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Adjuvant therapy in type 1 diabetes mellitus — choice or necessity in the COVID-19 pandemic?

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The COVID-19 pandemic has had a significant impact on the daily functioning of hospital and outpatient specialist centres. The restrictions we have been under since the spring 2020 have translated into difficulties in access to diagnostic and treatment procedures. As a result, telemedicine consultations have frequently become the only form of contact between a patient and a medical specialist. Further restrictions on the use of sports facilities, confinement at home, and remote working have translated into excessive food intake, lower physical activity and, consequently, weight gain, and deterioration of diabetes control. When conducting telemedicine diabetes consultations of frequently obese patients with uncontrolled glycaemia, a diabetologist is faced with the difficult task of modification of diabetes treatment in the case of a significant reduction in their physical activity and difficulties with the maintenance of a diet.

While next to insulin, new hypoglycaemic drugs are available for patients with type 2 diabetes (T2DM), intensive insulin therapy still remains the gold standard for type 1 diabetes treatment (T1DM).

A paper on the use of adjuvant therapy in type 1 diabetes was published in the previous issue of *Endokrynologia Polska* [1]. The search for new therapeutic options in patients with type 1 diabetes is the result of the increasing incidence of metabolic disorders in this group of patients, including obesity and, paradoxically, insulin resistance, which is particularly evident during the COVID-19 pandemic.

The aim of such treatment is to support exogenous insulin therapy to achieve the therapeutic goal and at the same time to reduce the risk of hypoglycaemia and to have a beneficial effect on body weight. Potential therapeutic options include metformin (which has been used for many years) and new hypoglycaemic drugs, such as SGLT2 inhibitors and GLP-1 analogues.

Metformin is the first-line drug in the therapy of T2DM, the main effect of which is to inhibit liver gluconeogenesis and reduce insulin resistance. It has been shown that the addition of metformin to insulin therapy in type 1 diabetic patients results in improved glycaemic control, reduces the need for insulin and the incidence of metabolic syndrome, and promotes the reduction and maintenance of normal body weight (1.4–6.0 kg, on average) [2, 3]. The use of metformin is not associated with an increased frequency of hypoglycaemic episodes or the risk of lactic acidosis, and the main adverse effects include gastrointestinal disorders typical of this group of drugs [2–3]. Metformin is particularly important in the treatment of patients with T1DM and polycystic ovary syndrome (PCOS), in whom insulin resistance is often associated with obesity [4, 5]. In women with PCOS, metformin has a beneficial effect not only on carbohydrate metabolism, but also on lipid and hormone metabolism. It increases the secretion of oestrogen and sex hormone-binding globulin (SHBG). It also reduces the production of ovarian and adrenal androgens [6]. Metformin has not been approved by the FDA for T1DM. However, both the American Diabetes Association and many European societies recognize the potential benefits of metformin as an adjuvant for insulin therapy, particularly in overweight and obese patients [7]. As a result, metformin is the most commonly prescribed drug to supplement insulin therapy in patients with T1DM and is used in about 8% of patients [7].

Sodium–glucose cotransporter 2 inhibitors (SGLT2) lower glycaemia independently of insulin. Inhibition of SGLT2 results in a number of beneficial effects, including loss of excess calories in urine (and consequently reduction in insulin requirements), weight loss, increase in insulin sensitivity, reduction in blood pressure, slowing the progression of albuminuria and diabetic nephropathy. Empagliflozin, canagliflozin, dapagliflozin, and ertugliflozin are approved for the treatment of T2DM, and some of them have also shown beneficial effects on the cardiovascular system and kidney function [8–10]. Sotagliflozin, approved also for the treatment of T1DM, is a dual SGLT1 and SGLT2 inhibitor. Additionally, it inhibits intestinal glucose reabsorption, thus reducing postprandial hyperglycaemia.

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Adding an SGLT-2 inhibitor to the therapy of T1DM has been shown to be associated with a significant reduction in HbA1c concentrations, a decrease in body weight and glycaemic variability, and an increase in time-in-range (TIR). The systolic blood pressure and daily insulin requirements are also reduced [11]. The possible adverse effects of adjuvant therapy with SGLT2 inhibitors in T1DM should also be considered (e.g. clinically significant urogenital infections and euglycaemic ketoacidosis). These adverse effects not only hinder the treatment process, but also require careful selection of patients undergoing such treatment [12]. In 2019, The European Medicines Agency (EMA) approved dapagliflozin 5 mg to be an adjuvant drug for the treatment of T1DM in patients with BMI no less than 27 kg/m^2 in whom insulin therapy alone does not result in optimal metabolic control. However, the American Food and Drug Administration (FDA) has not approved this treatment. Dapagliflozin (5 mg) is also used in Poland. Sotagliflozin has been approved by the EMA for T1DM. However, the FDA has not approved it [12].

GLP-1 analogues are effective and safe in patients with T1DM, as indicated by clinical trials. Liraglutide is a member of this class of drugs. GLP-1 analogues mimic the action of endogenous glucagon-like peptide-1 (GLP-1), which is an intestinal hormone released in response to food intake, thus regulating blood glucose levels. It acts by increasing insulin secretion by β cells of the pancreas in a glucose-dependent mechanism. It inhibits glucagon secretion, slows gastric emptying, and inhibits appetite. In addition to achieving improvement in glycaemic control and reduction in HbA_{1c} concentrations, liraglutide has been shown to lower arterial pressure, and reduce body weight and insulin requirements. Additionally, it has a positive effect on the quality of life. A significant weight loss (of 2.5 to 6.5 kg) was demonstrated in patients with T1DM who received liraglutide 1.8 mg [13, 14]. Data on the influence of other GLP-1 analogues on T1DM are limited.

In conclusion, in addition to insulin, more therapeutic options are available for patients with type 1 diabetes, particularly for those with obesity and metabolic syndrome.

When establishing a treatment regimen, the following must be considered: indications included in the summary of product characteristics and the related limitations (off-label therapy), the lack of indications for reimbursement, which results in high costs of treatment and the possibility of adverse effects, which requires careful selection of patients and their thorough education.

Conflict of interest

None declared.

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Expression of kisspeptin and KISS1 receptor in pituitary neuroendocrine tumours — an immunohistochemical study

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Abstract

Introduction: Pituitary neuroendocrine tumours (PitNETs), traditionally designated as pituitary adenomas, show relatively frequent invasive growth with exceptional metastatic potential, the causes of which are not entirely elucidated. Kisspeptins, which perform their activity through KISS1 receptor (KISS1R), are recognised as metastatic suppressors in many malignant tumours. This study aimed to investigate the immunohistochemical expression of kisspeptin and KISS1R in different types of PitNETs and to compare it with the expression in the normal anterior pituitary, using tissue microarray.

Material and methods: The experimental group consisted of 101 patients with PitNETs, with 45 (37.3%) being of gonadotroph, 40 (33.9%) somatotroph, 4 (3.4%) corticotroph, 4 (3.4%) thyrotroph, 3 (2.5%) lactotroph, and 6 (5.1%) null-cell type. The control group consisted of anterior pituitary tissue accidentally removed during the surgery for PitNETs in 17 patients.

Results: Kisspeptin expression was observed in both experimental and control groups, without statistically significant differences in the staining intensity. Negative kisspeptin staining was detected in 10 (9.9%), weak in 79 (78.2%), and moderate in 12 tumours (11.9%); none of the tumours had strong staining intensity. The weak staining intensity was predominant in all PitNET types except thyrotroph tumours. Significant statistical difference in terms of kisspeptin expression between types of PitNET and the control group was not observed. Immunohistochemical expression of KISS1R was not observed in the control group or in the experimental group.

Conclusions: We conclude that immunohistochemistry, as a method, cannot confirm the involvement of kisspeptin in tumourigenesis and aggressiveness of PitNETs, but potentially supports its antimetastatic role. The absence of KISS1R immunohistochemical expression in all anterior pituitaries and PitNETs in our cohort needs verification through the use of different procedures designed for the detection of the presence and localisation of proteins in the cell. **(Endokrynol Pol 2021; 72 (2): 91–96)**

Key words: *kisspeptin; KISS1 receptor; pituitary adenoma; PitNET; immunohistochemistry*

Introduction

Pituitary neuroendocrine tumours (PitNETs) [1], or traditionally pituitary adenomas, are the most common but heterogenous tumours of the adenohypophysis, with relatively frequent invasive growth and with exceptional metastatic potential [2]. The invasiveness of PitNETs, which leads to the significant morbidity, is a complex and still unclear process, at least partially governed by angiogenesis, degradation of extracellular matrix, epithelial-to-mesenchymal transition, and hypoxia [3], and related to PitNET-type [4]. The essence of aggressive biological behaviour and malignant transformation of PitNETs is still not entirely understood [5].

Kisspeptins are products of the *KISS1* gene [6, 7]. They are cleaved into peptides 54, 14, 13, and 10 amino acids long, which function through binding to the same receptor [8], named KISS1R (originally named GPR45) [9]. Many studies showed that *KISS1* plays the role of a metastasis suppressor gene, loss of which was observed during progression and metastasis in many malignant tumours (including melanoma, gastric, ovarian, endometrial, and bladder carcinoma) [10]. Nevertheless, these conclusions are not unanimous, because the elevated expression of kisspeptins in hepatocellular

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carcinoma was correlated with worse prognosis [11], and the association of kisspeptin expression and progression in breast carcinoma is dependent upon the presence of receptor status [12, 13].

Further investigations of kisspeptins and KISS1R in pituitary gland revealed that they also play an important physiological function in the hypothalamic–pituitary–gonadal signalling axis. Being produced by KISS1 neurons of the hypothalamus, kisspeptins activate hypothalamic gonadotropin-releasing hormone (GnRH) neurons through KISS1R, which stimulates production of follicle stimulating hormone (FSH) and luteinising hormone (LH) in the pituitary gonadotropic cells, giving signals for puberty and sexual maturation [14].

Previous studies of kisspeptin and KISS1R performed on the tissue of human PitNETs analysed the presence of their mRNA, using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). KISS1R transcripts were observed in the normal pituitary and PitNETs in both investigations [15, 16]. Results regarding kisspeptin were contradictory; kisspeptin transcripts were detected in both anterior pituitary and PitNETs in one investigation [15], whereas they were absent in the other [16]. To the best of our knowledge, the presence of kisspeptin and KISS1R in human anterior pituitary and PitNETs has not yet been explored by immunohistochemistry (IHC).

The aim of this study was to explore the immunohistochemical expression of kisspeptin and KISS1R in tissue of different types of PitNETs and to compare it with the expression in the normal anterior pituitary.

Material and methods

The samples of PitNETs were provided from patients treated neurosurgically at the Neurosurgery Clinic, Clinical Centre of Serbia, Belgrade.

Tumours were classified according to WHO classification [4], regarding IHC expression of anterior pituitary hormones and transcription factors, steroidogenic factor-1 (SF1), and pituitary-specific transcription factor 1 (Pit-1). The antibody for the transcriptional factor T-Pit (T-box family member TBX19) was unavailable.

The experimental group consisted of 101 patients, 54 of whom were male (53.5%) and 47 female (46.5%). The age of patients at the moment of surgery ranged between 20 and 80 years (mean 53 ± 13.9). Forty-four patients (43.6%) had gonadotroph tumour, 40 patients (39.6%) had somatotroph tumour, 6 (5.9%) had null cell tumour, 4 (4%) had corticotroph tumour, 4 (4%) had thyrotroph tumour, and 3 (2.9%) had lactotroph tumour.

The control group consisted of tissue of the anterior pituitary accidentally removed during the surgery for PitNETs in 17 patients (4 males [23.5%] and 13 females [76.5%]) with the age ranging between 34 and 70 years (mean 47.6 ± 12.3). The experimental and control groups were matched by age ($p = 0.095$).

All the experiments were reviewed and approved by the local Ethics Committee of the Medical Faculty, University of Belgrade, Belgrade, Serbia and were in accordance with the Declaration of Helsinki.

Preparation of the tissue and tissue microarray (TMA)

The fixation of the samples of both the experimental and control groups was performed in buffered 10% formalin, dehydrated in graded ethanol, and submerged in paraffin blocks. Three representative areas of each sample were recognised on haematoxylin-eosin-stained slides and selected for TMA construction with 1.2-mm cores [17]. The presence of at least one core was regarded as sufficient for the analysis.

Immunohistochemistry

As advised by the manufacturer, the IHC was done on the 5-μm sections of TMA with the following antibodies: anti-cytokeratin 8 (CK8) (Leica Biosystems, clone TS1, 1:50), anti-growth hormone (GH) (DAKO, polyclonal, 1:400), anti-prolactin (PRL) (DAKO, polyclonal, 1:300), anti-FSH (Immunotech, polyclonal, 1:3000), anti-LH (DAKO, clone C93, 1:50), anti-adrenocorticotropic hormone (ACTH) (DAKO, clone 02A3,1:100), SF-1 (DAKO, Invitrogen, clone N1665, 1:200), Pit-1 (DAKO, Novus Biologicals, polyclonal, 1:500), anti-kisspeptin antibody (ab72804 polyclonal, 1:100), and Anti-KiSS1 receptor antibody (ab140839 polyclonal, 14 mg/mL). Appropriate positive controls were used in order to standardise all immunostains (tissue of anterior pituitary for the pituitary hormones, SF-1, Pit-1, and CK8 and placental tissue for kisspeptin and KiSS1R). Immunohistochemistry for SF-1 and Pit-1 was performed using DAKO autostainer. For the remaining antibodies, IHC was performed manually, using an Ultravision Detection System, Large Volume, Anti-Polyvalent HRP (Thermo Scientific). Chromogen for all the stains was 3,3′-diaminobenzidine. Negative control was achieved omitting the primary antibody. Staining on the same run was performed for all antibodies to avoid inter-assay variability.

Quantification of the IHC Stains

Immunohistochemistry stain was taken into account as cytoplasmic for kisspeptin and membranous for KISS1R. Two pathologists (EMG and MM) established the definition of the kisspeptin staining intensity and chose a few representative cases of negative, weak, moderate, and strong intensity (Fig. 1).

Staining intensity for KISS1R was not established due to the negative staining in all cases in the experimental and control groups.

Statistical analysis

For the statistical analysis, for comparison of differences between the groups, Mann-Whitney U and Mantel-Haenszel chi-square tests were performed. P values less than 0.05 were considered significant. All the data were analysed by SPSS 20.0 (IBM corp.) statistical software.

Results

Cytoplasmic expression of kisspeptin was registered in both the experimental and the control group (Fig. 1). In the experimental group, staining was negative in 10 tumours (9.9%), weak in 79 (78.2%) tumours, and moderate in 12 tumours (11.9%); strong staining intensity was not observed. In the control group, staining was weak in 14 (82.4%) and moderate in 3 (17.6%) samples; negative staining was not observed. Statistically significant differences between the experimental and the control group, in terms of kisspeptin expression, were not observed ($p = 0.199$).

Regarding the expression of kisspeptin in different types of PitNETs, weak staining intensity was predomi-

Table 1. *Kisspeptin immunohistochemical expression in the anterior pituitary (control group) and types of pituitary neuroendocrine tumours (PitNETs), and the results of the comparison of the kisspeptin staining intensity between anterior pituitary (control group) and types of PitNETs*

Groups:	Kisspeptin expression			p value (control vs. experimental)
	Negative	Weak	Moderate	
Control				
Anterior pituitary	0 (0.0%)	14 (82.4%)	3 (17.6%)	
Experimental				
Lactotroph	1 (33.3%)	2 (66.7%)	0 (0.0%)	0.148
Gonadotroph	5 (11.4%)	36 (81.8%)	3 (6.8%)	0.095
Corticotroph	1 (25.0%)	3 (75.0%)	0 (0.0%)	0.185
Thyrotroph	0 (0.0%)	1 (25.0%)	3 (75.0%)	0.053
Null-cell	0 (0.0%)	4 (66.7%)	2 (33.3%)	0.576
Somatotroph — all subtypes	3 (7.5%)	33 (82.5%)	4 (10.0%)	0.297
Sparsely granulated	2 (11.1%)	14 (77.8%)	2 (11.1%)	
Densely granulated	1 (4.5%)	19 (86.4%)	2 (9.1%)	

*Results are presented as count (%)

nant in all PitNET types except thyrotroph tumours (Tab. 1, Fig. 1). The comparison between types of PitNET and control group in terms of kisspeptin expression did not reach statistical significance (Tab. 1). Comparison between the different types of PitNETs was not performed due to the small size of some subgroups, which would potentially lead to incorrect results.

Differences between genders regarding kisspeptin IHC expression in PitNETs were not observed ($p = 0.212$).

Immunohistochemical expression of KISS1R was not observed in the control group (anterior pituitary) or in the experimental group, although intensive positive staining was present in the external positive control (placental tissue) (Fig. 1).

Discussion

Immunohistochemical analysis in this study revealed predominantly weak positivity of kisspeptin and the absence of KISS1R expression in PitNETs, without significant differences in staining compared to the expression in the anterior pituitary (control group).

Although PitNETs are classified as benign neoplasms, they can show aggressive biological behaviour through the invasion of the surrounding structures, which is especially seen in some types (lactotroph PitNETs in males, silent corticotroph PitNETs, sparsely granulated somatotroph PitNETs, plurihormonal Pit-1 positive PitNET, and Crooke cell adenoma) [4]. Incomplete understanding of the causes of the aggressive biological behaviour of basically benign neoplasms, associated with exceptional development of metastases, inspired us to investigate how kisspeptin, widely known to participate in metastasis suppression, plays a role in PitNETs. Predominantly low kisspeptin immunohistochemical expression in PitNETs and anterior pituitary, which was without statistical difference compared to the control group of anterior pituitaries, suggests that the IHC method cannot confirm its role in the process of tumourigenesis of PitNETs. The decrease of kisspeptin expression in tumour tissue, compared to normal tissue, previously observed in the colorectal carcinoma [18], non-small cell lung carcinoma [19], and bladder cancer [20] advocates the involvement of the loss of kisspeptin expression in cancerogenesis. In our study, negative kisspeptin expression was observed only in the minority of tumours, including 1 lactotroph in a male, 5 gonadotroph tumours, 1 corticotroph tumour, and 3 somatotroph tumours (of which 2 were sparsely and 1 was densely granulated) (Tab. 1). Bearing in mind the small proportion of kisspeptin-negative PitNETs in our cohort, of which only some belong to potentially aggressive types [4], and that the aggressiveness of the other kisspeptin-negative PitNET cannot be excluded due to the lack of the clinicopathological correlation in our investigation, we speculate that the IHC method is not able to endorse the involvement of kisspeptins in the aggressiveness of PitNETs. However, the presence of kisspeptin positivity in 90.1% tumours in our study (although the majority of them were of weak intensity) potentially supports the antimetastatic role of kisspeptin in PitNETs, considering that their metastatic potential is extremely low in spite of the aggressive biological behaviour of some subtypes. Further investigation of the involvement of the kisspeptin/KISS1R system

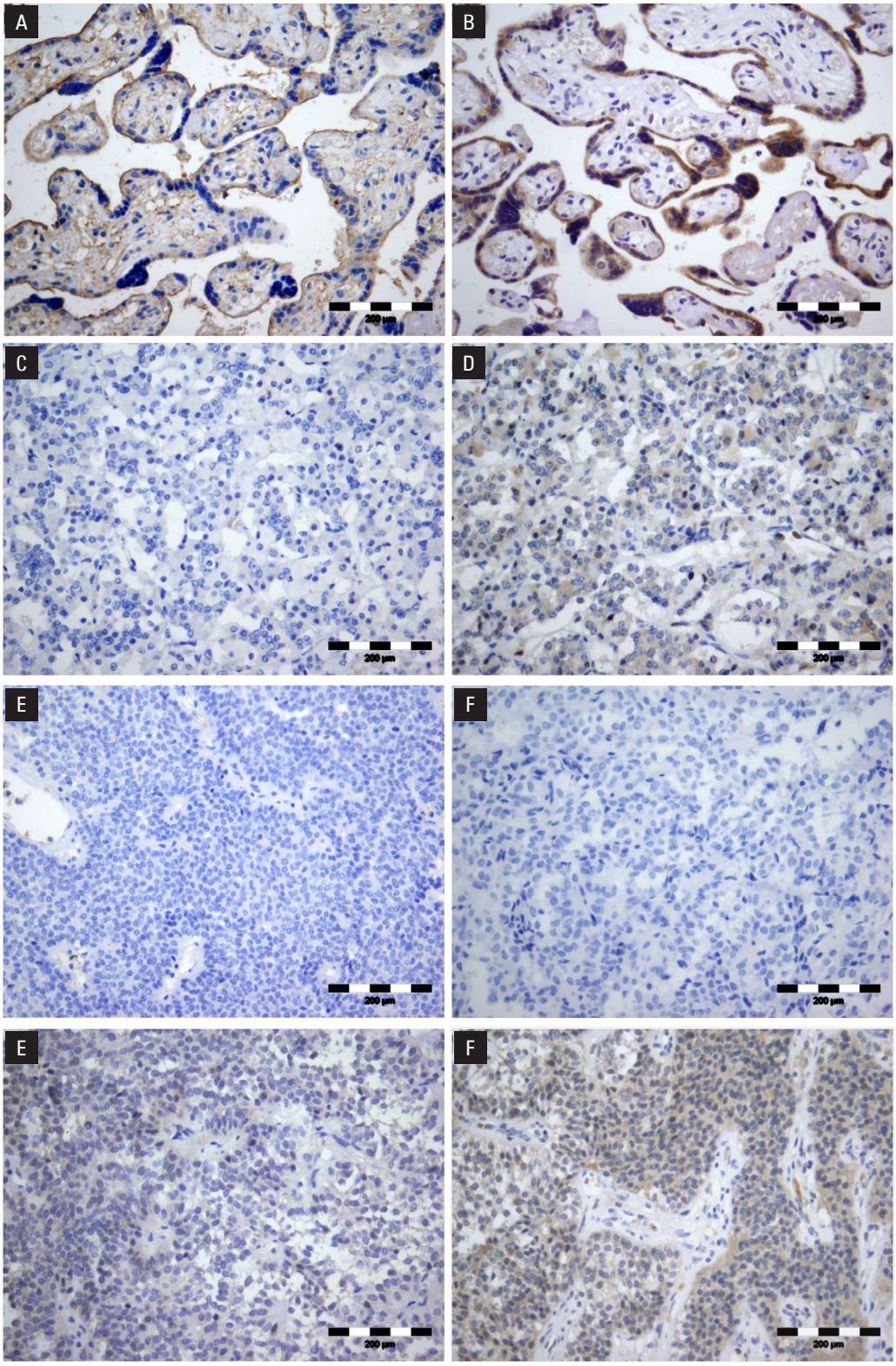

Figure 1. Immunohistochemical expression of kisspeptin and KISS1R in the placental tissue (control), anterior pituitary, and pituitary neuroendocrine tumours (PitNETs). Placental tissue showed intensive membranous positivity for KISS1R (**A**) and cytoplasmic for kisspeptin (**B**). The anterior pituitary showed negative staining for KISS1R (**C**), whilst kisspeptin positivity was predominantly weak (**D**). PitNETs were immunonegative for KISS1R (**E**). Kisspeptin immunopositivity in PitNETs was negative (**F**), weak (**G**), and moderate (**H**). All microphotographs were performed with magnification ×400

in PitNET types with clinically confirmed aggressive biological behaviour and pituitary carcinomas, which were not included in this study, would be advised.

The tumour microenvironment and its intrinsic biology are likely to be crucial factors for the influence of kisspeptins and KISS1R on tumour behaviour,

invasiveness, and metastatic potential [10, 21], which is particularly seen in thyroid tumours, where the aggressiveness and stage of different types of thyroid carcinomas could be related to kisspeptin/KISS1 expression [22, 23]. PitNETs are rather heterogenous types of tumours, classified according to their lineages of differentiation and hormone production [24]. Even though statistical analysis of the potential differences in IHC expression of kisspeptin between PitNET types was not performed in our study (due to the bias risk from small sample numbers in some subgroups, which could lead to incorrect conclusions), predominant weak positivity was observed in all groups. This finding suggests the potential absence of the correlation between the lineage of differentiation and/or hormone production and IHC expression of kisspeptin in PitNETs.

Kisspeptins perform their function presumably through KISS1R [8]. Intriguingly, investigations in cell culture models suggest that the effect of kisspeptins to metastasis suppression is not necessarily driven through KISS1R [25]. Our immunohistochemical findings of the absence of KISS1R in both anterior pituitary and various types of PitNETs are in disagreement with the previous immunohistochemical investigations of KISS1R in anterior pituitary in rats [26] and cell culture of human pituitary adenoma cells [15], discouraging potential treatment of aggressive PitNETs with kisspeptin analogues, previously proven in cell lines and preclinical models of cancers [10]. Nevertheless, it is known that of the many commercial antibodies for IHC only a few have gained the trust of pathologists and are used in routine practice. Even though the polyclonal antibody against KISS1R used in our investigation gave negative results in experimental tissue with positive control in placental tissue, it would be beneficial to perform additional immunohistochemical studies with multiple types of IHC antibodies against KISS1R, to verify the presence of KISS1R on the cell membrane of PitNETs.

The investigation of the presence and the function of any protein is very complex, requiring the use and validation by multiple detection systems. The discrepancy in the expression of KISS1R in PitNETs and anterior pituitary between our investigation, where it was not detected by IHC, and previous investigations, where KISS1R mRNA was detected by RT-PCR [15,16], could be explained by differences in methodological approach. Namely, the amount of mRNA in a cell is not in direct proportion to the amount of its transcribed protein in a cell, because some mRNAs remain untranslated and some are translated suboptimally. Furthermore, translation rates are also diverse among different mRNA species [27]. On the other hand, IHC is a highly sensitive and specific method for the detection of proteins in the tissue, whose results could be obscured by preanalytical conditions, such as the quality of tissue fixation, or mis-interpreted, because read-outs of the results of IHC are prone to subjective interpretation [28, 29]. Nevertheless, disagreement in kisspeptin expression in PitNETs and anterior pituitary in our IHC study with one [15], but not with the other [16], RT-PCR study emphasises the need for further investigation, in which simultaneous detection of mRNA (by RT-PCR) and its transcript protein (by IHC) would be performed.

We conclude that immunohistochemistry, as a method, cannot confirm the involvement of kisspeptin in the tumourigenesis and aggressiveness of PitNETs, but potentially supports its antimetastatic role. The absence of KISS1R immunohistochemical positivity in all anterior pituitaries and PitNETs in our cohort needs verification through the use of different types of antibodies and procedures designed for the detection of the presence and localisation of proteins in the cell.

Acknowledgements

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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 diagnosis). 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 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 diagnosed 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 (fT4), free tri-iodothyronine (fT3), prolactin (PRL), oestradiol (e2), adrenocorticotropic 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 NFPAs, while in 13 patients with NFPAs 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 then 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]
Patients with non-functioning pituitary adenoma (n = 30)							
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
Control subjects (n = 20)							
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

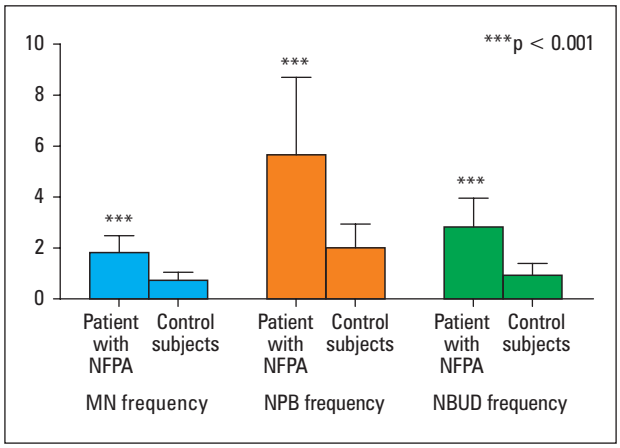

Figure 1. *The frequencies of micronucleus (MN), nuclear bud (NBUD), and nucleoplasmic bridge (NPB) in patients with non-functioning pituitary adenomas (NFPA) and control subjects*

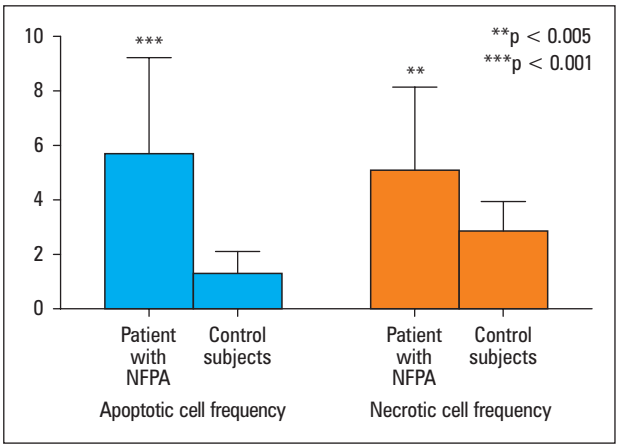

Figure 2. *Apoptotic and necrotic cell frequencies in patients with non-functioning pituitary adenomas (NFPA) and control subjects*

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].

