial cancer; Nesfatin; Molecular factors.

## Introduction

Endometrial cancer (EC) is the most prevalent gynecological cancer in economically developed countries. Over the last decade, a growing prevalence of the tumour type has been observed in several European countries [1-3].

In 1983, Bokhman was the first to present two types of EC, differing in etiology and clinical course [4]. Type I EC, which is more common, is detected in approximately 80% of patients and includes ECs of a slow course and good prognosis, linked to unbalanced estrogen stimulation and metabolic syndrome. Metabolic syndrome consists of several abnormalities, such as diabetes mellitus, reduced tolerance to glucose, insulin resistance, arterial hypertension, dyslipidemia, excess weight or obesity, and these factors also lead to abnormal hormonal regulation [5].

Typical molecular traits of EC type I have been described, and these include mutations in several genes, the presence of estrogen (ER) and progesterone (PR) receptors, disturbances in signalling pathways [6-9]. In studies by Colombo *et al.* [1] and those by Kreizman-Shefer *et al.* [8] the expression of ER and PR was lower, in parallel to the stage of advancement according to FIGO, as well as in cancers of lower histological maturity (G).

Type II EC involves a non-endometrioid cancer, encompassing serous, clear cell, undifferentiated cancers, and other less frequently occurring cancers (e.g. carcinosarcoma). It is thought that serous cancer (USC) should be distinguished separately: it manifests an aggressive course and frequent relapses, which results in an unfavourable clinical course. The cancer has been shown to carry typical mutations [10-14].

Molecular studies, including an analysis of ten cohort studies and 14 case control studies, including over 14,000 EC type I and II cancer cases, demonstrated that the etiology of the two cancer types may be similar; the etiology of EC type I may be not fully dependent on estrogens while EC type I of low differentiation (G3) clinically manifests a similar aggressive course and a high risk of relapses resembling USC type II [15]. No such relationship was noted by other investigators, particularly in the early stages of cancer development [16].

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Table 1. — *Clinicopathologic characteristics of studied patients with endometrial carcinoma.*

| Stage of advancement according to FIGO and grading | Number of patients |
|---|---|
| **Adenocarcinoma endometrioides (n=115)** | |
| IA | 33 |
| IB | 32 |
| II | 27 |
| III | 14 |
| IV | 9 |
| G1 | 36 |
| G2 | 56 |
| G3 | 23 |
| **Adenocarcinoma serosum G3 (n=18)** | |
| IA | 4 |
| IB | 3 |
| II | 7 |
| III | 4 |
| **Adenocarcinoma clarocellulare G3 (n=11)** | |
| IA | 1 |
| IB | 1 |
| II | 2 |
| III | 4 |
| IV | 3 |
| **Adenocarcinoma mucinosum (n=2)** | |
| II G1 | 1 |
| IV G2 | 1 |

The results of ER expression in EC type II are contradictory: according to some investigators ER expression is linked to a significantly worse prognosis, while others claim it represents an independent favourable prognostic index [10, 12]. The effect of other molecular factors, which modulate the expression of ER and PR may be significant.

The frequently controversial date presented above led to the genomic analysis of EC type I and type II cancers (TCGA) published in 2013. The analysis identified at least four subtypes of the cancers [17].

One of the recently described molecular factors involves nesfatin 1. The 82 amino acid adipokine, a derivative of nucleobindin 2 (NUCB2) participates in reproduction and is associated with obesity and diabetes type II. It participates in the regulation of hunger and fat storage, and is associated with glucose homeostasis and insulin resistance. In investigations by Takagi *et al.* [18] NUCB2 has been found to manifest prognostic significance both for PFS and for OS in endometrial cancer type I; the immunoreactivity correlated with an augmented risk of relapse in the disease and a more severe clinical course. Nevertheless, the expression of NUCB2 or its relationship to the status of estrogen receptors α and β and the PR were not examined in EC type II.

## Materials and Methods

This was a multi-centre, retrospective study. The investigated material involved archival histological preparations of endometrium obtained from 146 patients with a diagnosis of EC who underwent primary surgery from 2007 to 2014 in eight Polish gynaecological clinics.

In the studied group, endometriod adenocarcinoma (type I) was diagnosed in 115 (78.8%) patients, and non-endometrioid subtypes (type II) in 31 (21.2%) patients, including 18 (12.3%) serous ECs, 11 (7.5%) clear cell cancers, and two (1.4%) mucinous cancers .

Thirty-eight (26%) patients were diagnosed in Stage IA of the cancer, grade IB was identified in 36 (24.7%) patients, Stage II in 37 (25.4%) patients, Stage III in 22 (15%) patients, and Stage IV was disclosed in 13 (8.9%) patients (stages according to FIGO).