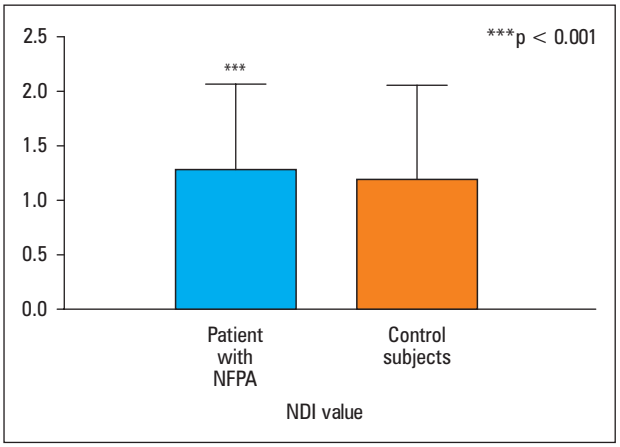

Figure 3. *Nuclear division index (NDI) values in patients with non-functioning pituitary adenomas (NFPA) and control subjects*

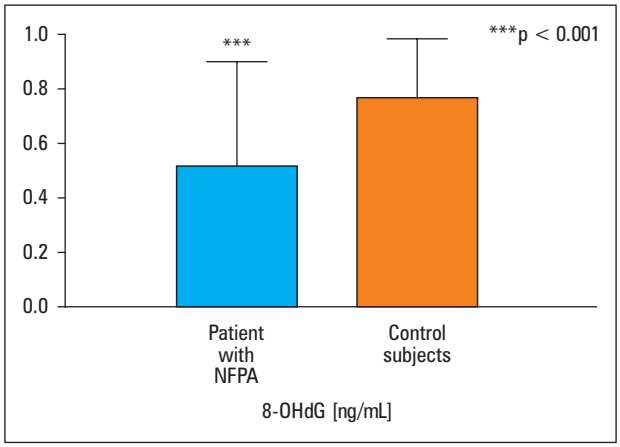

Figure 4. *Plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in patients with non-functioning pituitary adenomas (NFPA) and control subjects*

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 be 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.

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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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Is there a common cause for paediatric Cushing's disease?

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Abstract

Introduction: According to recent literature, somatic mutations in the ubiquitin-specific protease 8 (*USP8*) gene are the most common changes in patients with Cushing's disease (CD). Data on the frequency of these mutations in the paediatric population are limited. The aim of the presented study was to determine the frequency of the *USP8* gene mutations in a group of paediatric patients with CD treated at the Children's Memorial Health Institute (CMHI).

Material and methods: Eighteen patients (nine females) with CD were treated at CMHI, Warsaw, Poland between 1993 and 2019. All patients underwent transsphenoidal surgery (TSS) as a primary treatment for CD. The average age of all patients at TSS was 13.10 years (5.42–17.25). DNA was extracted from formalin-fixed paraffin-embedded resected tumour tissue. Sanger sequencing was performed on DNA sequence corresponding to the exon 14 of USP8 gene.

Results: The mean age at diagnosis of CD was 13.08 years, and the average duration of symptoms before diagnosis was 2.96 years. All patients were operated at CMHI by the same neurosurgeon. Fifteen out of 18 patients (83.33%) had initial biochemical remission after a single TSS procedure (post-operative serum cortisol < 1.8 μg/dL). The result of genetic testing was negative for all samples at the hotspot area of the *USP8* gene.

Conclusion: The current retrospective study demonstrates that mutations in the *USP8* gene may not be as common a cause of paediatric Cushing's disease, as previously reported. **(Endokrynol Pol 2021; 72 (2): 104–107)**

Key words: *Cushing's disease; transsphenoidal surgery; USP8 gene mutations; molecular background*

Introduction

Paediatric Cushing's disease (CD) is characterised by growth retardation with concomitant weight gain as well as other classic clinical features of CD (skin changes, psychiatric disorders, decreased bone mineral density, weakness, and others) caused by excessive secretion of adrenocorticotropic hormone (ACTH) [1–3]. The first-line treatment is transsphenoidal surgical resection (TSS) of the pituitary adenoma [4–6].

According to recent literature, somatic variants in the *USP8* gene encoding ubiquitin-specific protease 8 (USP8), clustered into a hotspot region overlapping with the 14-3-3 binding motif, are the most important in CD pathogenesis. The frequency of these variants is estimated to be 31–63%, but the performed studies have mainly concerned adult patients [7–11]. The effect of this mutation is increased deubiquitination of epidermal growth factor and thus increased induction of proopiomelanocortin transcription and secretion of ACTH [7]. Literature data on the clinical significance of the presence of mutations in the *USP8* gene in children are limited. There has only been one study on children, made by Faucz et al. on a group of 42 children aged 6.1–18.7 years with CD, indicating a frequency of somatic variants in the *USP8* gene of around 30% [8]. At the same time, published results indicate a different course of disease in children than in adults, with an increased risk of recurrence in the group of patients with a variant in the *USP8* hotspot [8]. The need for additional research is emphasised to verify the observed correlation. Hayashi et al. also showed that tumours with *USP8* variants have more type 5 somatostatin receptors that can be a therapeutic target with specific somatostatin analogues such as pasireotide [11].

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Material and methods

Eighteen patients (nine females) with diagnosed CD were treated at the Children's Memorial Health Institute (CMHI), Warsaw, Poland between 1993 and 2019. All patients underwent TSS as a primary treatment for CD (all patients were operated by the same neurosurgeon at CMHI). The average age of all patients at TSS was 13.10 years (5.42–17.25). The Institutional Bioethical Commission (10/KBE/2019) approved this study. Written informed consent from the parents and assent from the minor patients were obtained.

After excluding 12 patients (from 30 patients with CD) because of lack of patient's consent for genetic testing and/or lack of inability to obtain patient's consent or lack of material because surgery was outside CMHI, our study was performed on 22 tumour samples from 18 patients. Two samples from TSS2 were analysed. An additional two samples were from TSS1 of the same patient. Genetic testing was performed at the National Institutes of Health, Bethesda, MD. The tumour samples were anonymised so that no personal data were revealed to the centre carrying out the tests. The genetic material was resected tumour tissue secured in the form of paraffin blocks. The obtained anthropometric parameters were presented as standard deviations (SDS) for BMI using the LMS method [12, 13]. Z-score values were calculated from the formula:

$$z - score(x) = \frac{\left(\frac{X}{M}\right)^{L} - 1}{LxS}$$

The L, M, and S values were taken from the tables of the reference system for a given age and sex [14, 15].

Cortisol levels at night were defined as the mean values of two measurements, respectively: cortisol level at 00.00 H and 00.30 H. High 24-h urine free cortisol was defined as free urine cortisol more than 80 μg/m^2 [16]. The disease onset was defined as the moment when the first symptom of the disease occurred.

DNA was extracted from microdissected tumour tissues with the Pinpoint™ Slide Isolation System (Zymo, Irvine, CA). DNA was extracted after confirming that the selected cut of the tissue originated exclusively from the corticotroph adenoma, to exclude the possibility of mixing the tumour DNA sample with DNA from normal cells.

Somatic DNA encompassing the exon 14 hotspot region of the USP8 gene (amino acids 718 to 721) was amplified by end-point PCR (GoTaq Green Master Mix — M7123, Promega, Durham, NC) using the following primers: forward, 5′-CTTGACCCAATCACTGGAAC-3′ and reverse, 5′- TTACTGTTGGCTTCCTCTTCTC-3′. PCR product was purified (ExoSAP-IT™ Express — 75001.1.ML, Thermofisher — Waltham, MA) and direct bidirectional sequencing (BigDye Terminator 3.1 Cycle Sequencing Kit — 4337456, Applied Biosystems, Foster City, California) using a 3500xL Genetic Analyzer (Applied Biosystems) was performed. Sequences were analysed using Geneious Prime software v.2019.0.4 (Biomatters, San Diego, CA).

Results

Patient characteristics

In our group of 18 patients there was no sex predominance (Tab. 1). The mean age at diagnosis of CD was 13.08 years, and the average duration of symptoms before diagnosis was 2.96 years. The mean midnight serum cortisol and 24-hour urinary free cortisol (UFC) levels were 23.17 μg/dL and 692.43 μg/24 hours, respectively. In every patient 24-h urine free cortisol was increased [16]. Two of the 18 patients (11.12%) presented with evidence of cavernous sinus invasion (in one patient cavernous sinus invasion was detected after TSS2). In one patient (5.56%) the tumour penetrated the cavernous sinus and did not infiltrate its walls but dislocated the sinus.

Transsphenoidal pituitary surgery

All patients were operated at CMHI by the same neurosurgeon. Fifteen out of 18 patients (83.33%) had initial biochemical remission after a single TSS procedure (post-operative serum cortisol < 1.8 μg/dL [17]). One of three patients who did not have biochemical remission after TSS1 had successful hypophysectomy one year after TSS1. The overall rate of remission following TSS was 88.9%. In one patient biochemical remission was achieved after radiotherapy.

Pituitary histology

Histopathological examination confirmed corticotroph adenoma in 16/18 (88.9%) patients. Histopathological examinations of two patients revealed focal corticotroph hyperplasia and normal pituitary gland, respectively.

Table 1. *Data of analysed group of patients*

	Average	Min	Max
Age at diagnosis [years]	13.08	5.5	17.3
Time from first symptom to diagnosis [years]	2.92	0.8	9.00
Female sex n (%)	50.0		
BMI [kg/m^2]	24.27	19.53	31.50
BMI SDS	1.43	–0.30	2.48
Midnight plasma cortisol [μg/dL]	23.17	9.1	52.14
24-hour UFC [μg/24 h]	692.43	199.3	2263.2
Max. morning serum ACTH	131.23	49	536
Invasion to cavernous sinus, n (%)	2/18 (11.12%)		

ACTH — adrenocorticotropic hormone; BMI — body mass index; BMI SDS — body mass index standard deviation score; UFC — 24-hour urinary free cortisol

Follow-up

During a mean of 3.37 years. (0.17–8.33) of follow-up at CMHI, 15 patients (83.3%) were in remission, and one patient (5.56%) had recurrence of the disease. One patient died one month after TSS2 as a result of postoperative complications, and one patient had a persistent disease after TSS1 and TSS2 and was undergoing radiotherapy at latest follow-up.

Frequency of USP8 gene mutations

None of the samples showed any pathogenic variants at the known *USP8* hotspot region. One rare synonymous variant (c.2154C>T / p.Ser718= / rs1261832527 / / 0.0007%) was identified in the hotspot area. Surrounding the *USP8* hotspot area a new missense variant (predict as benign by PolyPhen in silico tool — http://genetics.bwh.harvard.edu/pph2/) was also identified: the variant p.Thr723Ile is formed by a transition from a C to a T at position 2168 (c.2168C>T).

Discussion

Somatic variants in the ubiquitin 8 specific protease gene have recently been identified as the most common changes in patients with CD. Including the studies of Reincke et al. (17 patients aged 33–56 yrs) [9], Perez-Rivas et al. (145 patients aged 7–76 yrs) [7], Ma et al. (120 patients aged 26–46 yrs) [10], and Albani et al. (34 patients aged 39–61 yrs) [18], the total frequency of *USP8* hotspot variants varies between 35 and 63%. Until now the paediatric population has not been extensively assessed, and its real frequency remains uncertain. In a recent study by Perez-Rivas et al. the frequency of *USP8* variants was lower in children than in adults (17% *vs*. 36%) [7]. In the biggest and the only paediatric study, the frequency of genetic changes in *USP8* gene was 31% [8]. In the present study, we did not find any pathogenic variants within the known USP8 hotspot region, comprising the amino acids 718 to 721. It is possible that a different population in the presented study could have influenced the results.

From the two variants identified in exon 14 in the presented study one was synonymous and within the hotspot region (p.Ser718=) and another was located nearby (p.Thr723Ile). Both were somatic. Because the first one is synonymous it is probably not related to the phenotype; however, without functional analysis, a creation of a splice site, for example, cannot be ruled out. For the p.Thr723Ile, even though an *in silico* tool predicted it as benign, the variant is very close to the hotspot, and the amino acid is highly conserved. However, so far, all disease-causing variants are located in the hotspot region, and position 723 does not seem to affect the 14-3-3 binding motif; so probably this variant also does not contribute to the formation of the corticotropinoma. Despite the low possibility of those two variants cause Cushing's disease, in the absence of functional studies is difficult to know if they are somehow responsible for the development of Cushing's disease in those patients. Additional functional tests should be performed in those two variants to rule them out as Cushing's disease drivers. Unfortunately, there were no conditions to perform such tests in the present work.

The identification of a molecular pathogenesis of CD is important because identified mutations create the opportunity to use new treatment targets. The results of our study imply the need for further research into other possible causes of CD in children. Among the possible somatic changes found in patients with CD are mutations in the following genes: *GNAS1*, *TP53*, *NR3C1* (Nuclear Receptor Subfamily 3 Group C Member 1), *NR0B1* (Nuclear receptor subfamily 0 group B member 1), *Brg1* (brahma-related gene 1), and *HDAC2* (Histone Deacetylase 2) [19–22]. Moreover, transcription factors associated with progenitor proliferation and differentiation (such as *TPIT*, *PROP1*, *LHX3*, *LHX4*, or *HESX1*) as well as somatic mutations in genes that cause syndromes associated with pituitary adenomas (*MEN1, PRKAR1A, AIP*) can be associated with molecular changes resulting in CD [23, 24]. However, the frequency of mutations in the above-mentioned genes is very low, and only a few literature reports add them to the range of potential factors in CD. In a study by Williamson et al. a variant in the *GNAS* was identified only in 2/32 ACTH-secreting adenomas [19]. Riminucci et al. reported a case report of an 11-year-old girl with CD with activating mutation in the *GNAS* gene. The girl did not achieve remission after TSS but only after radiotherapy [25].

Among the germline variants in patients with CD there are some genetic changes in genes such as *MEN1*, *CDKN1B* (MEN4), *AIP*, *DICER1*, and *CABLES1*; however, they are not responsible for a significant number of CD cases. Stratakis et al. studied a group of 74 children with CD, and germline changes in *MEN1* were detected in only two patients with positive family history with MEN1 [26]. The disease in these patients was recurrent or difficult to treat [26].

According to the literature, the *USP8* mutational status could predict remission and/or recurrence in patients with CD [18]. Our objective was also to correlate the presence of somatic *USP8* mutations with the rate of recurrence after successful TSS. Unfortunately, the negative results obtained prevent us from making such an analysis and from comparing between groups with and without a variant in the *USP8* gene.

Conclusions

The presented retrospective study demonstrates that variants in the *USP8* gene may not be a common cause of paediatric Cushing's disease in the cohort population. The knowledge about molecular background of corticotroph adenomas is still not entirely known. Future genetic research is essential to determine the pathology of Cushing's disease.

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Declaration of interest statement

C.A.S. holds a patent on the PRKAR1A, PDE11A, and GPR101 genes and/or their function, and his laboratory has received research funding from Pfizer Inc. F.R.F., who holds a patent on the GPR101 gene and/or its function.

The other authors declare that there is no conflict of interest.

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X-linked hypophosphataemic rickets in children: clinical phenotype, therapeutic strategies, and molecular background

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Abstract

Introduction: X-linked hypophosphataemic rickets (XLHR) is the most common form of hypophosphataemic rickets (HR), which is caused by mutations in the *PHEX* gene. The aim of this work was to investigate the clinical phenotype, therapeutic strategies, and molecular background of HR in children hospitalised in our clinic.

Material and methods: Eleven patients aged 5.7–18.25 years were included in this study. Molecular analysis was performed using polymerase chain reaction (PCR) and direct sequencing. The *PHEX* gene was examined in all of the patients, whereas the *FGF23* gene was analysed in 5 patients. All of them were treated with alphacalcidol and phosphorus, and 3 were additionally treated with recombinant human growth hormone (rhGH).

Results: The mean age at HR diagnosis was 4.05 ± 3.35 years. The mean htSDS was –2.99 ± 1.19. In 2 of the 3 patients treated with rhGH the height gain was +0.4SD and +0.3SD, respectively. In 10 of 11 patients, *PHEX* gene mutations were found. In 2 children, novel mutations in the *PHEX* gene were identified: c.325_326dupCA, N110Ifs*7 in one patient and c.899_900delTG, M300Kfs*4 in the remaining one, which coexisted with a known polymorphism c.1769-10C>T, rs3752433. In one patient, a novel deletion of exon 14 and 2 polymorphisms were detected: c.1646-46T>C, g.180417T>C, rs3213493 in intron 15 (known) and g.189156C>T in intron 17 (novel).

Conclusion: We report 3 novel mutations in the *PHEX* responsible for HR. Additionally, this study reports the effects of rhGH therapy for growth promotion in HR. **(Endokrynol Pol 2021; 72 (2): 108–119)**

Key words: *hypophosphataemic rickets; hypophosphataemia; PHEX gene; recombinant human growth hormone therapy; rhGH*

Introduction

Hypophosphataemic rickets (HR) belongs to a heterogeneous group of rare diseases that are caused by phosphate deregulation due to excessive renal phosphate wasting and decreased mineralisation of the growth plate in the growing child [1]. The mode of inheritance of HR varies depending on the affected gene, and the clinical manifestations of HR vary in severity. Some patients are minimally affected even in the absence of medical therapy, whereas others suffer from very severe rickets. The predominant skeletal deformities include progressive bowing of the legs, anteromedial rotational torsion of the tibiae, and short stature [2] along with dental, periodontal, and ear problems, which may also occur in patients with HR.

The most common form of HR (1:20,000 births) is X-linked HR (XLHR, X-linked dominant, OMIM #307800) [3] caused by mutations in the *PHEX* gene (OMIM *300550, a phosphate-regulating gene with homology to neutral endopeptidases on the X chromosome) located on the X chromosome. The XLHR phenotype is characterised mainly by rickets with progressive bone deformities, flaring of the metaphyses, short stature, and dental anomalies [4], and the difference between the phenotypes of affected girls and boys may not be significant. XLHR belongs to FGF23-mediated rickets, where the level of intact FGF23 is increased in the serum. In turn, *FGF23* is expressed by osteocytes standing for a key regulator of phosphate reabsorption in the proximal renal tubules and which also inhibits the activity of vitamin D renal 1α-hydroxylase.

Early diagnosis of HR and its treatment with vitamin D and phosphorus supplementation, which is safe for patients, are crucial because they can help reduce the progression of bone deformations, prevent

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severe bone deformities, reduce the number of necessary surgeries, and improve the patient's final height. However, such treatment is not always effective, and careful monitoring to avoid toxicity is mandatory [2, 5]. The above-mentioned conventional treatment is limited by its renal (hypercalciuria, nephrocalcinosis) and gastrointestinal (abdominal pain, diarrhoea) side effects. Moreover, both phosphate and active vitamin D trigger further FGF23 expression in bones, resulting in a vicious circle, which may limit the efficacy and safety of the standard treatment [6]. Current outcomes of this therapy are still not fully satisfactory, and new therapies targeting the pathophysiology of the disease, i.e. FGF23 excess is the goal. In 2018, the US Food and Drug Administration and European Medicines Agency approved burosumab, a human anti-FGF23 monoclonal antibody that appears to be effective for the treatment of XLHR in children aged 1 year and older [7, 8]. In some cases, HR is diagnosed after infancy and may lead to severe growth deceleration. Recombinant human growth hormone (rhGH) therapy may be used along with supplementation with vitamin D and phosphorus because, if combined with insulin-like growth factor 1 (IGF-1), this therapy improves longitudinal growth and transiently stimulates phosphate reabsorption [9]. Several studies have shown that treatment with rhGH is able to improve growth in short children with XLHR for treatment periods of up to 3 years [10–13].

In addition to vitamin D analogues and phosphate supplements that improve tooth mineralisation, oral hygiene, active endodontic treatment of root abscesses, and preventive protection of teeth surfaces are recommended.

The aim of the study

The aim of this study was to investigate the clinical phenotype, therapeutic outcome, and molecular background of HR in children hospitalised in the Department of Paediatric Endocrinology and Rheumatology, Poznan University of Medical Sciences.

Material and methods

This study was carried out in accordance with the Declaration of Helsinki and was approved by the local Ethics Committee (Karol Marcinkowski Poznan University of Medical Sciences) (No. 504/13).

Patients

We analysed 11 patients (6 girls and 5 boys) aged 5.7–18.25 years (mean age 11.63 ± 4.31), who were diagnosed with HR due to their clinical and biochemical profiles. All of the patients were under care of the Department of Paediatric Endocrinology and Rheumatology, Poznan University of Medical Sciences, and were treated with alphacalcidol and phosphorus. The average period of observation was 4.5 years (range, 0–7.3 years). Three patients with a diagnosis of growth hormone deficiency were also treated with recombinant human growth hormone (Omnitrope, Sandoz) (Tab. 1).

Methods

The height standard deviation score (htSDS) for chronological age was calculated using Polish references [14]. Bone age was estimated according to the Greulich and Pyle [15] method, while predicted adult height was calculated in line with the Bayley and Pinneau [16] method.

Blood samples were collected and frozen at –20°C until analysis. Genetic analysis of *PHEX* and *FGF23* was performed in the Molecular Endocrinology Laboratory of the Department of Paediatric Endocrinology and Rheumatology, Poznan University of Medical Sciences, Poland. Routine biochemical measurements were performed in the Central Laboratory of Karol Jonscher's Clinical Hospital of the University using commercial kits. The studies were carried out using the standard polymerase chain reaction (PCR) method and DNA isolated from peripheral blood leucocytes using the QIAamp® DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. Oligonucleotides (Supplementary Table 1) were purchased from GenoMed, and their sequences were published previously [17]. PCRs were performed in 10-μl volumes using HotStarTaq® DNA Polymerase (QIAGEN) under the following parameters: denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 60 s, annealing at 52–62°C (depending on the primer pair) for 30 s, and elongation at 72°C for 30 s. A final amplification at 72°C for 10 min completed the PCR program.

The *FGF23* gene was analysed in the selected patients (Patient Nos. 1-5) and amplified using routine PCR and primers that encompass exon/intron sites (Supplementary Table 2) [18].

The PCR products were separated by electrophoresis on a 1% agarose gel in the presence of ethidium bromide (Merck), purified from the gel using a QIAquick® Gel Extraction Kit (QIAGEN) and directly sequenced on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Finally, sequences were analysed using Vector NTI 9.0 Software (Invitrogen) and compared to the NCBI Reference Sequences. Bioinformatic analysis of the DNA variants identified in the patients was conducted using Mutation Taster software (http://www.mutationtaster.org/). The DNA variant was annotated as a novel variant when it was not found in ExAC (http://exac.broadinstitute.org/), 1000G (http://www.internationalgenome.org/1000-genomes-browsers/) as well as gnomAD databases (https://gnomad.broadinstitute.org/).

Results

Clinical findings

The average time of HR diagnosis was from 1 and 1/12 to 9 years. In four patients (Nos. 2, 8, 9, and 11), rickets was recognised later (from 7 to 9 years), despite their mothers being affected. In all patients examined, deformities of the lower limbs were observed, and the dominant clinical feature was bowing of the legs with genu varum (10/11 patients, 91%).

In one patient, genu valgum was observed (1/11, 9%). The deformities of the lower leg were sometimes associated with other rickets symptoms, such as widening of the distal parts of the forearms, frontal bossing, or hyperlordosis. Characteristic symptoms of rickets in the examined patients are shown in Figure 1. In 4/11 patients (36%), periodontal problems were observed: gingivitis in two of the patients and advanced caries in two others. Short stature was observed in 9/11 (81.8%) patients. The mean htSDS was –2.99 ± 1.19 (min –4.7, max –1.3).

Table 1. *Clinical characteristics of the patients*

Patient No.	Sex	Age [years/ /months]	Age at diagnosis [years/ months]	HtSDS at diagnosis/ /1st stay at department	HtSDS at current/ /most recent visit	Clinical symptoms/ /family interview	Ear problems	Dental/ /periodontal problems	Other clinical features	Max GH levels after stimulation (normal range > 10 ng/mL)	Treatment at current/ /most recent visit	Follow-up time [years]
1.	Female	9 7/12	2 3/12	–3.7	–3.0	Bowing of the lower limbs, short stature **Affected father**	No	No	No	5.2	rhGH: 0.033 mg/kg/d, phosphorus: 66 mg/kg/d, alfacalcidol: 40 ng/kg/d	7 4/12
2.	Female	13 4/12	7	–2.6	–2.2	Bowing of the lower limbs, short stature, lumbar hyperlordosis **Affected mother**	No	No	No	7.4	rhGH: 0.029 mg/kg/d, phosphorus: 33 mg/kg/d, alphacalcidol: 40 ng/kg/d, orthopedic surgery of lower limbs	6 4/12
3.	Male	8	2 3/12	–2.3	–1.8	Bowing of the lower limbs, short stature, frontal bossing, widening of the distal parts of the forearms	No	Gingivitis	No	7.1	rhGH: 0.029 mg/kg/d, phosphorus: 14 mg/kg/d, alfacalcidol: 69 ng/kg/d	5 9/12
4.	Male	9 6/12	2 8/12	–4.3	–4.2	Bowing of the lower limbs, short stature	No	Gingivitis	Transient hypogammaglobulinemia increased muscle tension	11.4	Phosphorus: 37 mg/kg/d, alphacalcidol: 60 ng/kg/d	6 10/12
5.	Male	7	1 1/12	–3.6	–3.7	Bowing of the lower limbs, short stature, deformation of chest, widening of the distal parts of the forearms	No	No	Immunodeficiency suspected; left cryptorchidism	Not tested	Phosphorus: 45 mg/kg/d, alphacalcidol: 88 ng/kg/d, orthopaedic surgery on the lower limbs	3 8/12
6.	Male	18 3/12	11/12	–2.1	–3.55	Bowing of the lower limbs, genu varum, lumbar hyperlordosis **Affected mother**	No	No	Chronic rhinitis, obesity, low level of cholesterol and triglycerides (as in the father)	10.3	Phosphorus: 11 mg/kg/d, alphacalcidol: 50 ng/kg/d, orthopaedic surgery on the lower limbs	4 5/12

→

Table 1. *Clinical characteristics of the patients*

Patient No.	Sex	Age [years/ /months]	Age at diagnosis [years/ months]	HtSDS at diagnosis/ /1st stay at department	HtSDS at current/ /most recent visit	Clinical symptoms/ /family interview	Ear problems	Dental/ /periodontal problems	Other clinical features	Max GH levels after stimulation (normal range > 10 ng/mL)	Treatment at current/ /most recent visit	Follow-up time [years]
7. (sister of Patient No. 6)	Female	16 11/12	3/12 (therapy was started before clinical symptoms occurred)	–1.3	–3.0	Genu valgus, lumbar hyperlordosis **Affected mother**	No	No	Low level of cholesterol and triglycerides (as in the father)	14.3	Phosphorus: 15 mg/kg/d, alphacalcidol: 68 ng/kg/d, orthopaedic surgery on the lower limbs	4 3/12
8.	Male	11 9/12	8 2/12	–4.2	–3.2	Bowing of the lower limbs, lumbar hyperlordosis, asymmetry of the skull bones **Affected mother**	No	No	–	14.8	Phosphorus: 48 mg/kg/d, alphacalcidol: 57 ng/kg/d, orthopaedic surgery on the lower limbs	1 5/12
9. (sister of Patient No. 8)	Female	10 7/12	8 4/12	–4.7	–	Bowing of the lower limbs **Affected mother**	No	Advanced caries	–	Not tested	Phosphorus: 43 mg/kg/d, alphacalcidol: 68 ng/kg/d	Only one visit at age 8 4/12
10.	Female	5 8/12	2 7/12	–1.3	0.2	Bowing of the lower limbs, widening of the distal parts of the forearms	No	Advanced caries	–	Not tested	Phosphorus: 60 mg/kg/d, alphacalcidol: 41 ng/kg/d	3 1/12
11.	Female	17 4/12	9	–2.8	–4.5	Bowing of the lower limbs **Affected mother**	No	No	–	14.8	Phosphorus: 13 mg/kg/d, alphacalcidol: 40 ng/kg/d, orthopaedic surgery on the lower limbs	6 3/12

rhGH — human recombinant growth hormone

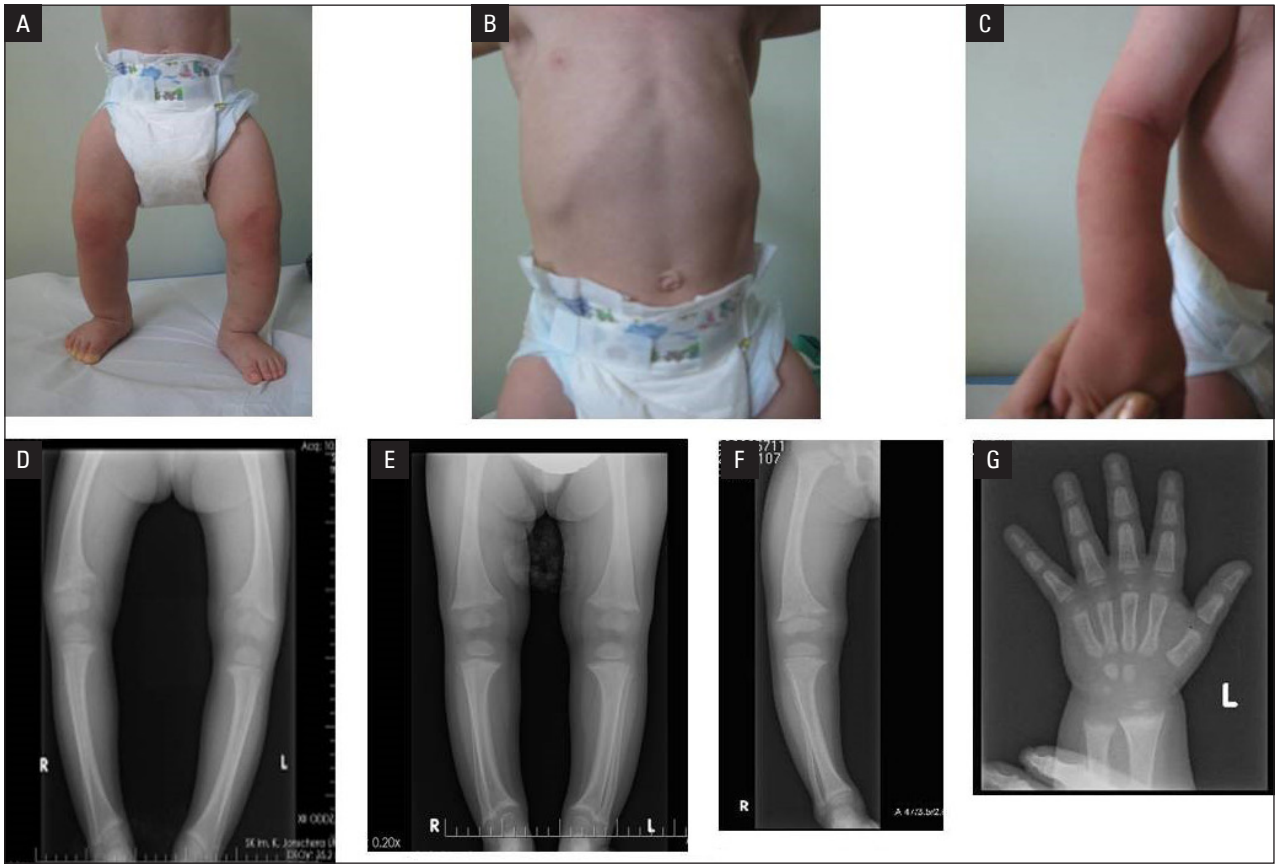

Figure 1. *Characteristic symptoms of rickets in the presented patients. Bowing of the lower limbs (***A***), deformation of the chest (***B***) and widening of the distal parts of the forearms (***C***) in Patient No. 5; X-ray of the lower limbs of Patient No. 1 (***D***), Patient No. 3 (***E***) and Patient No. 4 (***F***) at diagnosis, with characteristic bowing of the lower limbs and distal flaring of the long bones; X-ray of the left hand of Patient No. 3 with characteristic widening of the distal parts of the forearms (***G***)*

The clinical characteristics of the studied group are listed in Table 1.

The dominant biochemical abnormality at the time of diagnosis/during the first stay at the department was hypophosphataemia (11/11, 100% of patients) and high levels of alkaline phosphatase (10/11, 90.9%). In most patients urine phosphorus excretion was decreased (6/11; 54.5%), in 2 patients it was increased (2/11; 18.2%), and in 3 patients it was within the laboratory normal range (3/11, 27.3%). In all patients, tubular reabsorption of phosphate (TRP) at the time of the diagnosis/during the first stay at the department was normal but dropped below the range during follow-up. The concentrations of total calcium, parathyroid hormone, and 25(OH)D3 (a metabolite of vitamin D3) at diagnosis/during the first stay at the department were within normal ranges in most patients (10/11, 90.9%; 8/11, 72.7%; 10/11, 90.9%, respectively). Normalisation of alkaline phosphatase during therapy was observed in 6 children (6/11, 54.5%). The biochemical characteristics of the studied patients are presented in the supplementary materials (Supplementary Table 3).

In one patient (No. 3), due to the presence of frontal bossing, MRI of the head was performed, which revealed a Chiari type 1 malformation.

Six children underwent conventional orthopaedic surgery because of advanced deformities of the lower limbs, and in 3 patients (Nos. 1, 3, and 4) improvement of lower limb deformation was observed by applying only pharmacological treatment. The intercondylar distance changed from 12 cm to 2 cm in Patient No. 1 (Fig. 2), from 8 to 3 cm in Patient No. 3, and from 7 to 0 cm in Patient No. 4. In Patient No. 10 the intercondylar distance, despite an early diagnosis (2 years and 7 months) and 3 years of pharmacological treatment, decreased only from 8 cm to 5.5 cm, and because of severe bowing of the tibial bones, orthopaedic surgery is planned. In other patients, the effects of pharmacologic treatment were difficult to estimate because they underwent orthopaedic surgery or the clinical observation period at our department was too short.

Three patients (Nos. 1, 2, and 3) are being treated with rhGH because they presented GH deficiency. In two of them, we achieved an improvement in height

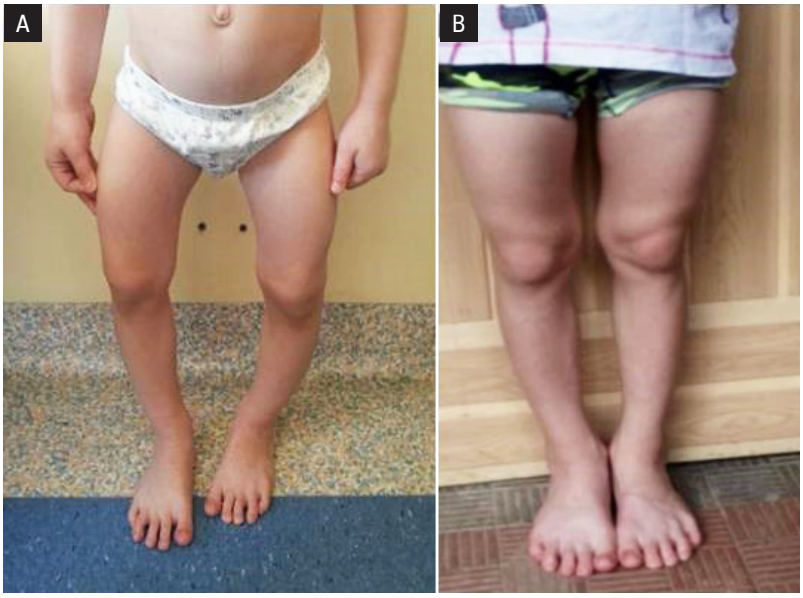

Figure 2. *Lower limbs of Patient No. 1. Bowing of the lower limbs at age 3 years and 3 months (***A***) and the current status at age 9 years and 7 months (***B***)*

Table 2. *Auxological parameters of patients treated with recombinant human growth hormone (rhGH)*

Patient	Sex	Age at the time of HR diagnosis [years, months]	Age at the onset of rhGH therapy [years, months]	Current age [years, months]	HtSDS at the onset of rhGH therapy	PAH at the onset of rhGH therapy [cm]	Current HtSDS	Current PAH [cm]
1	Female	2 3/12	6 9/12	9 7/12	–3.4	149.7	–3.0	152.6
2	Female	7	10 6/12	13 4/12	–2.0	153.6	–2.2	152.6
3	Male	2 3/12	4 9/12	8	–3.1	164.8	–1.8	168

HtSDS — height standard deviation score; PAH — predicted adult height

and adult height prediction. In one patient (No. 2), after nearly 3 years of rhGH treatment, the height deficit and the predicted adult height were slightly worse than they were at the starting point of the therapy. The auxological details of these patients are given in Table 2 and on growth charts (Fig. 3A–C).

Molecular findings

In 10 out of 11 patients studied, *PHEX* gene mutations were found, except for Patient No. 4, in whom only one known polymorphism in intron 15 (c.1646-46T>C, g.180417T>C, rs3213493) was detected and described as of unknown clinical significance. Unfortunately, we were not able to sequence the entirety of exons 12 and 19.

In 2 of the patients, we detected 2 novel mutations (Fig. 4): c.325_326dupCA, N110Ifs*7 in exon 3 in Patient No. 1 and her affected father, and c.899_900delTG, M300Kfs*4 in exon 8 in Patient No. 11. In 1 of these patients a novel mutation coexisted with a known polymorphism: c.1769-10C>T, rs3752433 in intron 17 (No. 11).

In one patient (No. 5), a novel deletion of exon 14 and two polymorphisms were detected: a known polymorphism in intron 15 (c.1646-46T>C, g.180417T>C, rs3213493) and a novel polymorphism (g.189156C>T) in intron 17, both of which may lead to aberrant splicing of the *PHEX* transcript and aberrant function of the PHEX protein (sequencing of the entire exon 19 was unsuccessful). This novel deletion is not shown in Fig. 4.

Moreover, in the remaining patients, known mutations were observed: c.663+1G>T in intron 5 in 2 siblings (No. 8 and No. 9) and their affected mother [19], and c.1483-1G>A in intron 13 in 2 other siblings (No. 6 and No. 7) and their affected mother [20].

In Patient No. 2, the c.1645+1G>A mutation in intron 15 coexisted with the polymorphism c.1769-10C>T, rs3752433 in the splicing site of intron 17. Both DNA changes, which may cause aberrant splicing of the *PHEX* transcript, were also found in the girl's affected mother. In Patient No. 3, c.1801_2250del encompassing exon 17-22, which leads to partial loss of the gene, was present.

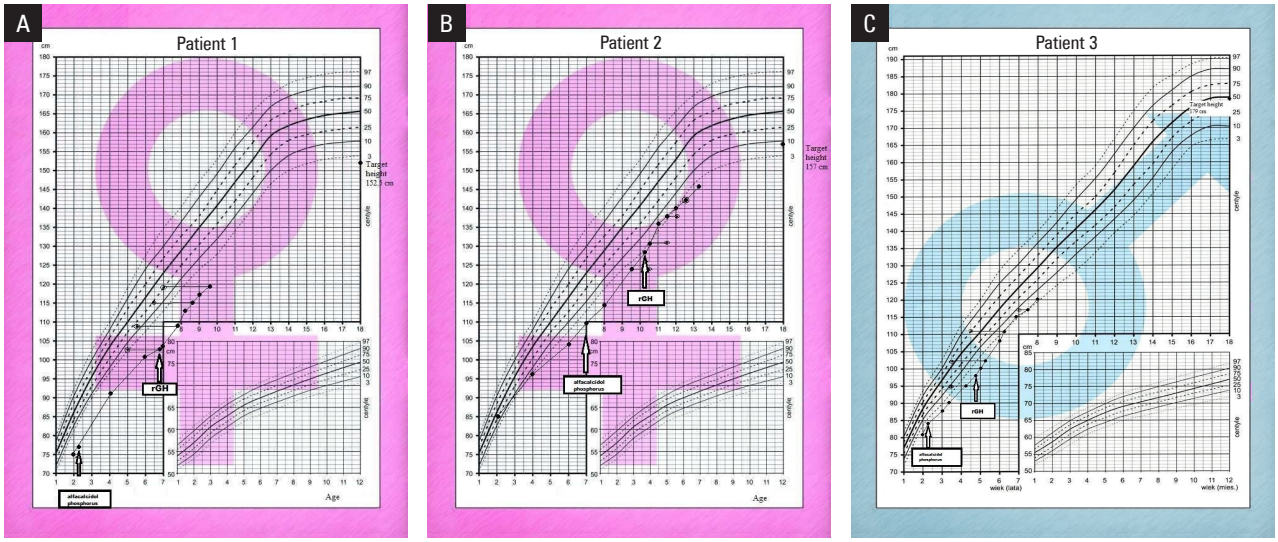

Figure 3. *Growth charts of patients treated with recombinant growth factor (rGH).* **A**. *Patient No. 1;* **B.** *Patient No. 2;* **C.** *Patient No. 3*

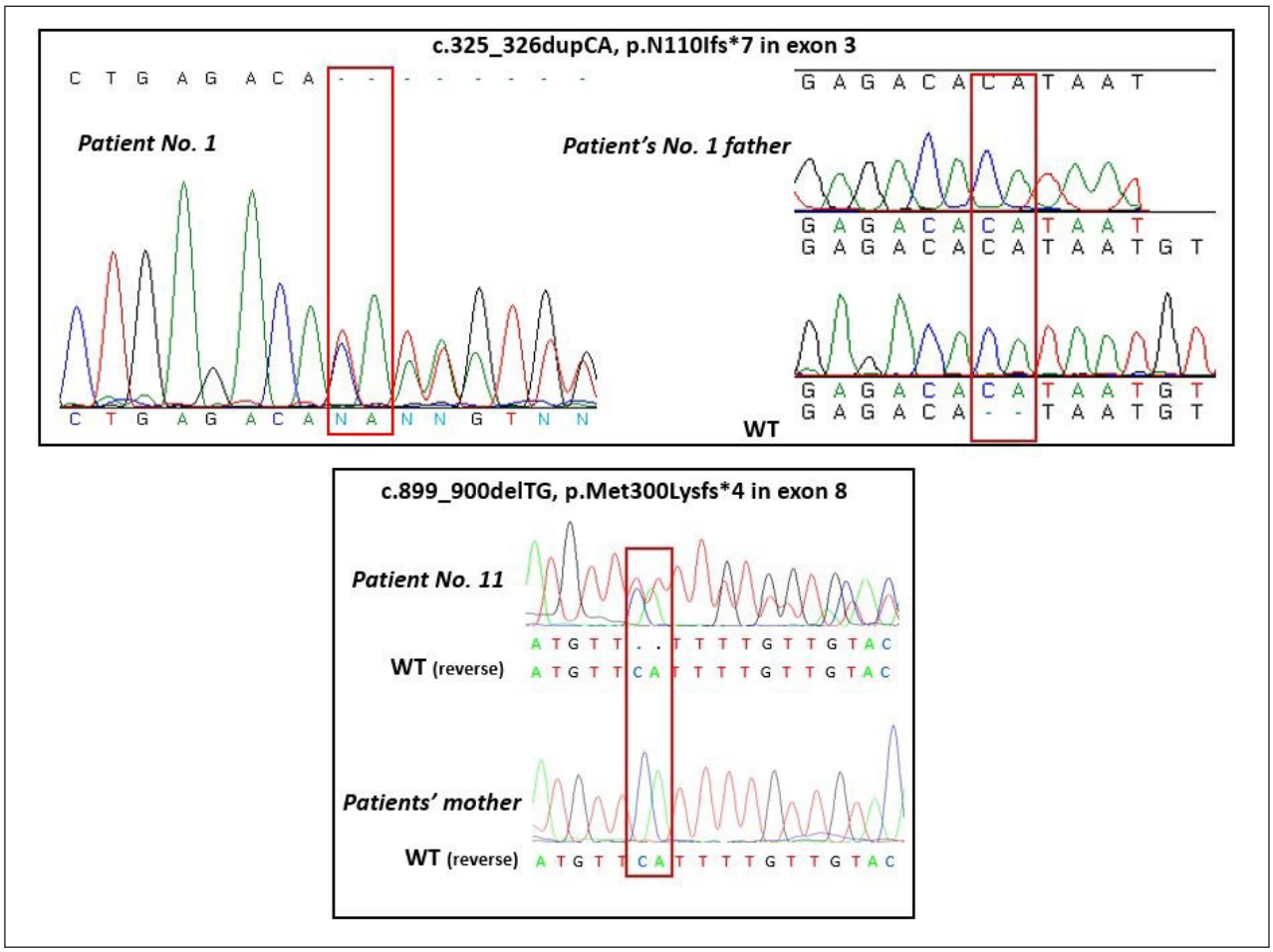

Figure 4. *Novel mutations found in the analysed patients. The figure shows only 2 point mutations found in Patients 1 and 11, except for Patient No. 5, in which a deletion of exon 14 was found. Polymorphisms are not shown*

In Patient No. 10, c.871C>T, p.R291* in exon 8 was found. The mutation leads to the formation of a premature stop codon and premature termination of PHEX protein translation.

Table 3. *Genetic characteristics of the patients. In patient No. 1, a known c.C716>T, p.T239M heterozygous polymorphism (rs7955866) in exon 3 of FGF23 was found, which was absent in the patient's father. The FGF23 gene was intact in patients Nos. 2-5; patients Nos. 6-11 were not tested for FGF23*

Patient No.	PHEX
1.	**A novel heterozygous mutation** (c.325_326dupCA, N110Ifs*7) in exon 3. This variant was found in neither the ExAC nor the 1000G database and was also present in the patient's affected father. The mutation causes a frameshift and premature termination of PHEX protein translation
2.	A known heterozygous splicing mutation (c.1645+1G>A) in intron 15 and a heterozygous polymorphism (c.1769-10C>T, rs3752433) in the splice site of intron 17. Both DNA changes, which may cause aberrant splicing of the PHEX transcript, were also found in the girl's affected mother
3.	A known hemizygous deletion (c.1801_2250del) leading to partial loss of the *PHEX* gene (exon 17 to exon 22)
4.	A known polymorphism in intron 15 (c.1646-46T>C, g.180417T>C, rs3213493), which might lead to aberrant splicing of the PHEX transcript and aberrant function of the PHEX protein (sequencing of the entire exon 12 and 19 was unsuccessful)
5.	A **novel** deletion of exon 14 and two polymorphisms were detected: a known polymorphism in intron 15 (c.1646-46T>C, g.180417T>C, rs3213493) and a novel polymorphism (g.189156C>T) in intron 17, both of which may lead to aberrant splicing of the PHEX transcript and aberrant function of the PHEX protein (sequencing of the entire exon 19 was unsuccessful)
6.	A known hemizygous mutation (c.1483-1G>A) in intron 13 leading to changes in the transcription of the *PHEX* gene (also present in the patient's affected mother and younger sister (Patient No. 7) [33]
8.	A known hemizygous mutation (c.663+1G>T) in intron 5 [32] and a known polymorphism (c.849+3A>G, rs200585038) in intron 7. Both mutations lead to aberrant splicing of the PHEX transcript and aberrant function of the PHEX protein. Both mutations were present in the patient's affected mother and younger sister (Patient No 9)
10.	A known heterozygous nonsense mutation (c.871C>T, p.R291X) in exon 8 leading to the formation of a premature stop codon and premature termination of translation of the PHEX protein
11.	**A novel heterozygous mutation** (c.899_900delTG) in exon 8, M300Kfs*4, a splicing mutation leading to a frameshift and premature termination of the PHEX transcript and a known polymorphism c.1769-10C>T, rs3752433 in intron 17

In one patient (No. 4), we found only one known polymorphism in intron 15; namely, c.1646-46T>C, g.180417T>C, rs3213493. The *FGF23* gene was intact, which suggests that HR in this case is caused by mutations in genes other than *PHEX* and *FGF23*.

Because HR can be caused by mutations in other genes a mutational analysis of *FGF23* was performed in the selected patients (Nos. 1–5), but we did not find any pathogenic variants in any of them. In one patient (No. 1), we detected a known c.C716>T, p.T239M heterozygous polymorphism in exon 3 rs7955866 (which was absent in the patient's affected father), without clinical significance. Thus, we concluded that the identified novel mutation c.325_326dupCA, N110Ifs*7 in exon 3 of the *PHEX* gene is a molecular cause of HR in this family. In patients Nos. 6-11, the *FGF23* gene was not tested because we did not have funds for further analysis, and the molecular background of HR was confirmed by mutations in *PHEX* gene.

The molecular results for the cohort are presented in Table 3.

Discussion

Clinical picture

The time of HR diagnosis was relatively early; however, it was late despite a positive family history in four patients. Most children are diagnosed with X-linked hypophosphataemia in the first year of life, provided that there is a known family history of the disorder [2]. Children presenting *de novo* HR symptoms are usually detected when poor weight gain and growth coexisting with progressive bowing of legs are observed [21]. Our cases show that the genetic counselling of affected families, and education of parents and general practitioners play a very important role in improving patient health and well-being. In the presented patients with late diagnosis and positive family history, the family members were not aware that rickets might be inheritable and that early diagnosis and treatment initiation improve the disease's outcome. Unfortunately, in three patients, the late diagnosis was associated with the necessity to perform several orthopaedic surgeries of the lower limbs. In affected mothers of three patients with a late diagnosis, the molecular tests were performed along with the genetic analysis of the children, and they were informed about the inheritance of the disease afterwards.

The dominant dysfunction in our patients was deformation of the legs (especially genu varum), which is in line with the current literature [2, 22]. General softening of the bone due to defective mineralisation, together with the weight of the child and muscles pulling on weak bones, led to lower limb bending [23]. The cancellous compartment of long bones, particularly the tibia, is undermineralised [24]. Some studies

show that females with XLHR have less related bone involvement than males [23]. Whyte et al. [25] failed to show any evidence for genetic heterogeneity or for effects based on gender, race, anticipation, or parent of origin on XLHR expression in children. Our case study also does not allow us to say that boys suffer in a more severe way. Early conventional medical treatment may prevent or reduce long-bone deformities and facilitate the healing of pseudofractures [26]. The objective in pharmacological treatment should be attained after 3–4 years of leg straightening, which is 1 cm in intercondylar distance every 6 months [22]. Unfortunately, early diagnosis and supplementation with phosphorus and alphacalcidol did not guarantee the reduction of skeletal abnormalities in our studied group. The patient's compliance should also be taken into account, especially because the phosphorus intake can be problematic. Oral phosphate supplements should be taken 4–5 times daily due to rapid absorption and excretion. The oral intake of phosphorus leads to a rapid increase in serum and reaches the baseline level within 1.5 hours [27]. The compliance of phosphorus intake in the studied group, especially at the beginning of therapy, could be not satisfactory, but evident irregularity in the treatment was not registered.

Almost all of our patients were short at diagnosis and during therapy. Only one girl (Patient No. 10) had a normal height (current htSDS 0.2) with the target height calculated on the basis of her parents' height estimated to be 167 cm. She is overweight, which probably has a promoting impact on her growth. Her height standard deviation score improved on conventional treatment. Somatic growth in obese patients appears to be mainly GH-independent [28]. Increased insulin action on the IGF-1 receptor [29] and insulin resistance suppressing IGF-binding proteins leading to greater IGF-1 bioavailability [30] are the mechanisms stimulating the growth process. Altered sex steroid concentrations and adipokines released by the adipose tissue could also play a role [28]. Leptin appears to be an additional factor for stimulating growth [31]. Furthermore, increased aromatisation of androgens into oestrogens in adipose tissue may be another mechanism regulating growth [32].Short stature in HR is secondary to growth restriction of the lower extremities rather than generalised growth failure [33]. The degree of growth impairment is not dependent on the magnitude of hypophosphataemia or the extent of leg bowing, and the chronic administration of phosphate supplements and alphacalcidol is usually not able to normalise the height [34]. Adults with XLHR have a final height that is significantly reduced by up to 20 cm, with a mean htSDS of –1.9 [352]. Adults who have begun earlier treatment with phosphate and calcitriol manage to grow taller despite having a similar degree of hypophosphataemia [36].

Our presented group is heterogeneous, and because of the different types of therapy and sometimes a short observation period, it is hard to clearly specify the conclusions regarding growth, time of diagnosis and laboratory findings.

In two out of three patients with an early diagnosis of HR and treated with rhGH, a reduction in the height deficit with improved adult height prediction and reduction in lower limb bowing were observed. One girl with a late diagnosis, who started rhGH therapy after pubertal onset, did not improve her height. It seems that the time of therapy introduction is the key point. Recombinant human growth hormone influences patient growth as well as bone mineral metabolism. The GH-related increase in phosphate reabsorption is thought to be mediated by IGF-1. In healthy individuals, the latter increases the renal production of 1,25(OH)2 vitamin D3 as well as intestinal and renal phosphate absorption [36, 37]. In view of the GH-induced rise in IGF-1 serum levels, this pathway seems to be still operational in XLHR, although this was not sufficient to normalise TmP/GFR (tubular maximum reabsorption of PO_4/ /glomerular filtration rate) and serum phosphate levels [13]. There are many studies concerning the usefulness of rhGH therapy in XLHR. The randomised study of Živičnjak et al. [13] resulted in a linear growth increase (+1.1 height SDS) in 16 short prepubertal patients with XLHR treated with rhGH for three years. Rothenbuhler et al. [38] also proved that two-year rhGH treatment is effective in treating short stature in XLHR children and that prepubertal children respond better to rhGH. However, the studies are still based on small groups, and there are doubts as to whether rhGH treatment has a positive impact on body proportions.

The initial dominant biochemical abnormality in our patients was hypophosphataemia and elevated alkaline phosphatase, which is a typical abnormality found in XLHR patients occurring in the first months of their life, as a result of FGF23-driven phosphaturia [24]. Urine phosphorus excretion was decreased, and TRP was normal at the time of diagnosis/during the first stay at the department in most patients. Low phosphorus excretion is a result of low chronic hypophosphataemia. Tubular reabsorption of phosphate improves as serum phosphorus and the filtered load of PO_4 declines, and the calculation of the tubular maximum reabsorption of PO_4 (TmP/GFR) is needed to identify the mutant renal phenotype [24].

In one of our patients, a Chiari type 1 malformation was revealed. XLHR children are at risk of developing cranial vault and craniovertebral anomalies, such as early closure of the cranial sutures and Chiari type

1 malformation. The association of craniosynostosis to rickets has been documented as early as 1964, and several reports of scaphocephaly in patients with rickets have been described [39]. Rothenbuhler et al. [40] found that 59% of XLHR children had a complete or partial fusion of the sagittal suture, and 25% of XLHR children showed protrusion of the cerebellar tonsils. A history of dental abscesses was related to craniosynostosis, which in turn was associated with abnormal descent of cerebellar tonsils. Only 2 patients from that study showed neurological symptoms. Our patient presented deformation of the head with frontal bossing and had a chronic problem with dental abscesses. Although he had an episode of seizures, there were no pathological signs in neurological evaluation, and epilepsy was finally excluded.

There are several limitations to our study. The first is the size of the study group and the relatively short time of observation. As bones' mineralisation continues beyond the period where the final height is reached, it would be valuable to extend the study to a longer period. Finally, we cannot exclude periodic non-compliance with phosphorus and alphacalcidol intake in some of our HR patients, affecting the results.

Molecular analysis

The *PHEX* gene consists of 22 exons [41, 42, 43, 44] and is translated into a 749 amino acid protein (https://www.uniprot.org/uniprot/P78562). Extensive mutation analysis showed that the spectrum of the *PHEX* gene mutations is very wide, including nonsense, missense, and splicing site mutations, as well as insertions and deletions in different positions. Current data from the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php; access date: 09.10.2020) include 588 mutations in *PHEX*. The most common types of mutations are missense/nonsense mutations whereas other types are less common. There are no hot-spot mutations, which makes the analysis more time-consuming and expensive. Moreover, the gene undergoes alternative splicing, which may lead to the production of several active forms of the PHEX protein (https://www.genecards.org/cgi-bin/carddisp.pl?gene=PHEX). It is possible that some of them might be involved in bone turnover and dentin formation, whereas others may play an important role in renal phosphate uptake and vitamin D3 metabolism. Considering that splice prediction programs cannot reliably predict the outcome of splice mutations, the effects of mutations in such, probably active alternative forms of PHEX, are very difficult to foresee.

There is also no clear genotype-phenotype correlation of the *PHEX* gene mutation in XLHR, and the severity of clinical symptoms does not strictly depend on the type of the *PHEX* gene mutation and its location. However, some trends in families with XLHR may be observed in the literature. For example, Popowska et al. [45] described 59 patients with XLHR and observed that hearing defects and dental abnormalities were correlated with mutations located at the beginning of the gene, whereas mutations located in its terminal region were associated with increased head length. Other studies showed more severe skeletal malformations in patients with nonsense mutations leading to a truncation of the PHEX protein [41] or with a mutation in the region encoding for its C-terminal part [42]. Lower 1,25(OH)2D3 levels and TRP have also been found in patients with nonsense mutations, which suggest that the phenotypic severity of the disease may be dependent on the type of *PHEX* mutation [46]. However, further and thorough molecular studies are needed to confirm this hypothesis.

Several studies have shown that elevated levels of circulating FGF23 in serum were associated with *PHEX* mutations in XLHR patients [47] and suggest that overexpression of FGF23 might stand for an ultimate linkage in the pathogenesis of XLHR. Unfortunately, we were unable to determine the serum level of FGF23 due to a lack of initial patient serum samples.

Conclusions

The study presents the clinical picture and biochemical profile of patients with XLHR. Three novel mutations in the *PHEX* gene responsible for HR are reported. Early diagnosis and implementation of conventional treatment and rhGH can improve patient height and minimise bone deformities. Molecular analysis is necessary to confirm the clinical diagnosis of HR and conduct appropriate genetic counselling in families with HR patients.

Authors' contributions

M.O.M. and A.R contributed equally to this paper.
All the authors have read the manuscript, accept responsibility for its entire content, and approve its submission. M.O.M — study concept and design, clinical evaluation of the patients, manuscript drafting; A.R. — study concept and design, molecular genetic studies, analysis and interpretation of molecular data, manuscript drafting, funding procurement; ZK — clinical evaluation of the patients; D.J. — molecular genetic studies, analysis and interpretation of molecular data; K.H.C.H. — clinical evaluation of the patients, analysis and interpretation of molecular data, critical revision of the manuscript for important intellectual content; M.N. — study concept and design, clinical evaluation of the patients, critical revision of the manuscript for

important intellectual content, funding procurement, study supervision.