In 38 patients (25%), ECs manifested were well differentiated G1; 59 patients (38.8%) manifested intermediate differentiated G2, while 55 (36.2%) patients manifested tumours of poor differentiated G3 (Table 1).

The mean age of the patients was 65.3 years. In the group of patients with EC, the mean age of patients was 64.7 (40-83 years), in women with a non-endometrioid cancer it was 67.6 (34-83 years) ($p > 0.05$).

The tissue material was fixed in 10% buffered formalin, pH 7.4. and placed in a processor. The tissue was embedded in paraffin at a temperature of 60°C, using standard histopathological techniques. The paraffin blocks obtained in this way and appropriately labelled, were cut on a microtome into 4-5μm thick sections, which were positioned on basic adhesive glass slides and left for an hour at a temperature of 60°C.

In the study, the immunohistochemical method was applied using the FLEX+system. In the paraffin sections, antigens were uncovered in the target retrieval solution. High pH, using PT-link apparatus, at a temperature of 97°C was utilized for 20 minutes.

Nesfatin-1 was estimated using Nesfatin-1/Nucleobindin-2 Antibody. The antigens present in the tissue material were demonstrated via the application of antibodies directed against: ER-α, ERβ-1, and Pg-R clone 636. The sections were incubated with an antibody for 20 minutes, at a dilution of 1/2000. Immunoperoxidase stainings were executed in the autostainer link 48 apparatus.

In order to estimate the intensity of staining manifested by NESF 1, ER α, ER β1, and PR, the following four-grade scale was used:

- 0 no reaction
- \+ reaction noted in 1 to 50 immunopositive cells (cell nuclei or cytoplasm)
- ++ reaction noted in 50-75 immunopositive cells
- +++ reaction noted in 75 to 100 immunopositive cells, all seen in 10 fields of vision.

Preparations manifesting ++ or +++ staining were taken as presenting positive reaction.

In statistical calculations tests of Mann-Whitney, Kruskal-Wallis and those of Spearman (STATISTICA) were used. Statistical significance was concluded when values of $p < 0.05$ were obtained.

## Results

A high expression of NESF 1, ER α, ER β1, and PR was detected in 53.1%, 40.4%, 26.7%, and 87 % and patients with eEC, respectively. The immunocytochemical reaction with antibodies directed at ER β1 was detected in both cell nuclei and in the cytoplasm. The PR protein and ER α manifested a nuclear reaction while nesfatin demonstrated a cytoplasmatic reaction (Figure 1).

In respect to the histopathological type, the patients were divided into two groups: endometrioid and non-endometrioid, the latter including patients with serous, clear cell, and

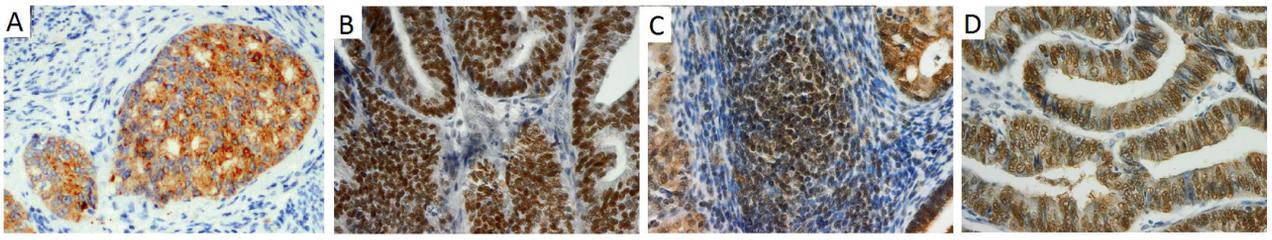


Figure 1. — High expression of A) NESF 1 B) ER α, C) ER β1, and D) PR in endometrial adenocarcinoma (×400).