Conflicts of interest

All authors have no conflicts of interest.

Informed consent

All patients and their parents were informed about the research, and written parental consent was obtained for the molecular analysis and publication of the data.

Availability of data and material

This manuscript contains previously unpublished data. The data support the findings of this study and are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

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Microwave ablation of autonomously functioning thyroid nodules: a comparative study with radioactive iodine therapy on the functional treatment success

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Abstract

Introduction: The objective of this study was to compare the efficiency of microwave ablation (MWA) and radioactive iodine (RAI) in the treatment of toxic adenoma (TA), and to investigate the functional treatment success of the used modalities for its remission.

Material and methods: Treatment outcomes — thyroid hormone levels and nodule characteristics — of 30 patients (23:7 F:M; 52.77 ± 11.13 years) treated by MWA were compared with the those of 35 patients (24:11 F:M; 61.43 ± 12.60 years) treated by RAI. The baseline characteristics of TAs, which are gender and pre volume, were analogous and did not show any statistical significance ($p > 0.05$). Thyroid hormone levels of patients treated with two different methods were measured after 9 months, and the obtained results were compared.

Results: Although there was no statistically significant difference in the nodule volume ($p > 0.05$), there was a greater volume reduction rate (VRR%) in the group treated with MWA rather than RAI ($p < 0.05$) at the end of the follow-up. In the MWA group, there was a higher increase in FT3 than in the RAI group ($p < 0.05$). Furthermore, no statistically significant difference in TSH ($p = 0.124$) and FT4 ($p = 0.144$) levels of the patients as treatment outcomes was observed. The therapeutic success was accomplished in 18/30 (60%) of the MWA group and in 24/35 (68.6%) of the RAI group.

Conclusions: Therapeutic success of MWA and RAI did not show any statistically significant difference ($p = 0.471$). However, the development of hypothyroidism in 7 of 35 patients treated with RAI was observed. On the other hand, no case of post treatment hypothyroidism was observed in patients treated with MWA. In this regard, MWA could be a great alternative to RAI due to its advantages in terms of non-exposure to radiation and lower risk of post-treatment hypothyroidism. **(Endokrynol Pol 2021; 72 (2): 120–125)**

Key words: *thyroid; nodule; toxic adenoma; microwave ablation; thyroid hormones*

Introduction

The second most widespread cause of hyperthyroidism after Graves' disease is autonomously functioning thyroid nodules (AFTN) [1]. Toxic adenoma (TA) and toxic multinodular goitre take place as an outcome of focal and/or diffuse hyperplasia of thyroid follicular cells [2]. AFTN can lead to the development of a series of functional abnormalities, including euthyroidism and overt hyperthyroidism [3]. Although euthyroidism can be achieved with thionamides, the symptoms of hyperthyroidism can be controlled rather than undertaking permanent treatment [4]. Antithyroid drugs (ATDs) can be an option in the long-term treatment of patients who do not want surgery or radioactive iodine (RAI) therapy [5]. The choice between radioiodine and surgery is based on individual patient factors and the regional availability of specialist thyroid surgeons. Permanent treatments for toxic thyroid adenomas are RAI treatment and surgery [6, 7]. Thionamides are often used before surgery and RAI to prepare patients for permanent treatments.

Several techniques have been applied up to now for the treatment of AFTNs, some of which are percutaneous ethanol injection (PEI) [8, 9] and laser ablation (LA) [10]. Due to their disadvantages such as less effectivity in the treatment of solid nodules and the requirement of multiple and tedious treatment secession for PEI and LA, respectively, alternative approaches have been tried in the last decades. In recent years, in particular, American thyroid associations have proposed radio frequency (RF) ablation as a new thermal ablation treat-

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ment modality for toxic adenomas patients who are not suitable or refuse to undergo permanent treatment options and ATDs [11]. The risk of major complications such as recurrent laryngeal palsy and hypoparathyroidism with these techniques is very low [12]. Also, its important advantages have been considered as good alternatives for patients unwilling to undergo surgery or RAI therapy due to benefits such as acceptable nodule shrinkage, the ability to be treated on an outpatient basis, and long-term effects [13].

In recent years, alternatively, microwave ablation (MWA) has been emerging as a new technology and is attracting increasing attention for the treatment of thyroid nodules because of its advantages including good cosmetic results, low complication rate, and nodule shrinking efficacy, especially for young patients who are preferring non-surgical treatments [14]. Although MWA therapy has been applied in several studies for the treatment of benign thyroid nodules, to the best of our knowledge, no study has been conducted up to now for the examination of the effectiveness of MWA in the treatment of AFTNs. Thus, the purpose of this study is to investigate the efficacy and safety of MWA ablation for AFTN therapy in a large population of patients.

Material and methods

Ethics statement

University of Health Sciences Antalya Training and Research Hospital Ethics Committee approved this study (Ethics Committee approval number and date: 12\19-13\8\2020). All patients gave their written informed consent before the procedure.

Patients

This study was carried out as a retrospective analysis of a prospectively collected data. From January 2017 to August 2020, 35 patients treated with radioactive iodine (RAI) for biochemically non-occult AFTN and 30 patients who underwent ultrasound-guided microwave ablation for biochemically non-occult AFTN were included in the study. The inclusion criteria of the MWA group were as follows: 1) patients with functioning solid thyroid nodules; 2) ineligibility or refusal to undergo surgery; and 3) benign nodules (fine needle aspiration biopsy results based on Bethesda categories). The exclusion criteria were as follows: nodules with increased risk for malignancy and malignant nodules (Bethesda categories ≥ 3).

Laboratory assessment

A Unicel TM Dxl 800 Access Immunoassay System (Beckman Coulter Inc., Brea, CA, USA) was used to measure serum thyroid function tests. The normal ranges of these tests were 0.61–1.1 ng/dL, 2.5–3.9 pg/mL, 0.34–5.86 μIU/mL, 0–4 IU/mL, and 0–10 IU/ml for free T4, free T3, thyrotropin (TSH), anti Tg, and TPO, respectively. Laboratory tests were performed 1, 3, and 9 months after treatment modalities.

Microwave ablation

A microwave ablation system (ECO-100AI3) with a microwave generator producing 30–40 W of power at 2450 MHz either continuously or in a pulse, a flexible cable, and internally-cooled 16-gauge thyroid antenna with 10 cm shaft length with a 3.0 mm active tip was used. Autonomous thyroid nodule ablation was applied on an outpatient basis under local anaesthesia without sedation. The patient was placed in a supine position with the neck mildly hyperextended. After determining the appropriate puncture side, a mixture of 30/70% lidocaine (Osel Pharmaceuticals, Istanbul, Turkey) and saline was applied along the puncture path from the skin to the thyroid capsule and then infused into the surrounding thyroid capsule to preserve vital structures adjacent to the thyroid nodule. An internally cooled thyroid microwave ablation antenna was positioned under ultrasound guidance via trans-isthmic approach or lateral cervical approach. A moving shot technique was used to ablate the target nodule throughout the procedure. Therapy was completed when the entire nodule was covered with hyperechoic echo, which is indicative of ablation. Vital signs were monitored during the procedure, and phonation was evaluated intermittently. After the procedure, all patients were followed up for 2 hours with cold compression to the neck to prevent haematoma. Before discharging the patients, an ultrasound examination was performed to evaluate the changes in MWA-induced focal complications.

RAI ablation

Scintigraphic images were obtained in all patients 15–20 min after the application of 75 MBq Tc-99m-pertechnetate and recorded with a scintillation camera (Mediso Nucline XR® TH / 22, Budapest, Hungary). Unifocal overactive areas with reduced or suppressed uptake in the remaining thyroid tissue were thought to be compatible with non-occult AFTN along with suppressed TSH. The patients were administered a fixed dose of 15 mCi (555 MBq) RAI ^{131}I without prior antithyroid therapy). Necessary information about radiation safety was given verbally and in writing, and then the patients were discharged.

Treatment efficiency and follow-up

The volumes of the nodules before, after, and during the follow-up process were assessed by ultrasonography examination via the use of a 5-14 MHz linear probe of a real-time ultrasound system (Aplio 500, Toshiba Medical Systems, Tokyo, Japan). The orthogonal diameters of the nodules before ablation, which are a, b, and c calculated from the equation $V = \varpi abc/6$, where V = volume, $\varpi = 3.14159$, a = the largest diameter, and b and c = the other two perpendicular diameters. In the calculation of the volume reduction ratio (VRR%), the following equation was used:

[Baseline volume – volume at 9 months] / [Baseline volume] 100.

The success of the therapy was accepted as euthyroid state at 6 months without antithyroid medical treatment. Antithyroid therapy was not used before MWA or RAI and during follow-up. Beta-blocker therapy was used if needed. With the aim of restoring euthyroidism, the levothyroxine treatment was administrated in the presence of a permanent hypothyroid state. Hypothyroidism, which is defined as high serum TSH concentration together with a low serum free T4 concentration, is a functional major side effect compared between two groups.

Statistical analysis

SPSS® 20.0 (Statistical Packages for Social Sciences; SPSS Inc, Chicago, Illinois, USA) was used to assess all the statistical tests. The normality and the homogeneity of the data were evaluated by Shapiro-Wilk and Levene's test of homogeneity. Qualitative variables were presented as percentages or frequencies, while continues variables were reported as mean ± SD. The data of the two groups were compared by the use of appropriate independent t, Mann-Whitney U, Wilcoxon rank, and Pearson chi-square tests. The significance level was considered as a p value of less than 0.05.

Results

Patients' data

The baseline characteristic data of the patients and the nodules according to their treatment grouping are summarised in Table 1. A statistical significance test was conducted to reveal the analogies between the two groups. Results revealed that gender, TSH, volume, and thyroid autoantibodies did not exhibit any statistically significant difference ($p > 0.05$). Also, before MWA therapy, positive thyroid autoantibodies were present in 4:30 patients, and 2:35 before RI therapy.

Treatment outcomes

Table 2 presents the treatment outcomes of the 6-month follow-up of the two groups. Investigation of Table 2 reveals that, at the end of the follow-up, there was no statistical difference between MWA and RI groups in terms of nodule volume ($p = 0.916$). On the other hand, it can be clearly seen that there was a higher volume reduction rate in the MWA group compared to the RI group ($p = 0.008$). In FT3 values, there was a greater increase in the MWA group rather than RI ($p < 0.001$). In addition, no statistically significant difference between the TSH ($p = 0.124$) and FT4 ($p = 0.144$) levels of the patients in the two groups was observed. The functional undesirable effect was 20% in the RI group in terms of clinical hypothyroidism percentage, whereas no undesirable effect was observed for the MWA group. Overall, none of the patients in both of the groups exhibited major complications in either of the groups.

The functional therapeutic success (FTS), defined as the restoration of euthyroidism, of the two groups was also investigated, and the detailed cross tabulation results are given in Table 3, which shows that FTS was achieved in 18/30 and 24/35 patients with a percentage of 60.0–88.6% in the MWA and RI groups, respectively, and there was no statistically significant difference between the values of them ($p = 0.471$).

Discussion

Our study showed that MWA is effective in treating TAs. While VRR% was higher in patients who underwent MWA, the therapeutic success rate was higher in the group receiving RAI. On the other hand, although

Table 1. *The baseline features of the patients and nodules according to treatment modalities*

Baseline	RAI (n = 35)	MWA (n = 30)	p value
Age	61.43 ± 12.60	52.77 ± 11.13	0.005
Gender (F:M)	24:11	23:7	0.467
FT3 (2.5–3.9 pg/mL)	3.34 ± 0.85	4.36 ± 0.76	0.000
FT4 (0.61–1.1 ng/dL)	0.96 (0.78–1.10)	1.12 (0.89–1.51)	0.006
TSH (0.34–5.86 μIU/mL)	0.05 (0.03 ± 0.13)	0.07 (0.04–0.22)	0.291
Volume (cc)	6.08 (4.24–8.82)	8.08 (4.03–12.50)	0.130
Thyroid autoantibodies			0.403
Positive:negative (%)	5:30 (14.3%)	4:26 (13.3%)	

FT3 — free triiodothyronine; FT4 — free thyroxine; TSH — thyroid-stimulating hormone; RAI — radioactive iodine; MWA — microwave ablation; $p < 0.05$ is significant

Table 2. *Treatment outcomes after nine months*

	RAI	MWA	p value
Volume [cc]	3.48 (2.00–5.43)	3.30 (2.16–6.10)	.916
VRR%	45.81 (20.60–45.81)	54.29 (45.23–59.61)	.008
FT3 (2.5–3.9 pg/mL)	3.14 (2.75–3.32)	3.79 (3.37–4.70)	.000
FT4 (0.61–1.1 ng/dL)	0.87 (0.78–1.00)	0.98 (0.78–1.06)	.144
TSH (0.34–5.86 μIU/mL)	1.20 (0.68–1.95)	1.04 (0.19–1.55)	.124
Functional undesirable effect			
Hypothyroidism (%)*	7/35 (20%)	0	

RAI — radioactive iodine; MWA — microwave ablation; *hypothyroidism was defined as high TSH concentration in association with low serum free thyroxine concentration; VRR — volume reduction ratio; FT3 — free triiodothyronine; FT4 — free thyroxine; TSH — thyroid-stimulating hormone; $p < 0.05$ is significant

Table 3. *Cross tabulation results*

	Functional therapeutic outcome (FTO)		Total
	Therapeutic success	Therapeutic unsuccess	
MWA Treatment			
Count	18	12	30
% within MWA	60.0%	40.0%	100.0%
% within FTO	42.9%	52.2	46.2%
RAI Treatment			
Count	24	11	35
% within RAI	68.6%	31.4%	100.0%
% within FTO	57.1%	47.8%	53.8%
Total			
Count	42	23	65
% within Treatment	64.6%	35.4%	100.0%
% within FTO	100.0%	100.0%	100.0%

RAI — radioactive iodine; MWA — microwave ablation; chi-square test. Pearson $\chi^2 = 0.519$, $p = 0.471$

hypothyroidism did not develop in any case after MWA, the development of hypothyroidism was statistically higher in the group that received RAI in the follow-up.

Hemithyroidectomy and RAI ablation are the permanent treatment used for TAs. The surgical process lowers the risk of permanent hypocalcaemia in the presence of damage to the affected side in the parathyroid glands. Furthermore, it ensures the maintenance of thyroid function by means of contralateral healthy thyroid lobe. All in all, the risk of permanent unilateral laryngeal nerve injury after surgical procedure is still between 0% and 2.1% [15].

The other alternative form of permanent therapy is RI, due to its high percentage of clinical efficacy between 3 and 12 months and its low cost [16]. However, RAI is absolutely contraindicated in pregnant or breastfeeding women [17]. The main disadvantage of RAI is the delay in conception for at least 6 months and the development of undesirable complications after exposure to radiation in both male and female patients [18, 19].

TAs lead to the investigation of alternative treatments, especially in selected patient groups, due to the disadvantages of both surgery and RAI treatment in their permanent treatments. A new development for the treatment of thyroid diseases — ultrasound-guided MWA — has the advantages of being minimally invasive, safe, and effective [20–22]. Huo S et al. reported that there was no adverse effect on the patient or foetus in the follow-up after MWA for an autonomously functioning thyroid nodule in a pregnant patient [23]. No study comparing MWA and RA has been reported in the literature until now. However, there are studies comparing RAI with other minimally invasive methods such as radiofrequency ablation and alcohol ablation [24, 25]. As in these studies, the end points in our study to evaluate the effectiveness of each treatment were volume nodule reduction and resolution of the hyperthyroid state 9 months after RAI and MWA. When evaluating through VRR%, the rate of volume reduction after RAI in TAs has been reported as 35–54% in the literature [16, 26, 27]. After radiofrequency ablation, this rate was reported as 52.1% and 79.7% [28, 29]. After percutaneous alcohol ablation in toxic nodules, the rate of thyroid nodule volume reduction was reported to be 66% in the 12th month [30]. In our study, although VRR% was evaluated at the 9th month after MWA, it was found that it was similar to VRR% in the 12th month of other minimally invasive methods. However, it was determined that VRR% was statistically higher in the MWA group compared to the patients who received RAI.

Hypothyroidism is less common after initial toxic adenoma radioiodine therapy. The risk of developing hypothyroidism after RAI is 12–32% after one year [31]. Focal nodule autonomy areas take up more radioiodine, while in the contralateral thyroid tissue suppressed by the hyperthyroid state, uptake is limited. As a result, radioiodine tends to destroy only autonomic areas, and most patients remain euthyroid after radioiodine administration [32]. Patients who develop hypothyroidism usually have incomplete suppression of iodine uptake in the extranodular tissue, or the patient has chronic lymphocytic thyroiditis with an autonomic toxic nodule [33, 34]. In our study, when the thyroid function of patients was evaluated after treatment, euthyroidism was achieved in 68.6% of the RAI group and 60% of the MWA group.

Although the rate of euthyroid patients was high in the RAI group, hypothyroidism was observed in

7 patients in the RAI group after treatment, while no hypothyroidism was found in the MWA group. In addition, 5 patients in the RAI group had antibody positivity (TPOAb), and all of these patients developed hypothyroidism after treatment. However, although 2 patients in the MWA group had antibody positivity, no hypothyroidism was observed after treatment. We think that this is because MWA affects only the autonomic nodule, and even if the antibody is positive, it does not destroy other healthy thyroid tissue. In a study by Cervelli R. et al. [24], although most of the patients (72%) treated with RAI converted to euthyroidism, clinical hypothyroidism was found in 5 patients. In addition, it was determined that 5 hypothyroid patients after treatment had positive TPOAb before RAI treatment. It has been reported that high TPOAb levels may be considered as a risk factor for hypothyroidism [35, 36]. Moreover, I^{131} retained by the autonomic nodule after RI treatment is taken up by the thyroid tissue surrounding the autonomic nodule and damages healthy thyroid cells [37]. Because thermal damage is created in the local area of the nodule with MWA, it does not damage healthy cells around the nodule.

The limitation of our study is that, due to the small sample size, large patient series with long-term follow-up are needed to better evaluate the efficacy after MWA treatment.

Conclusion

Although RI and MWA have similar effects in maintaining normal thyroid hormone function in patients with toxic autonomic thyroid nodules, post-treatment of RI, hypothyroidism is more common especially in patients with TPOAb(+). When it is desired to avoid hypothyroidism after treatment, MWA can be considered as a reliable method in toxic adenoma in patients with high antibody levels.

Disclosure statement

No potential conflict of interest was reported by the authors.

Authors' contributions

The study conception and design were by M.S.E, B.C, and M.C. M.S.E, B.C, M.C, and I.D.U performed the material preparation, data collection, and analysis. M.S.E wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Low incidence of focal lesions in the thyroid glands of patients with hereditary haemochromatosis — a single-centre study from Poland

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Abstract

Introduction: Hereditary haemochromatosis (HH) is a disease characterised by the excessive absorption of iron and its deposition in various organs. Late complications of this disease include cirrhosis, hepatocellular carcinoma, and endocrine disorders. Data from the literature on thyroid disorders in patients with HH are inconsistent and ambiguous, and no research has been done to determine the relationship between excessive accumulation of iron and the thyroid morphology. Therefore, the aim of this study was to characterise thyroid function and ultrasound images in patients with clinically overt hereditary haemochromatosis.

Material and methods: We studied 40 patients who were diagnosed with hereditary haemochromatosis with one of the mutations of the *HFE* gene and iron deposits in liver in specimen from liver biopsies (graded G2 to G4) or in MRI. To assess thyroid function, ultrasound examinations of the thyroid gland were performed and serum TSH concentrations were measured.

Results: We showed in our study that patients with HH have been diagnosed with thyroid focal lesions statistically less frequent than in the control group. We did not reveal any statistically significant difference in TSH concentration between patients with HH and the general population. However, patients with more severe iron deposits in liver showed lower TSH concentration.

Conclusions: Our results indicate lower incidence of focal lesions in thyroid gland in a group of patients with clinically overt hereditary haemochromatosis. **(Endokrynol Pol 2021; 72 (2): 126–132)**

Key words: *haemochromatosis; genetic diseases; iron overload; iron; thyroid; goitre; liver biopsy; MRI*

Introduction

Hereditary haemochromatosis (HH) is a disease characterised by the excessive absorption of iron and its deposition in various tissues of the body. It is one of the most common genetic disorders of metabolism in people [1]. The disease affects 0.24–0.5% of northern European residents. Among white people of western European descent, more than 80% of patients diagnosed with HH are homozygous for a C282Y mutation in the HFE gene. Increased iron absorption was also found in heterozygous carriers of C282Y/H63D and S65C/C282Y mutations [2, 3]. Other alterations in the HFE gene are of uncertain significance, but they may promote the accumulation of iron in the presence of certain factors that affect the physiological regulation of this chemical element [4].

Men are more likely to develop signs and symptoms of HH than women. This is because women naturally lose blood during their menstruation cycles. However, the risk of HH for women increases following menopause. The disease is mainly manifested by fatigue, weakness, decreased libido, joint pain, pain near the right costal margin, dyspepsia, enlarged liver, and moderately elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Late complications in untreated patients include cirrhosis, with a 100-fold higher risk of hepatocellular carcinoma (HCC), diabetes, endocrine disorders (hypogonadism, hypothyroidism, and infertility are among the most frequently quoted ones in literature), heart failure, and severe skin pigmentation disorders [5–8].

Hereditary iron disorders resulting from genetic abnormalities are determined by mutations of *HFE*,

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HJV, *HAMP*, *TfR2*, and *SLC40* genes, which encode, respectively: hephaestin, haemojuvelin, hepcidin, transferrin receptor 2, and ferroportin [9]. One of these proteins, hepcidin, is a hormone that is released in the liver in response to iron overload and inflammation. Decreased levels of hepcidin, observed in patients with hereditary iron overload syndromes, lead to the accumulation of iron in tissues. On the other hand, hepcidin overproduction is common in anaemia of chronic diseases. Another protein, ferroportin, serves as an iron exporter, which is present on the surface of absorptive enterocytes, macrophages, hepatocytes, and trophoblast cells. Interaction of hepcidin with ferroportin resulting in the degradation of hepcidin-ferroportin complex leads to the decrease in the export of cellular iron [10].

In patients suffering from HH and chronic liver disease with advanced fibrosis, the total amount of iron in the organism may exceed 25 g [11]. The iron is mainly deposited in the liver and glandular tissues, i.e. the pancreas, the pituitary gland, the thyroid gland, the gonads, and the adrenal glands. This process may be precisely visualised by using a non-invasive imaging method, i.e. magnetic resonance imaging (MRI) [12, 13]. Two laboratory parameters, ferritin and transferrin saturation, are also used to detect any abnormalities in the accumulation of iron. Not only may they indicate the need to extend the diagnostics for iron overload syndrome, but also, if the syndrome is diagnosed, help to determine the right treatment plan for the patient [14]. The main treatment for haemochromatosis includes bloodletting (therapeutic phlebotomy), a controlled removal of blood from the patient's body, that effectively reduces the amount of iron in the organism. The treatment helps to prevent irreversible organ damage and improves the prognosis and survival rate of patients [15].

Data from literature on thyroid disorders in patients with HH are inconsistent and ambiguous. In a publication from 1983, Edwards et al. showed a higher incidence rate of primary hypothyroidism in patients with HH — the disorder was diagnosed in 8.8% of male patients with HH [16]. However, more recent studies, published by Murphy in 2004, did not confirm that thesis [17]. International guidelines for medical practitioners developed by the European Association for the Study of the Liver (EASL) include recommendations to monitor both thyroid function and testosterone levels (in men) for patients diagnosed with HH [18]. So far, no research has been done to determine the relationship between excessive iron accumulation and greater thyroid volume, which could lead to the formation of goitres or focal lesions in this organ. It is also important to remember about the role of iron in the synthesis of thyroid hormones (TH). The presence of iron seems crucial for the proper functioning of thyroid peroxidase (TPO), because excess iron may stimulate TPO to synthesise TH. On the other hand, iron deposits in the pituitary gland may reduce the concentration of endogenic thyroid-stimulating hormone (TSH).

Thanks to the accessibility of genetic tests, it is currently possible to diagnose increasingly young patients, who have not yet developed signs and symptoms of serious organ pathology, including cirrhosis or heart failure. The assessment of pathological changes within the glandular tissue of these patients also have great significance in terms of their qualification for therapeutic phlebotomy. The aim of this study was to characterise thyroid function and ultrasound images in patients with clinically overt hereditary haemochromatosis.

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Material and methods

The aim of the present single-centre study was to show whether Polish patients diagnosed with, and treated for, primary HH (hereditary haemochromatosis) have a higher risk of thyroid disorders. The eligibility criteria included a C282Y/C282Y, H63D/C282Y, and H63D/H63D mutation in the *HFE* gene (confirmed by a PCR-based test), abnormal iron laboratory parameters in blood (elevated ferritin level of > 200 ng/mL in female patients and > 300 ng/mL in male patients and transferrin saturation > 45%), and excessive iron deposition in the liver (confirmed by either a core needle biopsy of the liver and staining with Prussian blue for presence of iron deposits in hepatocytes assessed at least at grade 2 or by abdominal MRI in which T2-weighted images revealed excessive iron accumulation in the liver*). Participants were informed about the aim of the study, and all of them gave written consent. The study was approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk (NKBBN/177/2016). The study included 40 participants (12 female patients and 28 male patients) aged from 21 to 73 years (the mean age of patients being 50.35 years), who were diagnosed with hereditary HH. The control group included healthy volunteers, matched for gender and age with the study group, who showed no abnormalities in iron laboratory parameters and no previous history of thyroid disorders. Table 1 presents the eligibility criteria for the study.

The homozygous C282Y/C282Y mutation was confirmed in 29 patients, whereas the H63D/H63D mutation — in 2 patients. Nine patients tested positive for the heterozygous C282Y/H63D mutation (Fig. 1). The excessive accumulation of iron was confirmed in all cases: 26 patients underwent liver biopsy: iron deposits in hepatocytes were assessed as grade 2 or grade 3–4 according to the Scheuer scale (as previously described) [19]. Nineteen specimens had an iron grade of G3/G4, and 7 specimens had an iron grade of G2. The remaining 14 patients underwent MRI of the liver, which confirmed in all cases the presence of iron deposits in this organ, which met the inclusion criteria. All patients were undergoing therapeutic phlebotomy and continued to be followed up in outpatient hepatology clinics. The assessment of patients included iron laboratory parameters in serum, measured at the qualification for the therapeutic phlebotomy and outpatient follow-ups, and the aminotransferase activity in serum, used at the stage of early diagnosis as an indicator of liver damage due to HH.

The distribution of mutations in patients with hereditary haemochromatosis (HH) is shown in Figure 1.

To assess the thyroid morphology and function, the ultrasound examination of the thyroid gland was performed and serum TSH concentrations were measured. The ultrasound examination was performed using a Logiq S7 expert device, and the parenchymal blood flow was visualized by means of colour and power Doppler.

Table 1. *Eligibility criteria for the study included the following: confirmed mutation in the HFE gene and fulfilment of one laboratory criterium and one iron deposition criterium*

Abnormal laboratory results	Confirmed iron deposition	Confirmed mutation in the *HFE* gene
Serum iron concentration > 150 ng/mL	Liver biopsy report, with the inflammatory grade of G2 at least	C282Y/C282Y
or	or	or
Serum ferritin concentration > 200 ng/mL in women or > 300 ng/mL in men	Excessive accumulation of iron on MRI in T2 sequence	H63D/C282Y
or		or
Transferrin saturation > 45%		H63D/H63D

MRI — magnetic resonance imaging

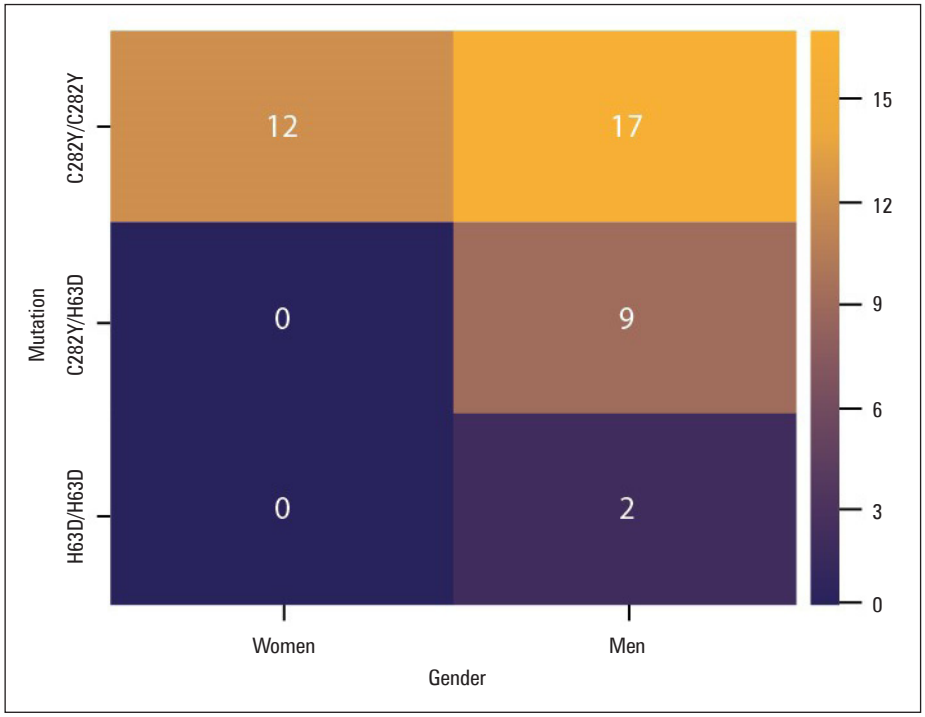

Figure 1. *Distribution of mutations in patients with hereditary haemochromatosis (HH)*

To prevent any errors in measurements, each ultrasound examination was performed by the same medical practitioner, using the same apparatus. The following parameters were evaluated during the examination: thyroid volume in millilitres, echogenicity of the thyroid parenchyma, vascularisation, and the number of focal lesions. The following characteristic features of autoimmune thyroid disease (AITD) were determined: abnormally low echogenicity of the thyroid parenchyma, presence of hyperechogenic bands indicative of fibrosis, areas of lower echogenicity, and abnormal blood flow. To evaluate the thyroid function in all study participants, serum TSH concentrations were measured by means of immunofluorescence. The patients were also asked to fill in a questionnaire, which gathered information about the time from the diagnosis of haemochromatosis and the frequency of bloodlettings. Moreover, the medical history was obtained with the focus on the following disorders of the endocrine system: hypothyroidism and hyperthyroidism, pituitary gland disorders, infertility, hypogonadism, and diabetes.

The characteristics of the study group: patient age, treatment data, and laboratory test results are presented in Table 2. Among 40 patients diagnosed with HH, six (15%) were found to have clinical symptoms of cirrhosis with nodular transformation of the liver and portal hypertension. Histopathological study performed on 26 patients who underwent liver biopsy revealed a fibrosis grade of G0 in eight patients (30.76%), G1 in 13 patients (50%), G2 in three patients (11.53%), and G3/G4 in two patients (7.69%). Twenty-three patients had endocrine disorders that had been diagnosed before the study enrolment, which are summarised in Table 3.

The control group included 20 patients (10 women and 10 men) with no previous history of thyroid disorder or family history of thyroid cancer. The mean age was 48.10 years (SD = 14.73). There was no statistically significant difference between the study group and the control group in this respect ($p = 0.05$, test U).

The results were statistically analysed using Python (version 3.7.4). Statistical tests were performed using SciPy (version 1.2.1), and the diagrams were created by means of Seaborn (version 0.9.0). Because the assumption of normal data distribution was not fulfilled, the groups were compared using nonparametric Mann-Whitney U test and, in the case of qualitative data, the Fisher's exact test. For the correlation between quantitative features, the Pearson's linear correlation coefficient was determined. The significance level was set at 0.05.

Table 2. *Study group — overview*

	Mean (SD)	Me	Min–max
Age at study enrolment [years]	50.35 (14.59)	51.5	21–73
Age at diagnosis	43.27 (12.75)	42.5	21–70
Number of phlebotomies	25.53 (42.49)	14.5	1–205
Maximum ferritin concentration [ng/mL]	918.40 (799.03)	689.0	126–3550
Mean ferritin concentration [ng/mL]	554.37 (509.06)	347.5	60–2183
ALT (0–41 U/l)	44.62 (27.27)	38.0	12–117
AST (0–40 U/l)	32.25 (15.28)	30.0	8–77
Transferrin saturation (20–40%)	79.25 (17.47)	86.5	30–100

ALT — alanine aminotransferase; AST — asparagine aminotransferase; SD — standard deviation; Me — median; min — minimum value; max — maximum value

Table 3. *Endocrine disorders diagnosed in the study group*

Number of patients (%)	
Hypothyroidism	4 (10)
Hyperthyroidism	1 (2.5)
Diabetes or pre-diabetes stage	14 (35)
Positive antithyroid antibody test results	3 (7.5)
Goitre	2 (5)
Status post strumectomy due to a goitre	1 (2.5)

Table 4. *Group comparison*

	Control (n = 20)	Study (n = 40)
TSH (0.35–4.94 uU/mL)	p = 0.239[a]	
Mean (SD)	1.10 (0.54)	1.37 (0.87)
Me	0.94	1.13
Min–max	0.48–2.33	0.44–4.58
Thyroid volume [mL]	p = 0.358[a]	
mean (SD)	12.81 (5.10)	16.37 (11.92)
Me	11.25	13.85
min–max	7.70–25.00	5.00–48.00
AITD	p = 0.304[b]	
n (%)	2 (10)	10 (25)
Thyroid focal lesions	p = 0.024[b]	
n (%)	9 (45)	9 (22.5)

AITD — autoimmune thyroid disease; TSH — thyroid-stimulating hormone; n — number; SD — standard deviation; Me — median; min — minimum value; max — maximum value; [a]Mann-Whitney U test; [b]Fisher's exact test

Results

Ultrasound examination of the thyroid gland in patients with haemochromatosis revealed the following: signs of AITD in nine patients (22.5%), thyroid focal lesions in seven patients (17.5%), and simple goitres in two patients (5%). One patient after strumectomy presented signs of AITD in the remaining thyroid parenchyma. In 22 patients (55%) the ultrasound findings were normal. In comparison, in the control group of 20 patients, ultrasound findings were normal in nine patients (45%), focal lesions were found in nine patients (45%), and signs of AITD were identified in two patients (10%). Thyroid volumes in the examined and control group did not differ significantly. Three patients with HH (7.5%) had elevated levels of antithyroid antibodies, as found in their medical records. None of the patients, however, required substitution with L-thyroxine (Tab. 4, 5).

In tests evaluating the thyroid function, the mean TSH concentration was 1.39 IU/mL (SD = 0.87) for the study group. Hypothyroidism requiring L-thyroxin supplementation was found in five patients (four patients [10%] due to AITD, one patient [2.5%] after strumectomy). In one patient, laboratory findings revealed hyperthyroidism due to a nodular goitre. One patient had a diagnosis of Graves' disease (currently in remission).

In the control group, the mean thyroid volume was 16.35 mL (SD = 8.90), and the mean TSH concentration was 1.10 IU/mL (SD = 0.54). No statistically significant differences comparing to HH patients were found for these two parameters. The ultrasound examination revealed nodular goitres in nine patients (45%) and signs of AITD in two patients (10%). In the remaining nine patients (45%), the ultrasound findings were normal. More specific gender-disaggregated data can be found in Table 5.

In the group of patients with severe deposition of iron in the liver, significant differences in TSH concentrations were found. In the group with iron grade of G3 or G4, there was a statistically lower TSH concentration (Fig. 2).

Discussion

The study attempted to analyse the thyroid dysfunctions in patients diagnosed with hereditary haemochromatosis. Due to the capacity of iron to deposit

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Table 5. *Comparison of the study group and the control group, based on selected parameters*

	Women		Men	
	Control (n = 10)	**Study (n = 12)**	**Control (n = 10)**	**Study (n = 28)**
TSH (0.35–4.94 uU/mL)	p = 0.346[a]		p = 0.264[a]	
Mean (SD)	0.94 (0.35)	1.01 (0.37)	1.27 (0.66)	1.52 (0.98)
Me	0.78	0.95	0.97	1.29
Min–max	0.48–1.56	0.44–1.80	0.50–2.33	0.45–4.58
Thyroid volume [mL]	p = 0.358[a]		p = 0.434[a]	
Mean (SD)	12.81 (5.10)	16.37 (11.92)	19.90 (10.65)	17.68 (5.34)
Me	11.25	13.85	18.65	18.00
Min–max	7.70–25.00	5.00–48.00	10.00–46.00	7.00–27.00
AITD	p = 0.015[b]	p = 0.304[b c]	p = 0.622[b]	
n (%)	0 (0.00)	6 (50.00)	2 (20.00)	4 (14.30)
Focal lesions	p = 0.192[b]	p = 0.024[b c]	p = 0.152[b]	
n (%)	6 (60.00)	3 (25.00)	3 (10.00)	3 (10.00)

TSH — thyroid-stimulating hormone; n — number; SD — standard deviation; Me — median; min — minimum value; max — maximum value; [a]Mann-Whitney U test; [b]Fisher's exact test; [c]comparison without gender disaggregation

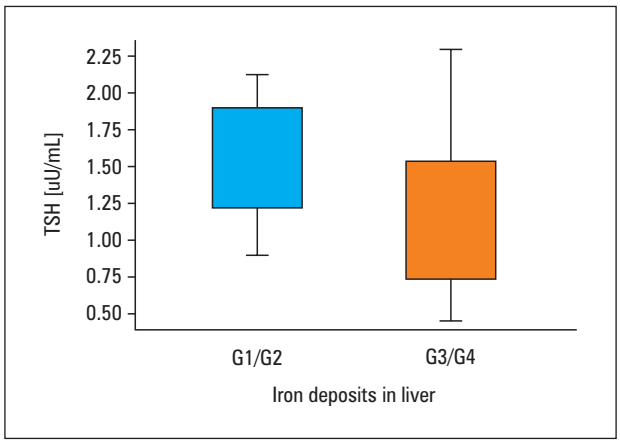

Figure 2. *In the group with severe iron deposition confirmed by liver biopsy (the iron deposits of G3 or G4), there was a statistically significant lower TSH concentration (p value = 0.005, test u)*

in the glandular tissue, including the thyroid gland, a hypothesis was formed that iron overload may be significant for the pathophysiology of thyroid disorders.

Thyroid diseases are a serious problem in Poland. According to the data of the Main Statistical Office, in 2006, 22% of the Polish population suffered from thyroid diseases, including 2% from hypothyroidism [20]. Based on thyroid ultrasound screening tests, the frequency of focal lesions in the thyroid gland is estimated at 19–67% [21]. The latest screening study in the Polish population — the Thyroid Disease Prevention Program implemented in the years 2006–2011 — allowed the diagnosis of thyroid focal lesions in 44.1%, and the frequency is very similar in our small control group [22].

In the study of the group of patients with HH, focal lesions were significantly less common than in the control group (22.5% vs. 45%) and in the general population [22], which is associated with a lower risk of neoplastic lesions. A study from 2018 found that iron deficiency impairs the synthesis of thyroxine (T4) and its conversion to triiodothyronine (T3) [23]. A cross-sectional study of schoolchildren in Iran published in 2002 showed that iron deficiency is associated with a high prevalence of goitre [24]. Our study put forward the hypothesis that in conditions of extremely high iron content in body, the opposite phenomenon may occur. This was reflected in the present study, in which the rate of thyroid focal lesions in patients with HH was significantly lower than in the control group.

It is also worth emphasising the role of iron in the production of peripheral thyroid hormones: iron is a microelement that has a potential influence on thyroid hormone deficiency. In the first two steps of thyroid hormone synthesis, thyroperoxidase, which is iron-dependent, acts as a catalyst [25]. Studies on human beings and animals showed that iron deficiency in the blood translates into a lower concentration of thyroid hormones, hinders the conversion of T4 to T3, and stimulates the pituitary gland to release thyrotropin. Adult patients with iron deficiency have lower concentrations of T4 and T3 and higher TSH levels when compared to the control group [26]. In the population at risk of iodine deficiency, iodine supplementation is much more effective with sufficient iron intake, which also has a protective effect against the formation of thyroid goitres in the group of patients who receive such supplementation [27].

A new study published by Polish authors in 2019 in Scientific Reports showed significant differences in hepcidin levels in patients with newly diagnosed Hashimoto's disease and after hormonal treatment. Along with administration of L-thyroxine, there was a significant decrease in serum hepcidin concentration, which proves that the activity of this protein, which is significantly involved in the pathophysiology of HH, changes in the case of thyroid dysfunction [28].

The present study did not reveal any statistically significant differences in TSH concentrations between patients with HH and the general population. The findings are consistent with the results of the HEIRS study, which showed that patients with a homozygous C282Y mutation are not at a higher risk of thyroid diseases than the general population. It should be noted, however, that the HEIRS study focused only on the genotype characteristic of HH and did not investigate the concentration of iron in internal organs. Patients with the C282Y mutation constitute from 10% to 30% of HH cases, and therefore participants of the present study had to fulfil laboratory, histopathological, or imaging criteria in addition to developing a haemochromatosis phenotype. This made it possible to analyse not only the mutation in the HFE gene, but also the influence of iron concentration on the thyroid morphology. Taking into account the deposition of iron in internal organs, it was shown that patients with a higher level of iron in the organism (numerous iron deposits confirmed by a biopsy and high transferrin saturation) have lower TSH concentrations. This could be related either to a more effective production of peripheral hormones, which lowers the TSH level, or to iron deposits in the pituitary gland, which lead to the destruction of thyrotropin cells and impair their releasing ability. A lower TSH concentration was also noted in patients with more severe iron deposition. The mechanism responsible for this phenomenon is unclear and requires further analysis.The role of iron in carcinogenesis, especially hepatocellular, is well known, but when it comes to the thyroid gland this issue can look very different. In the latest publication from 2020, it was demonstrated that silencing of transferrin-1 receptor (TfR-1) plays a role in inhibiting carcinogenesis pathways for follicular and anaplastic thyroid cancer. Under the conditions of high serum iron concentration, TfR-1 downregulation occurs due to IRP-IRE-related mechanisms (iron regulatory protein — iron responsive element), and carcinogenesis pathways are inhibited [29].

Autoimmune thyroid disease affects all age groups, and the incidence rate varies from 0.3 to 1.5%, which is probably underestimated – in pathology studies the disease is diagnosed in 14–17% of patients. The key role in diagnosing the disease is played by the following: 1 — TSH test, 2 — measurement of antithyroid antibodies (antithyroglobulin antibodies (aTg), anti-thyroid peroxidase antibodies (aTPO), and thyroid-stimulating hormone receptor antibodies (TRAb), and 3 — thyroid ultrasound. In the group of patients with HH, signs of AITD were more commonly detected during an ultrasound examination than in the control group (they occurred in 22.5% of all patients). However, given the small population sample, no statistical significance was found.

In a study from 1984 [16], 49 C282Y-homozygous patients were evaluated for thyroid disorders — the thyroxine and thyrotropin serum concentrations were also measured. It was shown that 3 out of 34 male participants (8.7%) had hypothyroidism. The patients were found to have elevated levels of antithyroid antibodies in blood tests and lymphocytic infiltrations in histopathological samples of the thyroid gland. The authors justified the greater rate of AITDs with the stimulating influence of iron on the autoimmune response in the glandular tissue. What is interesting, none of the 15 female patients in the study was diagnosed with any thyroid disorder. According to the authors, this may be related to a more intense iron overload in men diagnosed with HH than in women. However, due to the small population sample, no statistical significance was shown. In our HH group also we did not diagnose women with AITD. It would be necessary to conduct a further study, in which a larger sample of patients with HH would undergo thyroid function assessment and immune status evaluation.

A study by Murphy et al., published in 2004, questioned the correlation between thyroid disorders and HH. In the study, which involved a group of 154 patients with HH, haemochromatosis was confirmed by genetic tests, increased ferritin concentrations in the blood, and the presence of iron in liver samples. In the study, the patients were also treated with therapeutic phlebotomy. As part of the study, serum TSH concentrations as well as fT4 concentrations and the level of antithyroid antibodies were all assessed. Five patients were found to have an impaired thyroid function, and only one patient developed fully-blown AITD that required L-thyroxine supplementation. In two cases, the level of antithyroid antibodies was elevated. Based on the findings, the authors suggested that thyroid disorders are not so common in patients with HH. Thus, our study seems to shed more light on the links between iron overload and thyroid disorders by the use of ultrasound imaging of this gland.

Conclusions

The present study did not show any significant differences in the thyroid volume and TSH concentrations between patients with hereditary haemochromatosis

and the general population. However, patients with HH were less frequently diagnosed with thyroid focal lesions in USG. Nevertheless, the mechanisms behind this phenomenon remain unclear.

The role of iron in the pathogenesis of AITD and its connection with gender is yet to be examined. An abnormal ultrasound image of the thyroid gland, which could be responsible for AITD, was more common in the study group, but no significant difference was revealed. Therefore, it is not known whether this abnormality was caused by iron deposits in the organ. It would be necessary to conduct a further study, in which a larger sample of patients with HH would undergo thyroid function assessment, immune status evaluation, and imaging tests by means of ultrasound and MRI of the thyroid gland — an alternative method for diagnosing iron deposition in the thyroid gland.

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An assessment of the effectiveness of regional analgesia after VATS measured by an objective method for assessing testosterone, cortisol, α-amylase, sIgA, and β-endorphin levels — a randomised controlled trial

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Abstract

Introduction: Thoracic surgeries are associated with intense postoperative pain. General opioid analgesia is still the main anaesthetic method. Due to the large number of opioid-induced side effects, alternative methods of pain relief are sought. One of them is the use of balanced analgesia, which consists of regional analgesia, non-opioid painkillers, and small doses of opioids.

Material and methods: The objective of this study was to assess the effectiveness of preoperative thoracic paravertebral block (ThPVB) in the treatment of postoperative pain after video-assisted thoracic surgery (VATS) by measuring hormone levels in blood serum or saliva. It was a randomised, open-label study conducted in a single university hospital setting between May 2018 and September 2019. In total, 119 patients were scheduled for elective video-assisted thoracic surgery. Performed interventions included: preoperative thoracic paravertebral block with 0.5% bupivacaine, followed by postoperative oxycodone combined with nonopioid analgesics. Follow-up period comprised first 24 hours and one, two, and six months after surgery. Main outcomes were measured by pain intensity assessed using the Numerical Rating Scale (NRS) and the levels of the following hormones: testosterone, cortisol, α-amylase activity, sIgA, and β-endorphin.

Results: A total of 119 patients were randomised into two groups and, of these, 49 were subsequently excluded from the analysis. The final analysis included 37 patients from the study group and 33 from the control group. There were no statistically significant differences in the analysed parameters the relative change T1–T0. There was a tendency towards statistical significance in the relative change T2–T0 in testosterone levels. At rest, no statistically significant differences were found between groups and time in the percentage of patients with NRS ≥ 1. During cough, the percentage of patients with NRS ≥ 1 was higher at T1 and T2 time points in the ThPVB group. Of the factors considered, only α-amylase levels statistically significantly increased the chance for higher NRS score after a month [OR = 1.013; 95% PU: 1.001–1.025; $p < 0.01$].

Conclusions: ThPVB is effective and safe for patients undergoing VATS. It can be an effective alternative for general anaesthesia using high doses of opioids. **(Endokrynol Pol 2021; 72 (2): 133–142)**

Key words: *NRS; video-assisted thoracic surgery; acute pain; regional anaesthesia; testosterone; cortisol; α-amylase; sIgA; β-endorphin*

Introduction

Thoracic surgeries are usually associated with intense postoperative pain. Intraoperative tissue damage occurs mainly in the periosteum and pleura, which are very richly innervated with rami from the intercostal nerves, diaphragmatic nerve, and sympathetic trunk [1].

If not treated properly, postoperative pain may cause many complications. Sympathetic and neuroendocrine activation occurs. It has a deep negative influence on cardiovascular and respiratory systems. Neuroplastic changes in the central nervous system may cause chronic postoperative hyperalgesia [2]. Thoracic procedures are among those that frequently cause chronic pain syndromes. Persistent acute postoperative pain [3] not treated properly is the main risk factor. Adequate analgesia is a very important part of the therapeutic process and has significant influence on its outcome.

Video-assisted thoracoscopic surgery (VATS) is among the most common thoracic surgical procedures. VATS can be diagnostic or therapeutic. It is less invasive than classic thoracotomy and is associated with fewer complications [4] and lower-grade postoperative pain. Nevertheless, adequate analgesia may sometimes be challenging. Lately, oxycodone is one of the most frequently used opiates. It is pure agonist of MOR, DOR, and KOR opiate receptors. It has a very strong therapeutic effect and is safe (especially administered in patient-controlled analgesia — PCA method). In most of the cases, it is advisable to combine regional anaes-

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thesia (RA) techniques with opiates and nonsteroidal anti-inflammatory drugs (NSAIDs). Thoracic paravertebral block (TPVB) is one of the most popular and well described RA techniques. Thoracic paravertebral block requires administration of a local anaesthetic solution into the paravertebral space on the thoracic level. It is possible to anaesthetise the roots and rami of spinal nerves, pre- and postganglionic fibres, sympathetic nervous system, and proximal intercostal nerves. The block is unilateral — sensory, motor, and sympathetic. Effective TPVB with intravenous (*i.v.*) opiates and/or NSAIDs is state-of-the-art multimodal analgesia [5, 6], which minimises perioperative stress level in the patient.

It is possible to measure stress reaction, and therefore the quality of perioperative analgesia. One of the methods is to assess plasma and saliva levels of various endogenous substances that can be used as a measure of endogenous adrenergic activity. These include β-endorphin, secretory immunoglobulin A (SIgA), cortisol, testosterone, salivary α-amylase, and many more. Beta-endorphins are produced in the anterior pituitary gland. The substrate is proopiomelanocortin. Proopiomelanocortin is synthesised in response to corticoliberin, which is released from the hypothalamus as a reaction to stress or pain [7]. Beta-endorphins have an analgesic effect by binding to opioid receptors, mostly the mu-opioid receptors (MORs). This inhibits the release of tachykinins, mainly the P substance, and therefore blocks the conduction of pain stimuli. In the central nervous system, binding of β-endorphins to MORs causes release of gamma-aminobutyric acid (GABA), which is a well-known inhibitory neurotransmitter [8]. Secretory immunoglobulin A (SIgA) is an antibody that can be found on the surface of mucous and serous membranes. Because its levels drop during stress, it can be used to assess the body's response to pain [9]. Cortisol is a natural steroid hormone, often referred to as the stress hormone. It is the final product of stimulation of the hypothalamic–pituitary–adrenal axis (the HPA axis). The stimulation occurs in response to stress. Cortisol has many effects on metabolism [10]. The next substance is salivary α-amylase. It is an enzyme produced by salivary gland cells, and its levels are well correlated with the activity of the sympathetic nervous system — they increase as a part of stress response. Studies have shown that it is a reliable marker of the sympathetic nervous system response to stress stimuli [10]. Testosterone is the primary male steroid sex hormone belonging to the androgen hormone group. Endorphins secreted during pain or stress reduce testosterone levels by inhibiting the synthesis of gonadotropic hormone (GnRH), as well as by inhibiting the production of testosterone by Leydig interstitial cells in the testes [11]. Therefore, testosterone plasma levels are lower during stress and pain, chronic in particular [12].

The primary endpoint was evaluation measuring plasma or saliva levels of described hormones as well as measuring pain on the NRS scale in the perioperative period depending on the analgesia used after VATS.

Material and methods

This randomised, observational study was conducted in the Medical University of Silesia, Poland. With the approval of the Institutional Review Board (No. KNW/0022/KB1/138/I/17/18 of 13.03.2018) and after obtaining written informed consents, we enrolled 119 patients scheduled for elective VATS between May 2018 and September 2019. The study was registered on ClinicalTrials.gov under No. NCT04414488. All patients were aged between 18 and 75 years, had a body mass index between 19–30 kg/m^2, and had American Society of Anesthesiology (ASA) physical status between I and III. Lack of consent, significant coagulopathy, contraindication to ThPVB or drugs used in the protocol, history of chronic pain, chest wall neoplastic invasion, previous thoracic spine surgery, mental state preventing effective use of PCA device, and renal failure (GFR < 60 mL/min/1.73 m^2) were exclusion criteria.

Protocol

Patients were randomly assigned to one of two groups receiving different postoperative analgesic regimens:

— patient-controlled analgesia with oxycodone (control group);
— thoracic paravertebral block plus patient-controlled analgesia with oxycodone (ThPVB group).

Randomisation without stratification was based on computer-generated codes, which were kept in sequentially numbered opaque envelopes.

All patients were premedicated with oral midazolam at an adequate dose.

Fentanyl (FENTANYL WZF, Polfa Warszawa S.A., Poland) was used for surgical analgesia in both groups. Fentanyl at 1.5 μgkg^{-1}, followed by fractional doses of 1 to 3 μgkg^{-1} if heart rate (HR) or mean blood pressure rose more than 20% above the base-line value obtained just before surgery onset, was used for induction of anaesthesia.

In the ThPVB group, a single-shot ThPVB was performed at the Th3 to Th4 level, approximately 2.5 to 3 cm lateral to the tip of the spinous process before the induction of general anaesthesia. A pre-block ultrasound examination was performed to assess the depth of the transverse process and the pleura. An insulated 10-cm-long needle was used, and this was connected to a peripheral nerve stimulator with an initial set current of 2.5 mA. The current was gradually reduced as the needle was inserted until the appearance of visible intercostal muscle activity with a current of 0.3 to 0.5mA (paravertebral space identification). Plain bupivacaine (0.3 mL kg^{-1}) was then injected after a negative aspiration test for air or blood. The efficacy of the blockade to cold was checked after 20 min with a plastic ampoule of saline stored in the freezer. Testing was symmetrical on both sides of the thorax. A difference in the sensation to cold between the blocked and unblocked sides was checked to confirm an effective block.

General anaesthesia was induced in both groups with midazolam 0.1 mgkg^{-1}, propofol 2 mgkg^{-1}, and cisatracurium 0.15 mgkg^{-1}. Patients were intubated using a left-sided double lumen tube of an adequate size. Patients were then arranged in a lateral position. Anaesthesia was maintained with one minimal alveolar concentration (MAC1) sevoflurane. Patients awoke from anaesthesia in the post-anaesthesia care unit (PACU), where extubation was performed by an anaesthetist after administration of incremental doses of atropine and neostigmine, as required.

The postoperative pain management regimen was identical in both groups. Patients complaining of postoperative pain were given *i.v.* oxycodone by an anaesthetist before commencing the patient-controlled analgesia (PCA). This dose was titrated to achieve adequate analgesia or until side effects occurred. Each patient then commenced PCA. The PCA solution was oxycodone (1 mgmL^{-1}) and the PCA was programmed to allow a self-administered bolus dose of 1 mg oxycodone with a lockout time of 5 min. During the night, the basal rate oxycodone was 2–4 mg per hour. Additionally, patients were given 1 g intravenous paracetamol every 6 hours and 100 mg of intravenous ketoprofen every 12 hours, if needed.

Measurements

Demographic parameters such as age, sex, height, weight, BMI, as well as heart rate and blood pressure were recorded before surgery. After qualification for the study, blood and saliva samples were taken from each patient to determine the level of hormones: testosterone, cortisol, a-amylase activity, sIgA, and b-endorphin (T0). During anaesthesia, all patients were monitored: electrocardiography (3-lead), heart rate (HR; 1/bpm), non-invasive blood pressure (NIBP; mm Hg), end-expiratory carbon dioxide (EtCO2: mmHg) and sevoflurane (EtSev), and arterial blood saturation measured by pulse oximetry.

In the immediate postoperative period and the first 24 hours after surgery, the following data were recorded: heart rate (HR; 1/bpm) and arterial blood saturation measured by continuous pulse oximetry. Non-invasive blood pressure measurements and NRS pain levels were recorded every 6 hours. Six hours (T1) and 24 hours (T2) after surgery, each patient had their blood and saliva re-tested for hormone levels. Additionally, one, three, and six months after surgery, a telephone follow-up was performed and pain levels were determined on the NRS scale.

Obtaining material for biochemical assays

Saliva was collected from participants in order to perform laboratory tests, using a special disposable Salivette tube (Sarstedt AG & Co., Germany). Saliva was collected by placing a sterile tampon under the tongue or chewing it for 30–45 seconds. The soaked saliva pad was then placed in a suspended insert with a perforated bottom. The insert with the tampon was placed in a centrifuge tube and closed with a stopper. Next, the tube was centrifuged (1000 × g for 10 min) to obtain a ready-to-test saliva supernatant. Approximately 0.7 mL of the supernatant from every sample collected was used for further testing. Samples were frozen after centrifugation at –85°C until performing laboratory tests.

At the same time, blood was collected for laboratory tests from the ulnar vein. Blood for testing was collected using disposable equipment in a volume of 5 mL into a tube containing EDTA and aprotinin. Next, the tube was centrifuged (1000 × g for 5 min). After centrifugation and separation of morphotic elements, the obtained plasma was divided into two tubes and frozen at –85°C until laboratory tests were performed.

Biochemical analysis

Determination of alpha-amylase activity

Alpha-amylase activity assay was performed by a static method with an AMYLAZA kit (Aqua-Med Łodz, Poland). The samples were diluted 100 times using 0.9% chloride solution. This method uses 2-chloro-4-nitrofenylo-maltotrioside as a substrate. The reaction was performed in pH 6.0 MES buffer at 37°C, yielding a coloured reaction product. The product was then analysed via spectrophotometry at 405 nm. Results are expressed in salivary a-amylase activity units (U/mL). Measurement imprecision of the method was 4.1%.