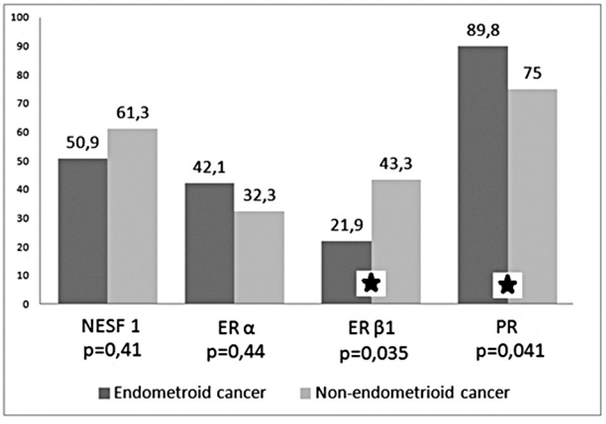


Figure 2. — Percentage of endometrial cancer cases manifesting a high expression of a given protein in EC type I and EC type II cells (intensity of staining : 3-2). * p< 0.05 EC type I and EC type II

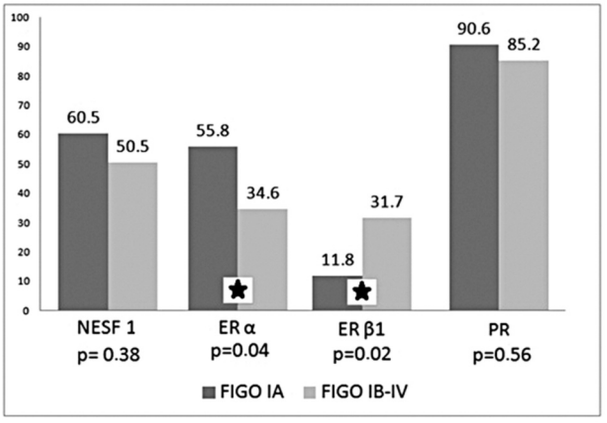


Figure 3. — Percentage of EC cases manifesting high expression of a given protein depending on clinical advancement of the neoplastic disease (intensity of staining: 3-2). *$p < 0.05$

mucinous cancers. A comparison of the two groups of women revealed no significant difference in the intensity of ER α expression in neoplastic cells ($p = 0.44$) – a high expression of ER α was noted in 42.1% of endometrioid cancers and 32.3% of non-endometrioid cancers. In patients with endometrioid cancer, a lower expression of ER β1 was disclosed in neoplastic cells (a high expression of the protein was noted in 43.3% cases of non-endometrioid and in 21.9% cases of endometrioid cancers, $p = 0.035$). In parallel, a higher level of PR expression was disclosed among women with EC type I (respectively: 89.9% and 75%, $p = 0.041$). A high expression of nesfatin was evident in 50.9% patients with EC type I and in 61.29% patients with EC type II. No statistically significant difference could be detected between the two groups ($p = 0.410$) (Figure 2).

Patients with EC were divided according to the clinical stage of the disease into early stage (IA) and later stages (IB-IV). Women with Stage IA were found to manifest a statistically significant higher expression of ER α (55.8% and 34.8%, $p = 0.04$). In parallel, the same group of patients manifested a reduced expression of ER β1 (11.8% in patients with Stage IA *vs.* 31.7% in patients with Stages IB-IV, $p = 0.02$). No differences could be disclosed in the intensity of PR expression or nesfatin expression which would be dependent on the FIGO stage of clinical advancement of the disease (p=0.56 and p= 0.38 respectively) (Figure 3).

Among all patients with EC, patients with moderately or well-differentiated EC (G1 and G2) manifested a higher expression of ER α, compared to patients with poorly differentiated cancer ($p = 0.011$). Also, the expression of ER β1 increased alongside a reduction in the histological differentiation of cells (G3 *vs.* G1, respectively, 9.1% *vs.* 38.5%, $p = 0.003$). No differences could be disclosed in the expression of PR or NESF in relation to the histological maturity of the cells (Figure 4).

In the analyses of patients with EC type I, similarly as in the entire group of patients, a statistically significant increase was noted in the expression of ER β1 in parallel to a decrease in cells differentiation ($p = 0.0436$). A high expression of ER β1 in patients with G1, G2, and G3 cancers amounted, respectively, to 9.09%, 26.53%, and 30.43%, respectively.

A division of patients with EC into patients with low G1 tumours *vs.* patients with a higher grade of histological malignancy (G2+G3) revealed a statistically significant reduction in NESF expression taking place in parallel to decreasing histological maturity of the tumour (a high expression of NESF for G1 cancers - 68.97% *vs.* G2+G3 - 45.45%, $p = 0.0487$).

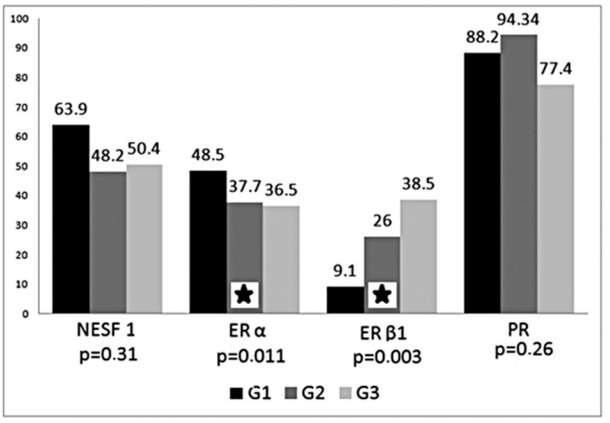


Figure 4. — Percentage of EC patients manifesting a high expression of a given protein, in relation to the histopathological grading of cells G1, G2, and G3 (intensity of staining: 3-2). *$p < 0.05$.

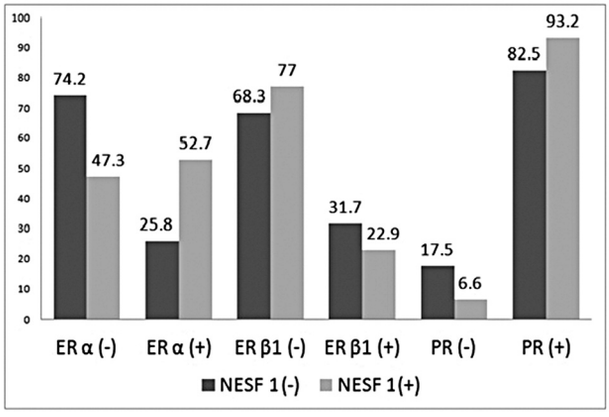


Figure 5. — Expression of nesfatin (NESF 1) in relation to the level of ER α, ER β1, and PR expression in the total population of patients.

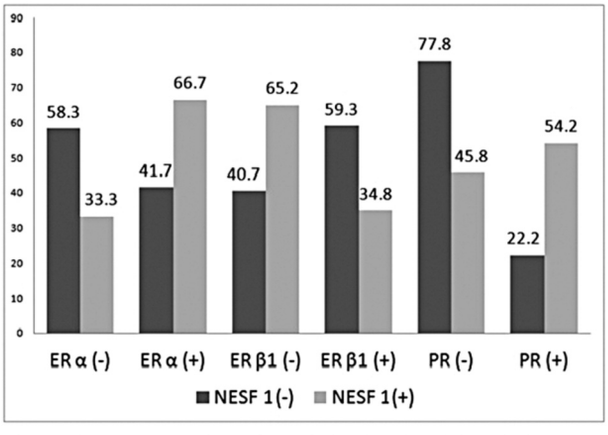


Figure 6. — Expression of nesfatin (NESF 1) in relation to expression of ER α, ER β1, and PR among patients with endometrioid cancer.

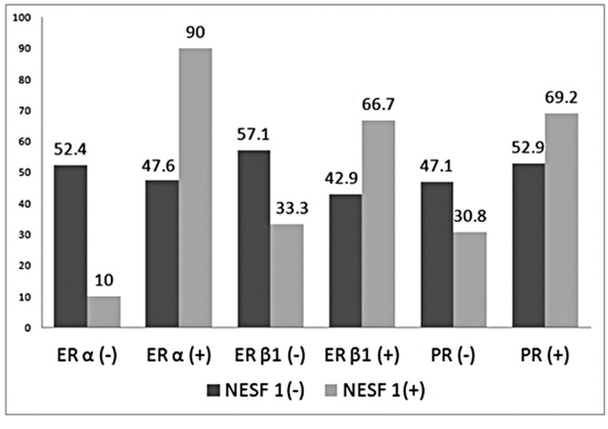


Figure 7. — Expression of nestafin (NESF 1) in relation to expression of ER α, ER β1 and PR among patients with non-endometrioid cancer.

The relationship between the expression of the studied proteins and the grade of histological maturity of cells in non-endometrioid cancers was not conducted as, by definition, the latter represents cancers of poor differentiation (G3).

Among all patients with ECs studied, a high expression of nesfatin correlated with a high expression of ER α ($p$ = 0.0026). When a high expression of ER α occurs, the chance for intense staining for nesfatin is 3.2-fold higher than when neoplastic cells manifest no ER α expression (95% confidence interval, 1.54-6.64 $p$ = 0.0018). No relationship could be demonstrated between the level of NESF expression and expression of ER β1 and PR in the total population of patients with ECs ($p$ = 0.0993). No correlation could be identified between the level of expression manifested by ER α and ER β1, ER α, and PR and between ER β1 and PR (Figure 5).