Determination of cortisol and testosterone levels

Commercial ELISA (Diapra, Italy) was used to determine the levels of cortisol and testosterone. The analytical procedure was in accordance with the manufacturer's instructions provided in the technical manuals supplied with the kits. Absorbance readings were taken using a μQuant reader (Biotek, USA), while results were processed using KCJunior (Biotek, USA). The sensitivity of the method was 0.12 ng/mL for cortisol and 3.28 pg/mL for testosterone. The method's imprecision was 6.2% and 7.9%, respectively.

Determination of sIgA concentration

Commercial ELISA kits (Immunodiagnostic AG, Germany) were used to determine the levels of sIgA. The analytical procedure was in accordance with the manufacturer's instructions provided in the technical manuals supplied with the kits. Absorbance readings were taken using a μQuant reader (Biotek, USA), while results were processed using KCJunior (Biotek, USA).

Determination of β-endorphin levels

Determination of b-endorphin levels was preceded by extraction on C18 Sep-Pak columns containing 50 mg C18, using trifluoroacetic acid (TFA) and elution buffer (i.e. 60% acetonitrile, 1% TFA, and 39% distilled water). The extracts obtained were lyophilised. To determine the levels of b-endorphins in the tested samples, lyophilisates were dissolved in an appropriate amount of buffer, and then commercial ELISA tests from Elabscience (USA) were used. The analytical procedure was in accordance with the manufacturer's instructions provided in the technical manuals supplied with the kits. Absorbance readings were taken using a μQuant reader (Biotek, USA), while results were processed using KCJunior (Biotek, USA).

Statistical analysis

Data on interval scale with a normal distribution were presented as mean ± standard deviation (SD), while data with a distribution deviating from the normal distribution were presented as the median and lower and upper quartiles. The normality of the distribution was assessed with the Shapiro-Wilk test and the quantile plot. Qualitative data are presented as numbers and percentages. In order to compare the variables on the nominal and ordinal scales, the χ^2 test was used. Comparison of two independent groups was carried out using Student's t-test for data with a distribution similar to normal or the U Mann-Whitney test in other cases. Data on the NRS scale were compared using the χ^2 test and the McNemara test (change in observation between T2 and T1 time). In the case of data analysis on a point scale, the Wilcoxon pairwise test was used. Time analysis of biochemical parameters was performed using mixed model analysis with contrast analysis with Benjamini-Hochberg correction for multiple comparisons. In the case of data deviating from the normal distribution, rank analysis of mixed models was used. Factors influencing the values of the NRS scale ≥ 1 were determined on the basis of univariate and multivariate logistic regression. The parameters were considered statistically significant when $p < 0.05$. The calculations were made using the following programmes: Statistica 13.0 (TIBCO Inc., Palo Alto, CA, U.S.) PL version, Excel of the MS Office suite, and the R (CRAN) environment.

Results

During the study period, 119 patients underwent VATS and were screened for this study. In total, 110 patients met the inclusion and exclusion criteria and were randomly assigned to the two study groups, with 55 patients in each group. Overall, 25 patients were excluded after randomisation: 13 patients from the ThPVB group (seven had conversion to thoracotomy; six had ineffective ThPVB) and 12 patients

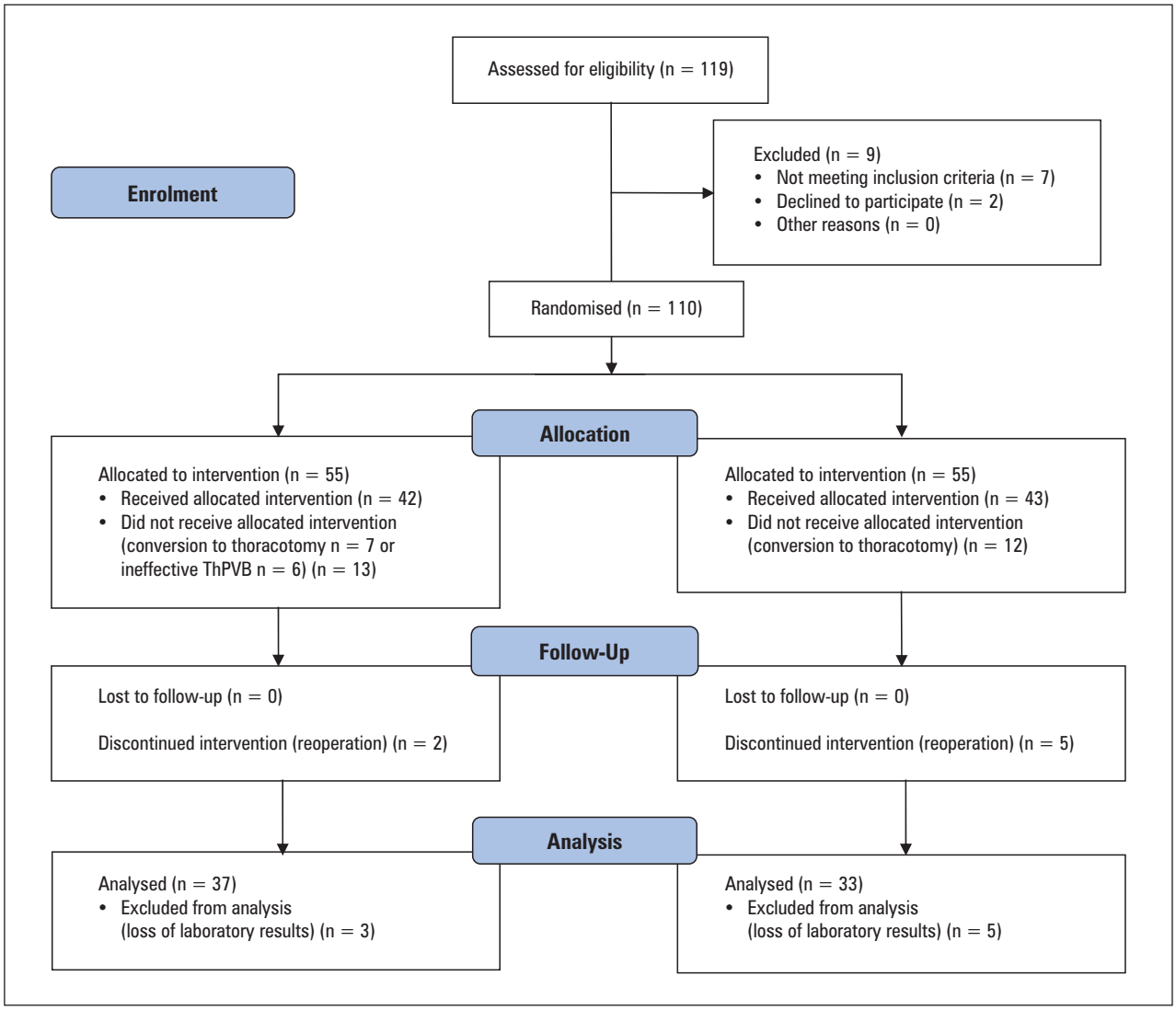

Figure 1. *Consort flow diagram*

from the control group (conversion to thoracotomy) (Fig. 1). Finally, 70 patients (33 men and 37 women) aged 62 ± 14 years and with a BMI of 27.4 ± 4.5 kg/m^2 completed the study. There were no significant differences between groups in gender, age, height, BMI, or ASA physical status. Moreover, no differences were found for surgery time and the operated site. Only lobectomy was statistically more frequent in the ThPVB group. The demographics and main clinical findings are presented in Table 1.

There were no statistically significant differences in the parameters analysed in the relative change T1–T0 (Tab. 2). There was a tendency towards statistical significance in the relative change T2–T0 in testosterone levels, with higher values observed in the ThPVB group (Tab. 3).

At rest, no statistically significant differences were found between the groups and time points in the percentage of patients with NRS ≥ 1 point. During cough, the percentage of patients with NRS ≥ 1 was higher at T1 and T2 in the ThPVB group. Simultaneously, a statistically significant reduction in the percentage of these patients over time was also observed in the ThPVB group. There were no significant differences in NRS scores during cough between the groups and, similarly, a significant reduction in NRS at T2 was observed in the ThPVB group. No significant differences were found between the groups in the percentage of people with NRS ≥ 1 during a follow-up after one, two, and six months (Tab. 4).

The analysis of β-endorphin, cortisol, sIgA, and α-amylase levels showed a statistically significant effect of time rather than the group on the levels of measured hormones. At the T2 time point, statistically significantly higher cortisol levels were found in the ThPVB group. A summary of descriptive statistics and ANOVA analysis, as well as the levels of measured hormones, are presented in Table 5 and 6.

Table 1. *Patient demographic characteristics*

Variables	ThPVB group (n = 37) (52.9%)	Control group (n = 33) (47.1%)	p
Female/Male N (%)	21/16 (56.8/43.2)	16/17 (48.5/51.5)	0.49
Lobectomy N (%)	25 (67.6)	12 (36.45)	< 0.05
Wedge resection N (%)	8 (21.6)	9 (27.3)	
Other N (%)	4 (10.8)	12 (36.45)	
Operated side R/L N (%)	18/19 (48.6/51.4)	23/10 (69.7/30.3)	0.07
Surgery time [min]	115.0 ± 45.8	103.6 ± 58.0	0.36
Age [yrs]	64 ± 11	61 ± 17	0.48
Weight [kg]	75.2 ± 12.0	79.3 ± 15.8	0.23
BMI [kg/m^2]	27.1 ± 4.3	28.0 ± 5.0	0.44
Height [m]	1.63±0.07	1.65±0.08	0.13
ASA class [I/II/III] (N)	2/22/13	2/19/12	0.49

Data are n, mean ± SD or %. ASA — American Society of Anaesthesiologists; L — left; R — right

Table 2. *Comparison of relative changes in the analysed parameters between T1 and T0 in both groups*

Variables	Δ relative (%) T_1–T_0		p
	ThPVB group	Control group	
β-endorphin	131.7 ± 103.1	104.6 ± 88.34	0.24
Cortisol	63.5 (–18.6–145.9)	12.7 (–17.6–151.5)	0.79
Testosterone	44.8 (12.6–147.3)	19.2 (3.2–64.8)	0.20
sIgA	–6.1 (–16.3–16.9)	9.5 (–16.6–39.3)	0.38
α-amylase	29.5 (20.3–51.9)	37.2 (26.6–63.3)	0.29
SBP	–13.9 (–22.4– –7.1)	–10.0 (–19.6– –4.0)	0.37
DBP	–12.6 (–20.0–3.3)	–12.5 (–22.1– –1.3)	0.80
MBP	–12.8 (–20.4– –3.1)	–12.5 (–19.2– –4.0)	0.98
HR	3.8 (–8.7–14.3)	0 (–6.2–4.8)	0.23

Data are Δ relative (%) T1–T0. sIgA — secretory immunoglobulin A; SBP — systolic blood pressure; DBP — diastolic blood pressure; MBP — mean blood pressure; HR — heart rate

Table 3. *Comparison of relative changes in analysed parameters between T2 and T0 in both groups*

Variables	Δ relative (%) T_1–T_0		p
	ThPVB group	Control group	
β-endorphin	198.3 ± 112.8	199.6 ± 124.5	0.96
Cortisol	35.3 (–22.7–107.6)	23.6 (–30.8–62.8)	0.11
Testosterone	30.60 (0.90–96.71)	5.82 (–6.42–30.34)	0.06
sIgA	14.53 (2.97–26.98)	12.66 (4.47–41.56)	0.32
α-amylase	26.58 (14.12–53.81)	45.09 (11.70–88.04)	0.26
SBP	–13.2 (–18.6– –4.6)	–11.7 (–22.6– –2.0)	0.91
DBP	–13.3 (–22.1–0)	–12.5 (–18.9– –7.5)	0.75
MBP	–11.2 (–22.1– –6.0)	–12.7 (–17.3– –2.4)	0.98
HR	–1.4 (–8.9–13.0)	4.2 (–5.6–8.6)	0.57

Data are Δ relative (%) T2–T0; sIgA — secretory immunoglobulin A; SBP — systolic blood pressure; DBP — diastolic blood pressure; MBP — mean blood pressure; HR — heart rate

Table 4. *Comparison of NRS scores between the groups*

NRS ≥ 1	ThPVB n = 37 (52.9%)	Control n = 33 (47.1%)	p
At rest			
T0 [N (%)]	0	0	–
T1 [N (%)]	19 (51.3)	15 (45.4)	0.62
T2 [N (%)]	14 (37.8)	8 (24.2)	0.22
p*	0.57	0.07	
During cough			
T0 [N (%)]	0	0	–
T1 [N (%)]	34 (91.9)	21 (63.6)	< 0.01
T2 [N (%)]	28 (75.7)	17 (51.5)	< 0.05
p*	< 0.001	0.44	
T1 [point]	3 (2–5)	1 (1–3)	0.10
T2 [point]	2 (0–5)	1 (0–3)	0.26
p#	< 0.01	0.10	
NRS ≥ 1			
1 month	23 (62.2)	23 (69.7)	0.51
2 months	8 (21.6)	7 (21.2)	0.97
6 months	5 (13.5)	5 (15.2)	1.00

p* — McNemar; p# — Wilcoxon signed-rank test

Table 5. *Descriptive statistics in the study groups in time for the levels of β-endorphin, cortisol, testosterone, sIgA, and α-amylases*

Group	Time	N	Mean	SD	Min	Max	Q_1	Me	Q_3
β-endorphin [pg/mL]									
ThPVB	T0	37	5.30	1.81	2.33	10.23	4.14	4.73	6.21
	T1	37	11.63	5.22	4.39	23.45	7.29	10.99	15.83
	T2	37	14.39	4.11	2.25	21.23	12.36	15.43	17.62
Control	T0	33	5.25	1.96	2.29	11.23	4.01	4.87	6.45
	T1	33	10.33	5.00	2.18	22.18	6.47	10.18	11.40
	T2	33	14.41	4.48	3.89	21.36	11.11	14.35	18.19
Cortisol [ng/mL]									
ThPVB	T0	37	12.22	3.86	7.06	21.90	9.20	11.20	13.76
	T1	37	19.54	8.69	7.34	35.80	10.50	21.30	27.40
	T2	37	16.76	7.19	7.33	34.10	9.89	16.70	20.10
Control	T0	33	12.08	3.16	6.21	17.89	9.59	11.74	14.25
	T1	33	18.86	9.75	7.29	38.10	10.30	14.55	27.60
	T2	33	13.47	5.75	7.34	30.10	8.87	11.22	17.80
Testosterone [pg/mL]									
ThPVB	T0	37	64.54	45.29	10.13	189.30	25.24	48.93	94.53
	T1	37	94.67	49.12	31.28	223.46	59.72	73.48	125.10
	T2	37	84.83	46.16	27.81	233.12	50.12	71.23	118.20

→

Table 5. *Descriptive statistics in the study groups in time for the levels of β-endorphin, cortisol, testosterone, sIgA, and α-amylases*

Group	Time	N	Mean	SD	Min	Max	Q_1	Me	Q_3
	T0	33	76.31	51.02	10.15	195.20	43.81	54.21	100.34
Control	T1	33	103.26	57.72	21.50	231.36	49.86	97.60	153.20
	T2	33	86.43	46.41	27.64	183.20	44.36	69.81	122.13
sIgA [ug/mL]									
	T0	37	112.94	13.06	75.56	130.22	102.54	115.98	123.07
ThPVB	T1	37	115.56	21.37	88.41	206.81	100.56	112.35	120.18
	T2	37	131.41	15.78	71.34	174.38	125.43	130.02	136.71
	T0	33	111.77	15.29	69.88	133.47	103.61	110.91	125.61
Control	T1	33	120.79	26.46	76.59	177.56	100.22	118.93	132.74
	T2	33	138.48	19.83	110.75	189.71	128.56	131.25	144.32
α-amylases [U/mL]									
	T0	37	91.10	28.13	6.59	126.70	76.18	99.89	108.45
ThPVB	T1	37	126.53	51.71	11.38	267.82	100.04	135.05	141.69
	T2	37	122.04	39.39	10.92	200.13	99.83	122.84	145.38
	T0	33	91.09	26.89	9.20	135.13	76.54	97.21	105.34
Control	T1	33	132.60	46.69	14.56	224.79	113.26	134.51	155.72
	T2	33	134.24	47.22	17.63	215.10	105.68	133.28	166.25

Data are: mean ± SD, n; Min — minimum; Q_1 — lower quartile; Me — median; Q_3 — upper quartile; Max — maximum

Table 6. *ANOVA analysis results with contrast analysis for β-endorphin, cortisol, testosterone, sIgA, and α-amylase levels*

Anova	p	Time	p_{Group}	Group	P_{Time}		$T_{0–1}$	$T_{0–2}$	$T_{1–2}$
β-endorphin [pg/mL]									
Group	0.46	T0	0.79	ThPVB	< 0.001	ThPVB	< 0.001	< 0.001	< 0.01
Time	< 0.001	T1	0.23	Control	< 0.001	Control	< 0.001	< 0.001	< 0.001
Interaction	0.39	T2	0.98						
Cortisol [ng/mL]									
Group	0.22	T0	0.98	ThPVB	< 0.001	ThPVB	< 0.001	< 0.01	< 0.01
Time	< 0.001	T1	0.61	Control	< 0.01	Control	< 0.01	0.46	< 0.001
Interaction	0.21	T2	< 0.05						
Testosterone [pg/mL]									
Group	0.60	T0	0.29	ThPVB	< 0.001	ThPVB	< 0.001	< 0.001	< 0.01
Time	< 0.001	T1	080	Control	< 0.001	Control	< 0.001	< 0.05	< 0.001
Interaction	0.18	T2	0.89						
sIgA [ug/mL]									
Group	0.19	T0	0.98	ThPVB	< 0.001	ThPVB	0.95	< 0.001	< 0.001
Time	< 0.001	T1	0.17	Control	< 0.001	Control	0.20	< 0.001	< 0.001
Interaction	0.48	T2	0.28						
α-amylases [U/mL]									
Group	0.48	T0	0.99	ThPVB	< 0.001	ThPVB	< 0.001	< 0.001	0.49
Time	< 0.001	T1	0.61	Control	< 0.001	Control	< 0.001	< 0.001	0.79
Interaction	0.37	T2	0.24						

Of the considered factors such as test group, sex, type of surgery, operated side, use of rescue analgesia, BMI, NRS ≥ 1 (at rest and cough at T1), and levels of measured hormones (at T1), only alpha-amylase levels statistically significantly increased the chance for higher NRS score after a month (OR = 1.013; 95% PU: 1.001–1.025; $p < 0.01$).

Discussion

In our study, we measured plasma or saliva levels of specific substances produced during stress generated by a thoracic surgical procedure (VATS). We also measured perioperative pain levels with the numerical rating scale (NRS). Analgesia was obtained either with opiates alone or opiates plus regional anaesthesia (TPVB).

No statistically significant differences were found between groups and over time in the percentage of patients with NRS ≥ 1 during rest. During cough, the percentage of patients with NRS ≥ 1 was higher at T1 and T2 in the ThPVB group. Simultaneously, a statistically significant reduction in the percentage of these patients over time was also observed in the ThPVB group.

There were no significant differences between groups in terms of sex, age, height, BMI, or ASA physical status. Moreover, no differences were found for surgery time and operated site. The only difference was that lobectomy was statistically more frequent in the ThPVB group.

Many studies show that regional anaesthesia (ThPVB) has a positive effect during the perioperative period when compared with systemic opioids and NSAIDs. Terheggen et al. compared ThPVB with general anaesthesia (GE) for breast surgery. The results of this study demonstrated that thoracic PVB resulted in superior postoperative pain relief compared with GA when used for minor breast surgery [13]. Zhi et al. preformed a systematic review and a meta-analysis to check the effect of thoracic paravertebral block on thoracoscopic surgery. The authors concluded that thoracic paravertebral block contributes to pain control after thoracoscopic surgery, with reduced incidence of adverse effects like nausea and vomiting, atrial arrhythmias, drowsiness, hypotension, and pneumonia, compared to systemic analgesics [14–16].

Casati et al. compared ThPVB (continuous) with thoracic epidural for patients undergoing thoracotomy. They concluded that continuous thoracic paravertebral blockade is as effective as thoracic epidural in controlling postoperative pain and is associated with fewer haemodynamic side effects. Furthermore, ThPVB had a lower risk of failure [17].

Haager et al. compared regional anaesthesia (thoracic paravertebral block or thoracic epidural anaesthesia) with a systemic opioid administration (patient-controlled analgesia — PCA). The primary endpoint was the postoperative pain level measured with VAS at rest and during cough. The results showed that resting VAS values were similar for all groups, although they were higher but comparable during cough in patients with PCA, except for the period 8–16 hours after the procedure. Intraoperative sufentanil administration was significantly higher in patients with no regional anaesthesia performed. These results show that PCA for VATS-lobectomy could be an acceptable alternative for regional analgesia [18].

The results discussed above suggest that the efficacy of all compared techniques is similar in terms of postoperative analgesia. Nevertheless, regional anaesthesia allows the avoidance of some adverse effects that occur during systemic opioid administration.

In our study, we also assessed plasma or saliva levels of specific substances to measure stress reaction, and therefore the quality of perioperative analgesia. These were β-endorphin, secretory immunoglobulin A (SIgA), cortisol, testosterone, and salivary α-amylase. There were no statistically significant differences between the groups of patients, although there was a tendency towards statistical significance in the relative change (T2–T0) in testosterone levels, with higher values observed in the ThPVB group. The analysis of the levels of β-endorphin, cortisol, sIgA, and α-amylase showed statistically significant differences between time points but not between the groups. At T2, statistically significantly higher cortisol levels were found in the ThPVB group. Of the considered factors (such as: test group, sex, type of surgery, operated side, use of rescue analgesia, BMI, NRS ≥ 1 — at rest and during cough at T1) and hormone levels (at T1), only alpha-amylase levels statistically significantly predicted increased chance for higher NRS score a month after the surgery.

Miecznikowski et al. compared two effective therapeutic methods for patients with cervical spine dysfunction (CSD). Secretory immunoglobulin A (SIgA) was one of the analysed markers of pain and stress response. Results revealed a statistically significant influence of the type of therapy on sIgA levels. In both tested groups, the final levels of salivary sIgA were higher than the initial levels before the beginning of the treatment [19]. These results confirm the efficacy of used therapies. Zhi-Yang Chen et al. assessed plasma levels of endogenous opioid peptides (also β-endorphin) in a group of patients undergoing elective surgical procedures under intravenous general anaesthesia combined with an epidural blockade. Plasma levels of β-endorphin were significantly lower at all time points (20, 40, 60, and 80 minutes after surgery) when compared with the baseline values [20]. The results of this study confirm adequate level of the

used analgesic methods during the surgery. Shirasaki et al. attempted to evaluate the usefulness of a portable salivary alpha-amylase analyser. They tested patients with chronic low back or leg pain and pain-free patients undergoing elective surgery under general anaesthesia combined with epidural analgesia (control group). There was a statistically significant correlation between the VAS pain scale and salivary alpha-amylase levels. The authors suggested that this biomarker could be a useful indicator for the objective assessment of pain intensity [21]. Yardenia et al. compared three pain-management techniques in patients undergoing lower abdominal surgery. These techniques were: intermittent opiate regimen (IOR), patient-controlled analgesia (PCA), and patient-controlled epidural analgesia (PCEA). The authors measured cortisol and prolactin levels during the first 48 hours after the procedure. The results showed that patients in the PCEA group had reduced postoperative pain and therefore lower activation of the HPA axis. This study showed that cortisol levels are changed by postoperative pain [22]. Also, studies have shown that elevated plasma levels of cortisol and ACTH might be an indicator of the magnitude of surgical trauma, and also could be modulated by the use of analgesia techniques [23, 24].

As mentioned before, our study showed a tendency towards statistical significance in the relative change (T2–T0) in testosterone levels, with higher values observed in the ThPVB group, which could suggest that this method of analgesia might be more effective.

The review of the above cited studies confirmed the efficacy of aforementioned hormones and endogenous opiates in the evaluation of pain level. Although not statistically significant, the results of this study might be a part of the process of evaluating pain and stress markers as a measure of adequacy of perioperative analgesia regimens. It should be mentioned that oxycodone might have an influence on testosterone and cortisol levels (as a result of pharmacological interaction rather than its analgesic action). Adequate analgesia technique is of utmost importance during the perioperative period. The efficacy of the chosen method and its safety are equally important. Many studies have confirmed the safety of regional anaesthesia techniques, and thoracic paravertebral block is no exception. On the other hand, opiates, which are also very effective, may cause significant side effects (respiratory depression, hypotension, bradycardia, nausea, and vomiting). These may diminish the effects of physiotherapy and extend the recovery process.

Conclusions

Preoperative ThPVB is effective and safe for patients undergoing VATS. The use of balanced analgesia which consists of regional analgesia (ThPVB), non-opioid painkillers, and small doses of opioids can be an effective alternative for general anaesthesia using large doses of opioids. No statistically significant difference between 6 hours and 24 hours after surgery in the levels of hormones (testosterone, cortisol, α-amylase activity, sIgA, and β-endorphin) confirms the efficacy of analgesia consisting of ThPVB and low doses of opioids.

Trial registration

ClinicalTrials.gov as No. NCT04414488.

Author contributions

Study conception: S.B., M.S., H.M. Literature search: M.S., A.M., H.M. Data extraction: M.S., S.B., D.C. Statistics: S.B., M.S. Drafting manuscript: S.B., A.M., H.M. Finalising manuscript: all authors. Responsibility for the paper as a whole: S.B.

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Patient outcomes following surgical management of thyroid nodules classified as Bethesda category III (AUS/FLUS)

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Abstract

Introduction: The Bethesda classification system for reporting thyroid cytopathology is the standard for interpreting fine needle aspirate (FNA). Because of its heterogeneity and inconsistent reporting, atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS), known as Bethesda category III, is the most controversial category. Thyroid nodules that fall within Bethesda categories III–IV have an overall risk of malignancy of between 15 and 40%. The aim of this study was to determine the malignancy rate in Bethesda III nodules.

Material and methods: A retrospective study was performed for 1166 patients who underwent thyroid surgery for multinodular goitre (MNG) or solitary nodular goitre (SNG) in our institution between June 2010 and May 2020. Data retrieved included demographic characteristics of the patients, FNB cytology, thyroid function test results, type of thyroidectomy, and final histology results.

Results: During the study period, 29.5% (344/1166) of patients with an FNA categorized as AUS/FLUS underwent thyroid surgery. Of these 344 patients, 190 were diagnosed with MNG and 154 with SNG. Incidental malignancy was found in 35 of 190 cases of MNG (18.42%) and 31 of 154 cases of SNG (20.13%). The most common malignant tumour type in either category was the follicular variant of papillary thyroid carcinoma.

Conclusions: The current study demonstrates that patients with a FNA categorized as AUS/FLUS may have a higher risk of malignancy than traditionally believed. Reconsideration may be necessary to guidelines that recommend observation or repeat FNA in this category of patients. **(Endokrynol Pol 2021; 72 (2): 143–144)**

Key words: *thyroid nodule; thyroid cancer; Bethesda classification*

Introduction

The incidence of thyroid cancer has increased dramatically in the last few decades, and it is now the fastest growing cancer in females, with papillary thyroid carcinoma (PTC) accounting for the majority of cases [1]. However, thyroid lesions are often found on the thyroid gland, and the majority of these are not malignancies. After an initial ultrasound (US), the next step in assessing the risk of cancer of a thyroid lesion is fine needle aspiration (FNA) [2]. The result of the biopsy can fall within one of six categories as defined by the Bethesda system for reporting FNA cytopathology results: I (non-diagnostic or unsatisfactory), II (benign), III (atypia of undetermined significance [AUS] or follicular lesion of undetermined significance [FUS]), IV (follicular neoplasm or suspicious for a follicular neoplasm), V (suspicious for malignancy), and VI (malignant) [3]. Because of its heterogeneity and inconsistent reporting, (AUS/FLUS), also known as Bethesda category III, is the most controversial category [4]. The aim of this study was to determine the malignancy rate in Bethesda III nodules in patients undergoing thyroidectomy for multinodular goitre (MNG) or solitary nodular goitre (SNG) in our institution.

Material and methods

Between June 2010 and May 2020, 344 out of 1166 patients who underwent thyroid surgery for multinodular goitre (MNG) or solitary nodular goitre (SNG) in our institution had a FNA categorized as AUS/FLUS and were considered for this retrospective study. Informed consent or approval by the local ethics committee was not obtained due to the observational nature of the study. Data were collected from medical and operating theatre records as well as from the hospital-coded database. Statistical analyses were done using SPSS for Windows 8.0. Student's t-test for normally distributed variables, Mann-Whitney U test for skewed variables, and chi-square test and Fisher's exact tests for comparison of results between groups. A p value < 0.05 was considered statistically significant.