Depending on histopathological type of the tumour, similarly as in the entire group, a correlation was disclosed between a high expression of nesfatin and a high expression of ER α; 66.7% of patients with endometrioid cancer manifested a high expression of ER α and NESF 1 proteins and only 41.7% of cancers presented a high expression of NESF 1 upon low expression of ER α ($p$ = 0.019) (Figure 6), and 91.67% of patients with non-endometrioid cancer and a low expression of nesfatin, manifested no expression of ER α; patients with a high expression of nesfatin, positive staining of ER α was detected in 47.37% of cases ($p$ = 0.0464) (Figure 6).

In the population of patients with endometrioid cancer, a tendency was noted towards a high expression of NESF 1 in women with an absence of ER β1 expression ($p$ = 0.0656). Among patients with a negative expression of ER β1, a positive expression of NESF 1 was detected in 59.26% cases, while in patients with a high expression of

ER β1, a high expression of NESF 1 was detected in 34.8% cancers. No relationship could be detected between level of nesfatin expression and expression of ER β1 among patients with non-endometrioid cancer ($p = 0.4651$) (Figure 7).

No relationship could be demonstrated between the extent of nesfatin expression and the expression of PR in the endometrioid cancer subgroup ($p = 0.0870$) and the non-endometrioid subgroup ($p = 0.3839$). Furthermore, no correlation could be disclosed between the level of expression manifested by ER α and ER β1, ER α and PR, or between ER β1 and PR in both patient subgroups (Figures 6 and 7).

## Discussion

Populational studies demonstrated a disturbed expression of nesfatin (NESF 1) in patients with diabetes type 2 and in those with polycystic ovaries syndrome [19, 20]. These two syndromes are linked to an augmented incidence of endometrioid cancer (EC type I). Takagi *et al.* [18], in studies on Nucleobinding 2 - NUCB2 (precursor of nesfatin) in population women with EC type I were the first to demonstrate its prognostic value. The expression of NUCB2 was found to represent a marker identifying group of patients with an unfavourable clinical course. However, studies by Setiawan *et al.* [15] showed that profiles of risk factors for endometrioid (EC type I) and non-endometrioid (EC type II) cancers manifest common etiological pathways and, therefore, in the present studies the authors defined the expression of nesfatin, the expression of hormonal receptors (ER α, ER β1, and PR) in EC types I and II.

The expression of NESF 1 manifested no differences between the two histological types of cancer nor in various advancement stages according to FIGO. However, nesfatin expression showed a reduction alongside a decreasing grade of histological maturity in EC type I cancers. No G-dependent difference in NESF 1 expression was detected in EC type II, since 29 out of 31 cancers involved poorly differentiated cancers (G3). Such a result is inconsistent with the data of Takagi *et al.* [18], who detected no difference between G status and expression of NUCB2.

The results presented in this paper, encompassing all ECs, revealed a correlation between NESF 1 expression and the expression of ER α in EC types I and II; high expression of NESF 1 correlated with a high expression of ER α. Expression of ER α was also significantly higher in the early clinical Stage IA, as compared to more advanced stages, and it was found to decrease in relation to reduction of cells' differentiation (G). The decrease in ER α expression in parallel to a reduction in histological maturity (G) was also documented by Kreizman-Shefer *et al.* [8], who expressed the opinion that this may point to an invasive character of ECs.

In the second, non-endometrioid type of EC, a high expression of NESF 1 correlated with high expression of ER α. According to Sho *et al.* [10], a high expression of ER α represents a negative prognostic factor. Therefore, the expression of NESF 1 may represent an additional parameter for ER α.

Expression of NESF 1 in EC type I was higher (even if insignificantly higher) in the group with absent expression of ER β1, the expression of which was significantly higher in G3 grade cancers.

At early stages of advancement according to FIGO, among all ECs, a significantly lower expression of ER β1 was detected than in advanced stages. The significance of ER β1 expression remains to be equivocal: according to some investigators, the expression of ER β1 remains unchanged in ECs [21], while others claim the progression of the cancer is followed by a reduced expression of ER β1 [8, 22].

The correlation between NESF 1 and ER α may point to their reciprocally modulating effect with NESF 1. Thus, NESF 1 in EC type I may be regarded to represent a prognostic factor.

No relationship could be disclosed between the expression of NESF 1 and PR, even if the expression of PR was higher in EC type I which, in the opinion of several authors, provides evidence for a good prognosis [8, 22]. The expression of PR did not decrease with a reduction of grade of histological maturity.

## Conclusions

Nesfatin in endometrioid cancer reflects favourable prognostic indicators: a high expression of ER α, the absence of ER β1 expression, and association with histological maturity of the cancer (G). The expression of NESF 1 is modulated by ERs, as indicated also by its correlation with ER α in EC type II. It is recommended that other molecular factors in ECs and nesfatin should be estimated to define their specific role in these cancers.

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