Results

During the study period, 29.5% (344/1166) of patients with a FNA categorized as AUS/FLUS underwent thy-

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roid surgery in our institution. Of these 344 patients, 190 were diagnosed with MNG and 154 with SNG. Total thyroidectomy was performed in 15.1% (52/344) of the nodules with AUS/FLUS, subtotal thyroidectomy in 81.1% (279/344) of cases, and thyroid lobectomy in 3.8% (13/344) of these patients. The demographic data of the patients with MNG and SNG are presented in Table 1. There were more female patients than male in both the MNG and SNG groups. The mean age of patients with MNG at the time of surgery was 48.6 ± 11.18 years, with no significant difference compared with the patients with SNG, at 47.9 ± 12.35 years. In addition, there was no significant difference regarding operation time, hospital stay, and rate of malignancy between the two groups. Incidental malignancy was found in 35 of 190 cases of MNG (18.42%) and in 31 of 154 cases of SNG (20.13%). The most common malignant tumour type in either category was the follicular variant of papillary thyroid carcinoma. There was no significant difference regarding histological subtypes of all other malignant tumours between the MNG and SNG groups.

Discussion

The results of our study demonstrate that the rate of malignancy is 18.1% for AUS/FLUS patients with MNG and 20.1% for those with SNG, and the most common subtype is follicular variant of papillary thyroid carcinoma (PTC). According to the literature, thyroid nodules that fall within Bethesda categories III and IV have an overall risk of malignancy of between 15 and 40%. If Bethesda III or IV lesions are found to be malignant, the most common histological subtype is the follicular variant of papillary thyroid carcinoma. This variant is generally less aggressive than classic papillary thyroid carcinoma. Furthermore, this subtype has been shown to have a lower risk of lymph node metastases, recurrence, and local extension, especially if it is encapsulated [3].

A retrospective analysis showed that ultrasonography (K-TIRADS 5) is the most influential independent predictor of malignancy in AUS/FLUS patients [5]. Another retrospective study showed that the rate of malignancy of 350 nodules with AUS/FLUS that went on to surgery was approximately 38%. They found that the most common malignancy was PTC (86.8%) [4].

Our study has several limitations because the patients were assessed retrospectively from a single centre. Our study indicates the need for a prospective randomized controlled trial of AUS/FLUS patients with MNG and SNG.

Table 1. ***Number of cases, gender, age, hospitalization, duration of surgery, and histological outcomes for the undetermined significance or follicular lesion of undetermined significance (AUS/FLUS) patients with multinodular goitre (MNG) and solitary nodular goitre (SNG). Values are presented as mean ± standard deviation (SD) with percentage in parentheses***

Variable	MNG	SNG	p
Number of patients (n = 344)	190 (55)	154 (45)	
Males/Females (67/277)	36/154	31/123	> 0.05*
Mean age (± SD) [yrs]	48.6 ± 11.18	47.9 ± 12.35	> 0.05
Hospitalization (± SD) [d]	4.3 ± 2.7	4.1 ± 2.9	> 0.05
Malignancies (n = 66)	35 (18.4)	31 (20.1)	> 0.05
Mean operative (± SD) time [min]	103.1 ± 59.1	101.7 ± 47.4	> 0.05

*statistically significant ($p < 0.05$)

Conclusions

In conclusion, the current study demonstrates that patients with a FNA categorized as AUS/FLUS may have a higher risk of malignancy than traditionally believed. Reconsideration may be necessary to guidelines that recommend observation or repeat FNA in this category of patients.

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Conflict of interest
None.

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Dysregulation of microRNAs as the risk factor of lymph node metastasis in papillary thyroid carcinoma: systematic review

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Abstract

Papillary thyroid carcinoma (PTC) has an excellent prognosis with a relatively low mortality rate, but a small portion of PTC patients suffer from an aggressive form of the disease. In such cases early detection of lymph node metastasis (LNM) is as paramount as it is problematic. The routine use of central neck lymph node dissection is not recommended. New methods to detect LNM are needed. MicroRNAs are a potential biomarker for diagnosis and prognosis of PTC. In this review we summarise the current knowledge regarding dysregulated miRNAs and their association with LNM in PTS patients.

The PubMed and EBSCO databases were searched using terms for "microRNA", "thyroid carcinoma", and "prognosis" by using Boolean operators. Based on eligibility and exclusion criteria, articles were screened and reviewed in full, methodological data of included studies were extracted, and risk of bias analysis performed.

In total, 446 unique studies were extracted from the mentioned databases, and based on inclusion and exclusion criteria 27 studies were included in this review. Of them 17 analysed tissue microRNAs, 5 analysed circulating microRNAs, and 5 studies analysed both tissue and circulating samples. MiRNA-146B, miRNA-221, miRNA-222, miRNA-21, miRNA-204, miRNA-451, miRNA-199a-3p, and miRNA-30a-3p were dysregulated in at least 2 separate studies. A sizable portion of studies failed to show statistically significant differences in miRNA expression between LNM-positive and -negative patients. Different methodologies and disparities of patient populations could explain these discrepancies.

This research supports the statement that specific up- and downregulated miRNAs are associated with LNM in PTC patients. However, the prognostic value of these miRNAs is limited. Additional targeted cohort studies are required to elucidate the role of miRNAs in defining individualised treatment strategies for thyroid cancer patients. **(Endokrynol Pol 2021; 72 (2): 145–152)**

Key words: *thyroid neoplasms; microRNAs; lymphatic metastasis*

Introduction

Thyroid cancer is the most frequently occurring endocrine malignancy, with an increasing rate of incidence over the last 3 decades [1]. It accounts for 1.7% of all malignant tumours worldwide [2]. Papillary thyroid carcinoma (PTC) accounts for up to 85% of all thyroid cancers [3]. Generally, PTC has an excellent prognosis with a relatively low mortality rate, but a small portion of PTC patients suffer from an aggressive form of the disease, with tumour invasion and metastasis [4]. The detection of neck lymph node metastasis (LNM) — especially subclinical — of thyroid cancer using imaging methods (ultrasound, computer tomography, magnetic resonance imaging) is often problematic [5]. In up to 60% of imaging lymph node-negative patients, cervical LNM are found on histology [6]. This could result in incomplete clinical treatment. The American Thyroid Association Management guidelines recommend the use of central neck dissection for patients with clinically involved central nodes, but the use of prophylactic central node dissection (pCND) in patients without clinically detected central neck lymph nodes remains controversial [7]. Prophylactic central node dissection significantly reduces locoregional recurrence in PTC patients with clinically uninvolved central neck lymph nodes, but pCND is often associated with postoperative complications. Patients with pCND have significantly higher chances of transient recurrent laryngeal nerve injury and transient or permanent hypocalcaemia [8]. Therefore, accurate identification of LNM is of crucial importance in optimising individualised PTC treatment.

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Table 1. *Detailed search retrieval from PubMed and EBSCO databases, including results for terms and their combinations*

No.	Search details in PubMed	Search details in EBSCO	PubMed	EBSCO
1	"thyroid cancer" [Title/Abstract] OR "thyroid carcinoma" [Title/Abstract] OR "thyroid nodule" [Title/Abstract] OR thyroid neoplasms [MeSH Terms]	(TI thyroid cancer OR AB thyroid cancer) OR (TI thyroid carcinoma OR AB thyroid carcinoma) OR (TI thyroid nodule OR AB thyroid nodule)	63,540	76,736
2	"micro RNA" [Title/Abstract] OR "miRNA" [Title/Abstract] OR „miR" [Title/Abstract] OR micrornas [MeSH Terms]	(TI micro RNA OR AB micro RNA) OR (TI miRNA OR AB miRNA) OR (TI miR OR AB miR)	114,487	134,624
3	"prognosis" [Title/Abstract] OR "prognos* "[Title/Abstract] OR "lymph node" [Title/Abstract] OR "metasta*" [Title/Abstract] OR prognosis [MeSH Terms]	(TI prognos* OR AB prognos*) OR (TI lymph node OR AB lymph node) OR (TI metasta* OR AB metasta*)	2,330,377	1,670,450
4	1 AND 2	1 AND 2	918	890
5	3 AND 4	3 AND 4	410	359

REVIEW

Many researchers are looking for new testing methods to identify LNM before surgery. Advanced new molecular genetic biomarkers could identify patients with aggressive PTC for more aggressive treatment options.

MicroRNAs (miRNAs) are a class of non-coding RNAs approximately 19–24 nucleotides in length that can function as oncogenes or tumour suppressors by inhibiting the translation of tumour suppressor genes or by blocking the translation of oncogenes [9]. MicroRNAs modify gene expression by binding to specific targets in the 3′ untranslated region, and they are implicated in tumourigenesis of a variety of tissues [10]. Regulation of classical oncogenes and tumour suppressor genes by miRNAs was easily identified as a hallmark of cancer research, transforming this class of small RNAs into potential targets for cancer diagnosis, prognosis, and therapy. With the availability of high-throughput, next-generation sequencing, miRNAs can be detected more accurately from controls compared with other miRNA microarrays, northern blots, and TaqMan microRNA Assays Human panel [11]. There has been a lot of interest in the feasibility of miRNAs as biomarkers for the diagnosis of thyroid cancer in recent years. Several miRNAs possibly associated with LNM were identified in patients with PTC [12].

In this review, we focus on miRNA expression in thyroid cancer to evaluate miRNA signatures associated with LNM. The objective of this systematic review is to summarise the current knowledge regarding dysregulated miRNAs and their association with LNM in patients with PTC.

Systematic literature analysis

Systematic literature analysis was performed following the Cochrane Handbook for Interventional Systematic Reviews. The Study was written in accordance with the guidelines proposed by the preferred reporting items for systematic review and meta-analyses (PRISMA) [13]. Search was performed by querying the PubMed and EBSCO databases with terms for "micro RNA", "thyroid carcinoma", and "prognosis" by using Boolean operators, for articles published prior to 2020-08-13 (Tab. 1). Two reviewers (R.L., V.J.) working independently using standardised forms screened titles and abstracts to identify potentially relevant studies. Articles of interest were further evaluated in their entirety (Fig. 1).

Studies were considered to be eligible if they: focused on patients with any type of papillary thyroid carcinoma or had information about clinicopathological patient characteristics. The effect of the dysregulated miRNA on cervical lymph node metastases was assessed. Exclusion criteria consisted of the following: non-PTC thyroid cancer studies, non-English articles, reviews, letters, comments, and studies using miRNAs only for differentiation between benign and malignant thyroid lesions or studies with a primary focus on biomolecular mechanisms of miRNAs.

It is of note, reviewers elected not to exclude studies if they satisfied inclusion criteria but failed to find statistically significant differences in microRNA expression among LNM positive and negative groups.

Risk of bias was assessed using a quality assessment tool for diagnostic accuracy studies (QUADAS-2). QUADAS-2 focuses on the quality of primary diagnostic accuracy studies, but not on the patient cohorts, control groups, or blinding procedures. It consists of 4 key domains covering patient selection, index test (in our case miRNA), reference standard, and flow of patients through the study as well as the timing of the index test and reference standard ("flow and timing"). These domains are assessed in terms of risk of bias

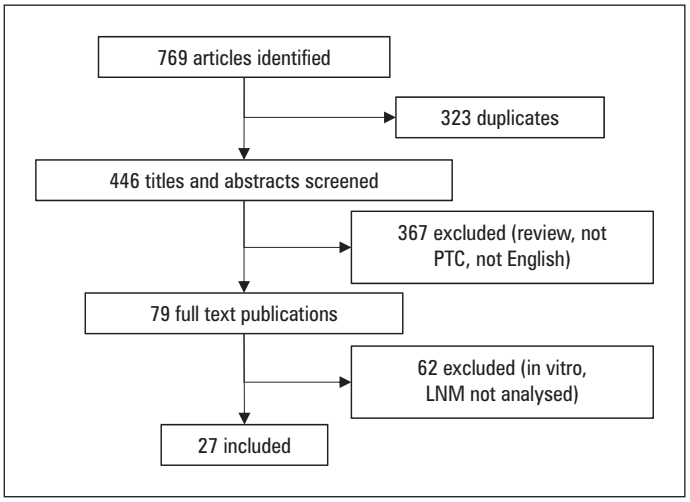

Figure 1. *Flowchart of study selection. PTC — papillary thyroid carcinoma; LNM — lymph node metastasis*

and the first 3 are also assessed in terms of concerns regarding applicability. To help reach a judgement on the risk of bias, signalling questions were included [14]. QUADAS-2 review questions and specific guidance for this review were discussed among the reviewers, and then evaluation was performed by 2 reviewers working separately.

Methodological and outcome data were extracted from the selected studies. Discrepancies between 2 reviewers in study selection (title and abstract screening, full article evaluation), data extraction, and risk of bias evaluation processes were discussed and a joint resolution was made.

Analysis results

The PubMed search yielded 410 possible studies and EMBSCO yielded 359. In total 446 unique studies were included in the further analysis after removing duplicates. Based on the inclusion and exclusion criteria, 367 studies were excluded after title and abstract screening (most were reviews and studies not addressing PTC or lacking clinicopathological data), and when the decision was not clear, the studies were analysed in full. Of the 79 full text publications, 27 studies were included in this review (Fig. 1). The 2 reviewers were in agreement regarding study eligibility. The earliest study was published in May 2010 [15] and the latest in June 2020 [16]. The included studies were from 7 countries: China (n = 20), Serbia (n = 2), Lithuania (n = 1), South Korea (n = 1), Taiwan (n = 1), Turkey (n = 1), and the USA (n = 1).

Study design

A methodological summary of the included studies is summarised in Supplementary File — Table S1.

From 27 studies, 17 analysed tissue microRNAs, 5 analysed circulating microRNAs, and 5 studies analysed both tissue and circulating samples. Among studies analysing tissue, 11 evaluated freshly frozen (snap-frozen) samples, 9 analysed miRNAs from formalin-fixed paraffin-embedded tissue (FFPE), one analysed tissue from fine needle aspiration (FNA), and in 1 case the method of specimen preparation/extraction was not specified. In studies analysing circulating microRNAs, 3 analysed plasmas, 4 — serum, 1 — blood (otherwise not specified), 1 — plasma exosomes, and 1 — both plasma and serum exosomes (Fig. 2).

For initial genome screening, 4 studies [15, 20–22] performed microarray, and 1 study [17] used Solexa sequencing, followed by qRT-PCR to validate results. However, of all the rest of the studies 21 performed qRT-PCR [16, 19, 23–41] and 1 study performed northern blotting [18] to quantify miRNA expression for *a priori* selected miRNAs from the literature.

The studies employed a variety of statistical methods. Twenty-one studies compared continuous miRNA expression between different LNM groups. When parametric test assumptions were met, Student's t-test and ANOVA were used, and when such assumptions were not met, Kruskal-Wallis H and Mann-Whitney U tests were used. Analysing associations between miRNA and LNM, one study [17] used Pearson's correlation coefficient to correlate miRNAs from blood and tissue, and 2 studies [26, 33] used multivariate logistic regression to assess independent predictors for LNM. Six studies divided miRNA expression into low/high groups based on mean or median values, and then compared those groups as categorical variables using the chi-square test to assess differences between miRNA expression and LNM status [16, 28–31, 37]. Four studies [32, 38, 39, 41] performed ROC analysis to evaluate the diagnostic utility of miRNA

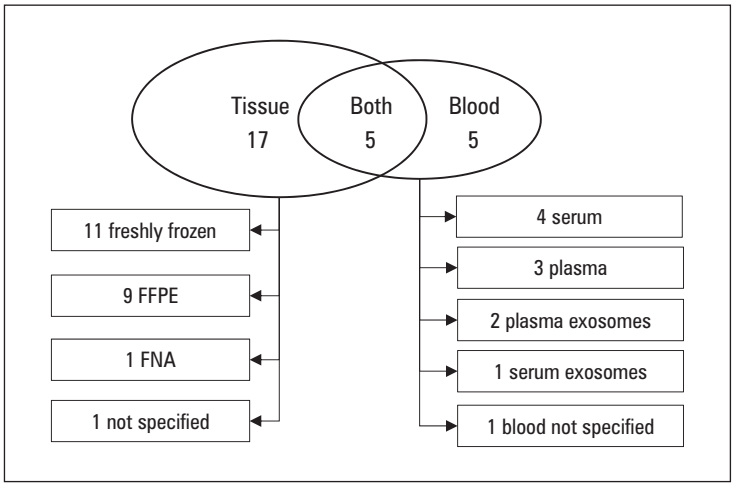

Figure 2. *Studies by analysed specimens. FFPE — formalin-fixed paraffin-embedded tissue; FNA — fine needle aspiration*

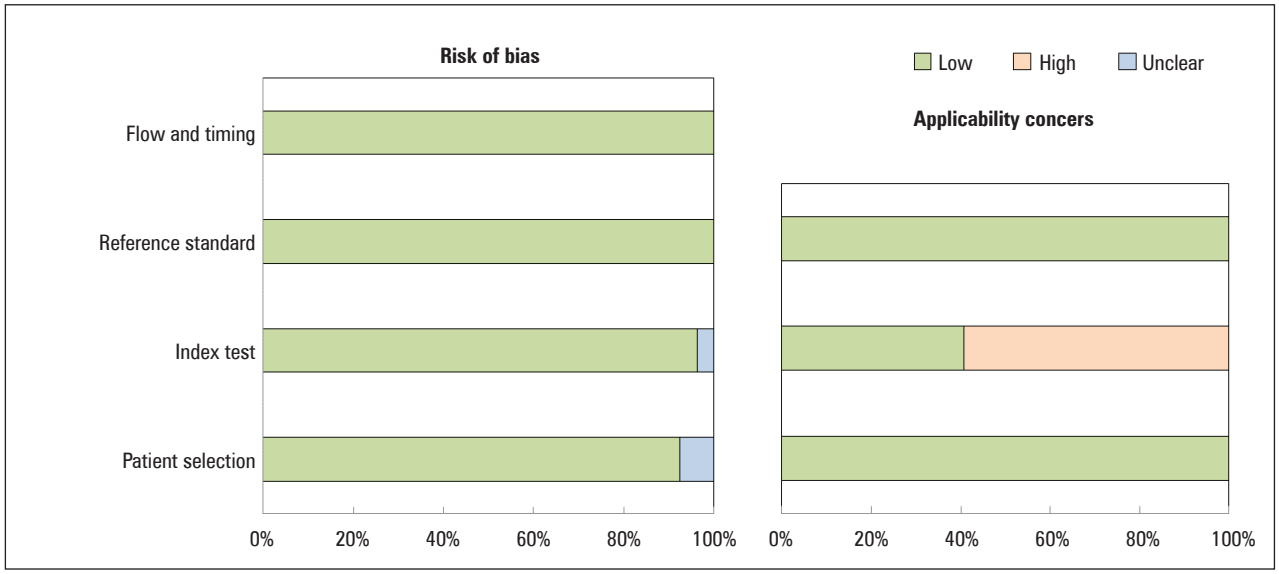

Figure 3. *Summary of risk of bias analysis by quality assessment tool for diagnostic accuracy studies (QUADAS-2)*

in predicting LNM. One study [38] used univariate and multivariate Cox proportional hazard model to analyse miRNA as independent risk factors for LNM.

A summary of the risk of bias analysis can be found in Supplementary File — Table S2, and Figure 3.

Most of the studies included in the analysis were at low risk of bias in all evaluated domains. However, in some studies the patient population and methodological descriptions were not fully clear. Several previous studies showed discrepancies between miRNA profiles in surgically resected tissues and FNA material, which led other authors to conclude that the results from surgically resected material cannot be extrapolated into preoperative use without further validation [42]. Therefore, this review attempted to find miRNA able to inform the extent of surgery, hence changes in miRNA from post-surgical specimens may not necessarily translate to changes measurable preoperatively (e.g. serum, FNA specimens). Additionally, all but one of the included studies performed clinically indicated or not specified cervical lymph node dissection, as opposed to prophylactic cervical lymph node dissection, and this could lead to a selection bias, favouring more advanced disease and missing early stages of cervical LNM. This could also explain why in a sizable portion of included studies there were more PTC patients with LNM than without LNM.

Of note, only one [26] of the included studies specified PTC subtype in a LNM vs. no LNM context, providing separate analysis for all variants combined and classical variant in particular excluding Tall cell and follicular PTCs.

Table 2. *Dysregulated miRNA mentioned in at least 2 separate studies*

MiR	Studies with ↑ MiR	Studies with ↓ MiR	No statistically significant difference
146b	[24, 26, **34**, **38**, 40]		[15, 19, 20, 22, **23**, **25**]
221	[18, 19, 24, 26, **38**, 40]		[15, 18, 20, **23**, 26]
222	[**17**, 19, 24, 26, 36, **38**, 40]		[15, 20, **23**, 36]
21	[**38**, 40]		[19. 26]
204	[**38**]		[26, **39**]
451	[21]	[**32**]	
199a-3p		[31]	[**38**]
30a-3p			[22, 35, **38**]

bold — specimens could be obtained preoperatively

MicroRNA expression and LNM

Dysregulated miRNAs associated with LNM in the included studies are listed in Supplementary Table S3 (with pre-operative specimens in bold). When the same miRNA was mentioned in at least 2 separate studies, they were listed in Table 2 (specimens obtained preoperatively are shown in bold).

miRNA-146B, miRNA-221, miRNA-222, miRNA-21, miRNA-204, miRNA-451, miRNA-199a-3p, and miRNA-30a-3p were mentioned in at least 2 separate studies. Similar at least two different studies excluding tissue specimen miRNA-221, miRNA-222, miRNA-146b, and miRNA-204 were found as well. Some studies analyzed not only differences between LNM-positive and -negative groups, but also tried to find differences in miRNA expression between groups based on LNM location in the neck lateral LNM vs. central LNM [21, 23, 36].

Most of the studies agreed on the presents of miRNA dysregulation; however, in some studies statistically significant differences were not identified (Tab. 2).

In one study upregulated miRNA-451 was detected in patients with LNM [21] and downregulated by another one [32].

The same trend remains even in studies where specimens were available preoperatively. This could be explained by different methodologies, groups sizes, and disparities in patient populations, because all but one of the studies lymphadenectomies were performed only by clinical suspicion or the indication was not specified.

miRNA biomarkers predicting LNM in PTC

Differentiated thyroid cells express a pool of miRNAs, alterations in the expression of which could induce neoplastic changes. A downregulation of miRNAs that ensure normal function of cells, or alternatively an upregulation of miRNAs that promote oncogenic effects, can lead to increased expression of oncogenes. That can result in the induction of the malignant effects of cell proliferation, differentiation, and apoptosis, leading to tumour growth and progression [43]. In this systematic review, we focused on exploring the utility of miRNA biomarkers predicting LNM in PTC.

MicroRNA-146

MicroRNA-146 is one of the most investigated miRNAs in thyroid cancer and has been shown to be reliably upregulated in PTC [24, 26, 34, 38, 40].

Functional analyses of miRNA-146 revealed its involvement in various cellular functions including proliferation, migration, and invasion [44, 45]. Chou et al. demonstrated that miRNA-146b expression was an independent risk factor for poor prognosis in PTC [46].

Chen et al. indicated that microRNAs can be identified not only in tissue samples but also in serum and plasma in a remarkably stable form. That makes miRNA expression detection possible in blood samples and serum, serving as potential biomarkers to detect various cancers. [47]. The clinical utility of using miRNAs as a biomarker in serum could be a diagnostic tool for identifying patients with LNM in PTC for more aggressive treatment options.

Eleven articles in our review described the association between miRNA-146 overexpression and LNM in PTC. Eight of these studies analysed tissue miRNA (4 FFPE, 3 FF, and 1 FNA) [15, 19, 20, 22, 24, 26, 34, 40], 2 — circulating samples (plasma exosome) [23, 38] and 1 — both tissue and circulating samples (FF and blood) [25].

Several independent groups of investigators examining microRNA in histological samples found the expression level of miRNA146b to be significantly elevated in patients with lymph node metastases [24, 26, 40]. However, no significant correlation was observed in other studies [15, 19, 20, 22].

Sun et al. found positive associations between levels of miRNA-146a expression in PTC tissues and

positive cervical lymph node metastasis. Nevertheless, the same study did not note significant differences in expression of miRNA-146b in the peripheral blood or in tissue between patients with PTC and LNM and patients with PTC but without LNM, which indicates that miRNA-146a and miRNA-146b expression in peripheral blood are not useful markers for LNM in PTC [25].

Yang et al. examined miRNA-146b expression in FNA, and malignancy was confirmed by histology, as a gold standard for accuracy. That study showed that levels of miR146b were significantly higher in PTCs with lymph node metastasis [34].

Jiang et al. explored the selected exosomal miRNAs as potential biomarkers predicting LNM in PTCs. Exosomal miRNA-146b-5p and miRNA-222-3p ROC analysis showed AUCs of 0.811 and 0.834, respectively. Combining exosomal miRNA-146b-5p and miRNA-222-3p significantly improved the diagnostic value and increased the AUC to 0.895, with a sensitivity and specificity of 85.1% and 80.0%, respectively [38].

The only study in our review by Han et al. examined miRNA expression in the patients undergoing prophylactic lymph node dissection. The specimens they used were FFPE. On FFPE specimens miRNA-146b-3p, miRNA-146b-5p, and miRNA-222 were identified as potential markers of LNM [26].

miRNA-221/222

Many PTC studies in our review identified expression of miRNA-221/222 as the most consistently upregulated miRNAs, with significant association with clinicopathological features. PTC with LNM showed higher tissue miRNA-221/222 expression in comparison to PTC without LNM [18, 19, 24, 26, 36, 40]. Jiang et al. revealed that a high serum miRNA-222 and miRNA-221 level has significant correlation with the presence of LNM [38]. Enhanced expression of serum miRNA-222 was also found in patients with cervical lymph node metastasis in a study by Yu et al. [17]. In an another study, Lee et al. [23] recruited PTC patients with and without LNM. Mean fold changes in plasma-derived miRNA-221 (–4.86 ± 0.051), and miRNA-222 (–5.36 ± 0.91) in patients with LNM group were slightly higher than that of miR-221 (–5.06 ± 0.074), and miR-222 (–5.45 ± 0.079) in patients without LNM. However, without statistical significance.

miRNA-451

Another important member of the microRNA family is miRNA-451, which has been shown to be downregulated in various human tumours [48]. It is dysregulated in multiple cancers and take part in various human physiological and pathological processes and cancer-related biological processes such as apoptosis, angiogenesis, proliferation, and metastasis [49–51]. It often acts as a tumour suppressor gene in various cancers. However, little is known about its role in PTC.

In the reviewed articles we found that the data about miRNA-451 association with LNM in PTC are controversial. The results of a study by Zhang et al. [32] indicated a strong correlation with lower miR-451 levels in PTC patients with LNM, suggesting that the tissue miRNA-451 level is also a "good" biomarker (AUC = 0.792) for lymph node metastasis. Expression of serum miR-451 in positive LNM patients' samples was only modestly (77%) lower than in LNM-negative patients. ROC analysis of serum miRNA-451 as a biomarker for evaluation of lymph node status was performed. A value of 0.690 in AUC would indicate that it is a possible "modest" biomarker for lymph node metastasis.

Conversely, the findings of Wang et al. [21] revealed that the expression of miRNA-451 tissue was significantly upregulated in PTC with LNM, compared to PTC cases without LNM. These findings suggest an oncogenic role for miRNA-451 in PTC. Further studies in larger cohorts are needed to explain the discrepant results on the role of miRNA-451 in thyroid cancer.

MicroRNA-199a-3p

MicroRNA-199a-3p is downregulated in a number of different cancers, such as ovarian carcinoma, colorectal cancers, and hepatocellular carcinoma [52-54]. The results of a study by Liu et al. [31] showed positive associations between levels of miRNA-199a-3p expression in PTC tissues and positive cervical lymph node metastasis.

The same study revealed that low miR-199a-3p expression was associated with LNM ($p = 0.036$) and recurrence of LNM ($p = 0.03$). These results suggest that the detection of miRNA-199a-3p in the PTC could reflect metastasis and predict prognosis.

Jiang et al. [38] recruited 136 patients with PTC. The expression level of serum miRNA-199a-3p was examined in patients with and without LNM. However, the difference between groups was not significant.

One of the factors limiting the comparison of miRNA changes between different publications is that most studies use their own rather subjective miRNA expression values, and no absolute levels of target miRNAs are set, which complicates the external validity usage of miRNA as a clinical tool.

In addition, as was noted in another review [55], analysis of miRNA expression and underlying mechanisms cannot be related to a single miRNA analysis. Due to non-canonical and partially complementary binding properties of miRNA, nearly half of the miRNA targets contain binding sites for at least 2 miRNAs. Therefore,

miRNAs binding to the same target can synergise and/or antagonise the expression of target genes, making studies on single miRNAs questionable.

As discussed by Han et al. [26], statistical significance does not necessarily predict the actual clinical applicability of molecular markers. The authors found that 3 miRNAs were able to predict LNM in a multivariate model. However, there was significant overlap in their expression in LNM-positive and LNM-negative groups. Only in a minority of cases were miRNA expression levels high enough to enable segregation of LNM-positive cases, providing poor sensitivity at acceptable levels of specificity.

Conclusions

This research supports the statement that specific up- and downregulated miRNAs are associated with LNM in PTC patients. However, the prognostic value of these miRNAs is limited in individual cases because the distribution of miRNA expression overlaps between patients with LNM and without LNM. Analysis of miRNA expression levels and detection of circulating or FNA miRNAs can be used for the pre-operative diagnosis of thyroid cancer cervical lymph node metastasis. To translate these data into clinical application, large cohort studies are required to examine the prognostic and diagnostic value of miRNAs panels. Improved standardisation of methods used to assay miRNAs will allow more extensive use of this approach in defining individualised treatment strategies for thyroid cancer patients.

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The rationale for selenium supplementation in patients with autoimmune thyroiditis, according to the current state of knowledge

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Abstract

Selenium (Se) supplements are commonly prescribed to autoimmune thyroiditis (AIT) patients by European endocrinologists, despite the lack of official guidelines. The majority of Europe is depleted of natural Se sources, and the daily population intake does not comply with recommended values. Optimal individual plasma Se concentration is reached when the selenoproteins (selenoprotein P, glutathione peroxidase) are fully saturated. However, Se intake has to be regulated because both Se shortage and overdose negatively impact health. In the case of AIT, Se may alleviate symptoms or prevent progression to hypothyroidism and postpartum hypothyroidism. Se supplementation in euthyroid, subclinical, or overt hypothyroid AIT patients decreased thyroid autoantibodies, lowered or maintained the TSH level, decreased the fT4/fT3 ratio, reduced the body's oxidative stress and inflammatory status, and amended quality of life and thyroid ultrasound structure and volume. In pregnant females, adequate Se intake protected them against miscarriages, preeclampsia/hypertension, preterm birth, small-for-gestational-age infants' birth, and improved child's neuropsychological development. In the elderly population, adequate Se supplementation decreased cardiovascular diseases and hypertension risk, but prolonged intake of excessive doses increased the all-cause mortality rate. Routine Se supplementation implementation requires from researchers and clinicians consideration of specific populational differences in natural Se and iodine supply, the patient's clinical situation (supplementation simultaneously or before levothyroxine treatment, AIT/non-AIT hypothyroidism), individual response to supplementation (Se and selenoprotein P assessment), predisposition (genetic testing), the status of other trace elements, and the interplay between those micronutrients. Moreover, the safety of commercially available Se formulations, doses, and duration of treatment should be determined. Proper guidelines are warranted to standardise the medical approach to Se supplementation. This article presents a comprehensive review of recent randomised-controlled trials, meta-analyses, and clinical trials concerning the risks and benefits of Se supplementation in different clinical settings and specific populations with particular emphasis on AIT in a practical manner. **(Endokrynol Pol 2021; 72 (2): 153–162)**

Key words: *selenium; thyroid; autoimmune thyroiditis; diet supplements; pregnancy*

Introduction

The influence of diet supplements on autoimmune thyroid disease (AIT) has been debated recently. Growing patients' interest in nutraceutical products as an alternative form of AIT treatment, wide availability and uncontrolled intake of supplements (over-the-counter in most countries) forces scientists and clinicians to expand and update the evidence-based state of knowledge. Although no positive recommendation for selenium (Se) supplementation in AIT has been provided by European or American Endocrine/Thyroid Societies, it is widely supported worldwide in daily clinical practice. According to an Associazione Medici Endocrinologi (AME) Survey completed by 815 doctors (91.6% endocrinologists) in 2016, the majority of doctors (79.4%) prescribe Se to euthyroid AIT patients, aiming for hypothyroidism delay or a decrease of thyroid antibodies (TA) titre. Interestingly, only 20% of doctors would advocate against Se if a patient requests any form of treatment instead of observation. In patients with subclinical hypothyroidism (SHT) and positive TA, two-thirds of respondents would prescribe Se, whereas only one third — with negative TA, regardless of additional levothyroxine (LT4) treatment. In euthyroid AIT women who are pregnant or conceiving, 40% of doctors decided on Se supplementation, intending to prevent postpartum thyroiditis (PPT). The preferable dose in all groups was 100–200 μg/day [1]. Surprising results of the previous survey encouraged investigators to expand the questionnaire within the European Thyroid Association (ETA) members (147 physicians,

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84% endocrinologists). Sixty-six per cent of them would use Se in AIT; however, only 20% recognised convincing evidence favouring Se supplementation. In the LT4-treated AIT group, 50% of responders would prescribe Se, where 71% would not recommend it in pregnancy. In AIT subjects not receiving LT4 physicians were more likely to support Se supplementation (69%), and a lower rate (67%) would not prescribe it in case of pregnancy. Besides the goals mentioned above, some respondents consider the quality of life (QoL) and thyroid ultrasound (US) image improvement as expected supplementation outcomes. The preferable treatment pattern was ≤ 200 μg/day of selenomethionine (SeMet) for weeks to months [2].

This article provides a comprehensive review of the potential role and usefulness of Se supplementation in various clinical settings based on recent randomised-controlled trials (RCTs), meta-analyses, and clinical trials and systemises knowledge concerning the role of Se in human health among different populations, with particular emphasis on AIT, in a practical aspect.

REVIEW

Natural sources and populations' supplies of Se

Populations' replenishment of Se is country-specific and largely depends on soil content and bioavailability to enter the food chain through plants. The vast majority of the Earth's rocks have a low Se content, but some sedimentary and volcanic rocks are enriched with this trace element, explaining the geographical differences [3]. The majority of Se in our body is obtained from consuming seafood (e.g. tuna, halibut, sardines, shrimps), animal organs and meat (e.g. ham, beefsteak, turkey, beef liver, chicken), cereals, grains and crops (e.g. pasta, brown rice, whole-wheat bread), milk or dairy products (e.g. cottage cheese, milk, eggs), and fruit/vegetables (e.g. spinach, bananas) [4]. However, the food with the highest Se content is Brazil nuts [5]. Importantly, its concentration in nuts is notably diversified depending on the place of origin and brand; it is non-standardized (no product information about Se concentration or geographic region of import) and variable (high seed to seed and batch variation). Analysis of 13 commercially available Brazil nut brands, only from Brazil, showed that the batch from the largest nut producer in the Se-rich Amazonas state exceeded by up to 3.5 times the tolerable upper intake level (UL) for Se in a standard serving of 6–8 nuts. Therefore, the authors opted to reduce the recommended portion of any Brazil nuts to 3 nuts, to avoid Se overdosing [6].

The National Institutes of Health (NIH) in the U.S. established 2 terms with regards to nutrient consumption: 1. recommended dietary allowance (RDA) - a value required to achieve optimal glutathione peroxidase (GPx) expression and in practice used to plan an adequate diet for healthy people, and 2. Upper limit (UL) — above which side effects were noted. In the case of Se, the recommended RDA-UL values from birth to 6 months of age is 15–45 μg/day (from breast milk), 6 months to13 years of age ranging from 20-45 to 40–280/day, and from 14 years until adulthood — 55–400 μg/day. The RDA is temporarily higher in pregnancy (60 μg/day) and during lactation (70 μg/day) due to increased foetus and newborn requirements [7]. Similarly, the European Food Safety Authority (EFSA) recommended a higher (70 μg/day) adequate Se intake for adults (including pregnancy) and in lactating women (85 μg/day) [8]. The value for adults (55 μg) is in accordance with the Scientific Committee for Food (SCF) of the European Commission, but the UL was set at a lower value of 300 μg/day. However, the Reference Nutrient Intake (RNI) for Se in the UK is 60 μg/day in women and 75 μg/day in men and lactating women [9]. Nevertheless, most European countries have a Se daily intake lower than preferable (30–90 μg/day) [10]. As a result, some countries are Se-repleted, including North America, Venezuela, Greenland, Japan, and certain parts of China, whilst others are Se-depleted — the majority of countries in Europe, the Middle East, and certain parts of China [4]. Most European countries, except parts of Norway and Finland, have insufficient natural Se supplies, with the lowest values recorded in Eastern Europe. For example, Polish students have a deficient Se intake, with daily dose average around 25 μg/day according to one study [11]. In the last decades, progressively lower Se concentrations have been reported in Polish women, regardless of their pregnancy status, probably due to decreased food Se intake. Moreover, in Polish pregnant females, Se serum status was lower than the European average (31% had Se level < 50 μg/L *vs.* 4.5% in other European countries) [12]. A multicentre comparison between Italy, Greece, Romania, and Austria showed suboptimal Se levels in each cohort, with the highest serum Se concentrations in Italy [13]. Se status has also declined in the UK in the last decades, which coincides with the bread-making industry switching from high-Se wheat imported from North America to low-Se wheat grown in Europe or the UK, using fertilizers rich in S instead of Se, decreasing use of coal (Se-rich rock) as a fuel, and an increasing preference of a vegetarian diet over meat consumption [3]. An interesting example is northern Germany, where the division of countries with moderate Se in the west and those with low Se in the east follows the instated West-East German border. Such differences may be explained by the use of fertilisers with different Se content. The national program of implementing

sodium selenate-rich fertilisers in primarily Se-depleted Finland in the early 1980s resulted in improvement of Se intake in the Finnish population from 30 mg/d to 100 mg/d in the late 1980s and promoted Finland to the list of European countries with adequate Se intake [14].

Se status assessment

Assessment of blood Se concentration may be misleading due to unspecified substitutions of SeMet for methionine in many proteins, where it is functionally inactive. Functional and individual Se status assessment may be evaluated using 2 markers, the activity of which is related to Se body supply: GPx concentration in whole blood, plasma, erythrocytes or thrombocytes and Selenoprotein P (SePP) in plasma/serum, where the latter is more accurate due to the crucial role in tissue Se distribution and higher required plasma Se concentration (100–120 μg/L) than for GPx (90 μg/L) to be fully expressed. Therefore, the optimal plasma/serum Se concentration of 120–125 μg/L reflects the maximal selenoprotein activity and sufficient Se storage. The positive correlation between blood Se and SePP exists in subjects until adequate Se concentration fully saturates the selenoprotein. However, both serum biomarkers are not tissue-specific and do not reflect the thyroid Se concentration [8, 15, 16]. Of note, there are no proposed region-specific reference ranges for plasma Se, which would be especially beneficial in the context of thyroid diseases, and no reliable thyroid biomarkers for Se status assessment are currently available.

Se optimal supply and health

According to Rayman et al., only Se-deficient individuals are reasonable candidates for substitution of this micronutrient due to the proposed U-shaped relationship between Se and health effects, where neither Se deficiency nor Se overdosing are beneficial [17].

Except for AIT, selenopenia is associated with increased risk for Keshan and Kashin-Beck disease, colorectal cancer in females and prostate cancer in males, infertility/reproduction complications, mental decline, more flawed immune defence, increased viral virulence, and higher mortality. Conversely, excessive Se intake produces selenosis (nail and hair loss, garlic breath odour, tachycardia, gastrointestinal and neurological symptoms), dermatitis, non-melanoma skin cancer, and increased prostate cancer risk and mortality. According to some studies, prolonged Se supplementation may also facilitate diabetes mellitus type 2 (DM2) development [18,19]. Nevertheless, the latest meta-analysis from 3 RCTs (SELECT, NPC, and Selenium Trial) analysing 20,290 participants with 200 μg Se daily intake for 3–7.7 years, showed only a nonsignificant increase in DM2 occurrence between the study and placebo group (OR = 1.18, 95% CI: 0.95–1.4). However, observational studies have linked high Se status with increased DM2 incidence (OR = 2.03, 95% CI: 1.51–2.72) [20]. Of note, the tendency was observed only in the highest Se concentration quartiles, in which sufficient Se supply was exceeded, and it mainly focussed on males [21]. On the other hand, DM2 or insulin resistance may trigger the liver to overproduce SePP, which alters per se plasma Se concentration. To explain whether high Se is a cause or effect of impaired glucose metabolism, convincing large RCTs and meta-analyses are needed [22]. Some authors associate Se deficiency with development of metabolic syndrome, obesity, and DM2 as a first-line consequence [21].

Se in diet supplements

The bioavailability, bioactivity, and potential toxicity of Se in the diet largely depend on the type of food and chemical properties of associated micronutrients. Se is present in the diet primarily in the organic form of selenocysteine (SeCys) and SeMet, which is most likely absorbed via transcellular diffusion in the small intestine [23]. On the other hand, the inorganic form of Se, selenite or selenate, is present in food to a lesser degree. SeMet is a form of Se that is most readily absorbed. At the same time, SeCys, selenate, and selenite are less bioavailable [24]. Supplementation of Se is frequently achieved using SeMet or Se yeast (60% of which is SeMet) [25]. If supplementation is recommended to a patient, preference should be given to the organic form, SeMet, rather than inorganic. An attractive therapeutic option may be the patented Danish product by Pharma Nord (SelenoPrecise®), used in large RCT (UK, Danish, Swedish PRECISE), which contains organic Se yeasts (100 μg of Se per tablet, stable Se content: 66% of SeMet and < 1% of inorganic forms) with high bioavailability of 88.7%, proven by the EFSA [26].

Se supplementation in euthyroid AIT patients

The idea of Se supplementation in thyroid diseases (mainly AIT) arose from epidemiological studies, in which Se-depleted regions had a higher number of AIT patients. A Chinese study of 3038 people from Se-rich and 3114 subjects from low-Se provinces proved increased prevalence and higher odds ratio (OR) for AIT (OR = 0.47, 95% CI: 0.35–0.65), SHT (OR = 0.68, 95% CI: 0.58–0.93), overt hypothyroidism (OR = 0.75, 95% CI: 0.63–0.90), and goitre (OR = 0.75, 95% CI: 0.59–0.97) in the latter group ; altogether 18 *vs.* 30.5% ($p < 0.001$)

[27]. In addition, in a comparator multi-centre study from 4 European low Se-equipped countries, the Se concentrations were significantly lower in AIT patients than in patients with non-AIT thyroid diseases [13]. The obtained results suggest that Se supplementation might reduce the incidence of thyroid diseases. The majority of studies concerning Se supplementation were performed on euthyroid AIT patients. The commonly measured primary outcome in those studies was a decrease in TA. However, some authors underlined the need to find clinical evidence, expressed by thyrotropin (TSH) decrease (or no progression to overt disease) or lowering LT4 dose in the course of combined LT4 and Se treatment. US echogenicity, oxidative and inflammatory body status, or QoL are frequently considered as secondary outcomes. Assessment of TA (the higher the level, the better the response), individual Se level at baseline (substitution or supplementation), geographical area of living, and iodine status (iodine deficiency exacerbates the effects of Se deficiency) should be taken into account in the interpretation of results. In the latest published meta-analysis (2017) concerning data from European and Brazilian patients, the authors failed to find the disease progression (expressed by TSH rise) in LT4-untreated subjects after 3, 6, or 12 months of Se supplementation [28]. Another meta-analysis (2016) by the same authors revealed that levels of anti-thyroid peroxidase antibodies (a-TPO) and anti-thyroglobulin antibodies (a-TG) declined significantly after 3 months in the group of patients who did not receive LT4. In the LT4-treated group a-TPO decreased at 3, 6, and 12 months, while a-TG decreased only after 12 months, which is in accordance with the previous meta-analysis results. Se supplementation favourably impacted the mood and general well-being of participants in this study [29, 30]. The countries that were analysed in these studies had a baseline plasma Se concentration below the normal range and reached a Se serum concentration of 70–85 μg/L and 37 μg/L upon conclusion of the European and Brazilian studies, respectively. Both meta-analyses underline no justification for routine Se supplementation in AIT euthyroid patients, due to lack of clinical evidence for improvement of the disease course. The adequately Se supplied AIT population without LT4 treatment (basal serum Se 110 ± 16 μg/L in the study group and 123 ± 19 in healthy controls) had significantly decreased a-TPO, but not a-TG and TSH, after 3 months of 200 μg/day Se treatment in comparison to healthy controls. Additionally, the oxidative marker panel (total antioxidant capacity, superoxide dismutase, malondialdehyde) indicated an oxidative stress pattern in AIT patients compared to healthy controls. This improved significantly after Se intervention in the first group. The authors concluded that Se alleviates the thyroid autoimmune process by improving body antioxidative status [31]. Se supplementation (60 and 180 μg/day) significantly reduced proinflammatory cytokine release mediated by INF-γ (CXCL-9 and 10) in AIT euthyroid females who were not treated with LT4 in comparison to the placebo group. This serves as evidence for a Se-positive immunomodulatory effect and may prove advantageous in cases of an undetected change in thyroid parameters [32]. An Italian group (2016) found fT3 increases at 3 and 6 months and an fT4 decrease 3 months after Se supplementation in patients at the time of AIT diagnosis without LT4 treatment. The results of peripheral hormones were explained by the enhanced iodothyronine deiodinase activity due to incorporation of Se, which led to the augmented conversion of fT4 to fT3 [33]. Although Se supplementation resulted in a 9.9% a-TPO decrease after 6 months in AIT patients in one study, its discontinuation led to an a-TPO level rise of 4.8% at 12 months. In contrast, the continuation of Se supplementation reduced a-TPO further by up to 21% at the above-mentioned time point. Interestingly, the observed a-TPO level in the group who continued the treatment at the end of the study was more reduced among non-smokers [34]. Nevertheless, no effect on a-TPO and TSH was seen in a well-designed, randomised, placebo-controlled, and double-blind study from an iodine-sufficient region, after 200 μg/day sodium selenite for 6 months given to Se-deficient (not fully expressed SePP) euthyroid AIT females without LT4 treatment. The possible explanation may be that iodine-sufficient subjects are less sensitive to immunosuppressive Se properties [35]. Interindividual variations in response to Se supplementation among AIT patients may be explained by genetic variation. Genotyping revealed that single nucleotide polymorphism in r25191g/a of SePP gene (*SEPP1*) is expressed in Se responders, which may be associated with a more prominent a-TPO decline [36].

Se supplementation in hypothyroid AIT patients

The results of the "SETI" study were published in 2020. The research included patients with SHT, in whom TSH was within the range 4.26–10.00 mIU/L, due to AIT (positive a-TPO), with no history of LT4 treatment. After 4 months of treatment with 83 μg/day of SeMet, 48.9% of participants (responders) re-established euthyroidism (TSH ≤ 4.2); the rest (non-responders) remained hypothyroid (TSH > 4.2). At the end of the study, Se levels in serum significantly increased, while fT4 concentrations significantly decreased in

both groups versus baseline. Six months after Se withdrawal, the TSH level was controlled again; in the non-responder group 14.2% of patients achieved euthyroidism, while in the responder group this was up to 83.3%. The authors indicated that 4 months of Se supplementation allows for normalisation of TSH levels. Thus, Se is a promising therapeutic option for patients with SHT [37]. The aforementioned observation is pursuant with a previous study (2016), which was conducted on a group of patients with mild SHT due to AIT. The time of treatment and the dose of SeMet were the same. At the end of the study, 17.2% of participants were again euthyroid. Follow-up studies conducted 5 months later showed that euthyroidism was maintained mainly in the Se group [38]. These studies included patients with no history of thyroid hormone therapy, but Se treatment may also be useful as an adjunct to LT4 for patients with AIT and SHT. SeMet supplementation with 200 μg/daily with LT4 for 6 months caused a significant reduction in a-TPO concentration: 46% after 3 months and 55.5% after 6 months. The group receiving only LT4 had a decrease of a-TPO of 21% after 3 months and 27% after 6 months. There were no significant changes in a-TG and thyroid hormone levels. The reduction of a-TPO levels might be the result of the influence of Se on the immune system and oxidative stress [39]. Se supplementation with LT4 is also effective in reducing the a-TPO level in AIT hypothyroid patients. Three months of treatment with 200 μg sodium selenite/day + LT4 resulted in a significant reduction in a-TPO and a-TG levels [40]. Se added to LT4 in euthyroid and hypothyroid AIT patients decreased a-TPO and a-TG more than in patients treated exclusively with LT4. Moreover, euthyroid AIT patients were less Se deficient at baseline than hypothyroid ones, and the latter subgroup reached lower TA levels after Se treatment. These results showed that Se supplementation in addition to LT4 may be beneficial in the later disease stage. Additionally, Se intake significantly ameliorated levels of proinflammatory cytokine (IL-2) and therefore corrected disturbed immune balance in AIT patients, which was not observed in the group without Se supplementation [41]. However, another study showed that SeMet supplementation (200 μg/day) had no significant effect on the a-TPO level; however, this resulted in a substantial reduction in a-TG after 3 months of treatment [42]. The reason for the different responses to Se supplementation remains unclear. However, the duration of the treatment and the dose of Se preparations may affect the results. It is also possible that the initial concentration of TA, Se level, and thyroid disease duration play a crucial role in the outcome.

Se supplementation in AIT and healthy pregnant women

Se supplementation in pregnancy is currently not supported by the American Thyroid Association (ATA) due to conflicting results of studies, omitted impact of baseline Se and iodine status, and potential DM2 risk [43]. European Thyroid Association (ETA) guidelines do not raise the issue of Se supplementation for the management of subclinical hypothyroidism in pregnancy and children. Nevertheless, the majority of studies justify its potential advantages. Considering the physiological Se decline throughout pregnancy (partially due to haemodilution), the risk of developing severe Se deficiency is higher in this group [44]. In healthy pregnant females from Poland, Se concentrations physiologically decreased by 24% from the first trimester to the end of pregnancy [12]. However, Se deficits in AIT pregnant females were not more intensified than in healthy pregnant controls from a Se-depleted, mildly iodine-deficient area [45]. The question of whether low Se status has a negative impact on the course of AIT in pregnancy remains unanswered. In pregnant women, similarly to the general population, Se supplementation reduces the TA titre. Taking into account the narrow therapeutic index of Se, interventional studies on pregnant females are frequently characterized by lower Se proposed doses in comparison to the rest of the studied populations. The "SERENA" study (2019) demonstrated a significant reduction of a-TPO in the third trimester of pregnancy and 6 months after delivery in euthyroid females with AIT taking 83 μg of SeMet since their first trimester. Although an a-TPO decrease during pregnancy is a natural immunosuppressive body reaction, preventing rejection of the foetus, TA tends to rise dramatically after delivery, as observed in the placebo group. The above-mentioned results provide evidence that Se supplementation during pregnancy reduces the risk of postpartum thyroiditis (PPT) [46]. This is in accordance with the previous finding, where 200 μg of SeMet given to AIT women (with or without LT4) from the 10th gestation week until the postpartum period significantly reduced the incidence of PPT (28.6% *vs*. 48.6% for placebo) and persistent hypothyroidism (11.7% *vs*. 20.3%). Additionally, the researchers observed an improvement in US echogenicity of thyroid parenchyma in the Se-treated group [47]. However, in euthyroid AIT patients with no additional LT4 treatment recruited for "SPRINT" (double-blind, randomized, placebo-controlled study), in which the group of 114 women from a mild-to-moderate iodine-deficient country received a lower dose of Se (60 μg/day) from the 12th week of pregnancy, no reduction in a-TPO (baseline lower median levels than

in the previous study) was observed (54.2 *vs.* 65.6% in placebo). Nevertheless, Se modulated thyroid function by increasing fT4 ($p < 0.029$) and decreasing TSH ($p < 0.050$) throughout the pregnancy more in the Se group than in the placebo group. This may be explained by the additive Se immunomodulatory effect on the AIT course [48]. An interesting interventional clinical trial on the influence of immunomodulatory factors (including low Se dose) on intracytoplasmic sperm injection (ICSI) effectiveness in infertile AIT euthyroid patients was registered in 2019; however, the results have not been published yet [49].

Moreover, Se intake may also contribute to an uncomplicated pregnancy course. Within the "SPRINT" group, Se supplementation in Se-depleted subjects resulted in reduction of OR for developing pregnancy-induced hypertension or preeclampsia [50]. Women with the lowest Se serum concentration in the 10th-14th gestation week (from quartile Q_1) were 3 times more prone to deliver small-for-gestational-age (SGA) newborns (OR = 3.02, $p = 0.019$) than in other quartiles [51]. Maternal Se-rich food intake was shown to decrease the risk of preterm birth [52]. In the population of women with spontaneous abortions, Se levels were significantly lower than in controls, potentially due to the associated risk of increased placental oxidative stress and trophoblast damage in early pregnancy [53].

Encouraging evidence originates from infertility studies of men and women, in which Se supplementation of < 200 μg/day positively impacted sperm motility and enhanced oocyte cycle evolution, possibly by reducing oxidative stress [54].

Future double-blind placebo-controlled studies raising the issue of Se supplementation in pregnancy are warranted, especially in high-risk pregnancies in women from Se-depleted regions. Concomitant assessment of SePP as a marker of individual response for adjusting correct Se doses and the mutual interplay between different micronutrients may be relevant [55, 56].

The impact of Se on quality of life

Few clinical trials have assessed the effect of Se supplementation on QoL. According to these studies, Se supplementation (as sodium selenite or SeMet 200 μg/day for 3-6 months) resulted in improved well-being and mood and diminished fatigue in a group of patients with AIT, compared to controls [39, 57, 58]. More insight is expected from the "CATALYST" study, which is scheduled to be completed in December 2021. The aim of the study is to assess whether 200 μg/day Se supplementation for 12 months alongside LT4 will have an impact on QoL in AIT patients [59].

The impact of Se on the US image

Se influence on thyroid US remains questionable. Supplementation of 50 μg SeMet per day for 3 months among children with AIT led to thyroid volume regression by over 30% [60]. In a study of a large group of patients (1100 women; 792 men), an inverse association between Se level and thyroid volume was shown ($p = 0.003$). In a group of tested women, an increase in serum Se concentration by 1 μmol/L caused a decrease in the thyroid gland volume by 0.18 mL. Moreover, low Se status was significantly related to goitre risk (OR 0.07, 95% CI: 0.008–0.6) and thyroid hypoechogenicity (OR = 0.2, 95% CI: 0.06–0.7), but not to thyroid nodules. In the male group, there was no relationship between Se level and thyroid structure or thyroid volume [61]. Another study showed that low Se concentration increases the risk of developing multiple nodules in the thyroid gland ($p = 0.087$) [62]. A Se dose of 200 μg/day with LT4 for 3 months in AIT patients improved thyroid US echogenicity [58]. On the other hand, in studies where Se was supplemented in a dose of 80 μg, 160 μg, or 200 μg for 3–12 months there was no change in echogenicity and volume of the thyroid gland at the end [32, 63]. Further research is essential to find the optimal dose and time required for Se to exert its beneficial effects. It is necessary to investigate patterns of thyroid US in patients' who would benefit most from the supplementation.

Se supplementation in non-AIT hypothyroidism

Dietary Se deficiency may cause hypothyroidism, which was identified in 3 cases of children with SHT and no signs of infection. The thyroid US picture was unaffected. The plasma Se concentrations in subjects were 40 μg/L, 32 μg/L, and 43 μg/L. Each child received 10 μg/kg daily of sodium selenite. After 4 weeks, clinical symptoms improved, and thyroid gland parameters and Se levels became normal. The authors concluded that the direct cause of hypothyroidism was Se deficiency and reduced activity of type II 5'-deiodinase, which confirms the vital role of Se in the aetiology of hypothyroidism, even without concomitant AIT [64]. With that said, high-quality evidence is needed to confirm the above observation. This effect may be applicable to hypothyroidism coexisting with other end-stage chronic diseases, in which fT4 to fT3 conversion is impaired — for instance, in renal failure [65]. Determination of hypothyroid aetiology (AIT *vs.* non-AIT) and distinguishing these 2 subgroups would be advantageous in future clinical trials.

Se supplementation in a paediatric population

A Se deficit in childhood is a considerable risk factor for poor physical and cognitive development [66]. Se plays an important role in synaptogenesis, myelination, and neuronal cell differentiation by regulating thyroid hormones [67]. Maternal erythrocyte Se (Ery-Se) concentrations were associated with neonatal development measures at 1.5 years [68]. An increase in maternal Se by 0.50 μg/g haemoglobin was associated with improved language comprehension by 3.7 points (0.5 SD, 95% CI: 0.40–7.1, p = 0.028) and increased psychomotor development by 12 points in girls (0.9 SD, 95% CI: 4.3–19, p = 0.002) at 1.5 years of age. In a follow-up study, a positive association was also observed between maternal Ery-Se in early pregnancy and the outcomes in children at 10 years [69]. An increase of maternal Ery-Se from 5th to 95th percentile was associated with a full developmental score by approximately 8.1 points (95% CI: 3.8–13). Similarly, a Polish study reported a significant positive association between Se levels in blood collected during the first trimester of pregnancy and motor skill development at 1–2 years of age and cognitive development at 2 years of age, and borderline association (= 0.2, p = 0.05) was observed for language development at 2 years of age [44]. A similar association between maternal urinary Se (23 ± 8.6 μg/L) and cognition at 4 years of age was also reported in Greece (n = 575) [70]. Conversely, a recent study from Spain suggests an inversion of this relationship between maternal Se concentration and neuropsychological development at 1 year of age when the Se concentration in the first trimester exceeds 86 μg/L [71]. However, a study conducted in the U.S. did not identify an association between high levels of Ery-Se in pregnant women (206 μg/L, n = 872) and children's cognitive function at 7.7 years of age [72]. Assessment of preschool children in Ethiopia revealed that low serum Se was associated with lower cognitive scores as validated tests [66]. Another body of research focuses on the effects of Se nutritional intake in children with autism spectrum disorder (ASD). A recent literature review of 4 studies found significant differences in Se tracing in the hair of children with ASD, compared to unaffected children [73]. Two of these 4 studies found increased Se levels in the hair of children with ASD, and the other 2 studies reported the opposite finding. With that said, more studies will be needed to evaluate the significance of Se and ASD association and its clinical implication. Additionally, Se deficiency was associated with the development of Keshan disease in children between the ages of 2 and 7 years and women of reproductive age. The possible aetiology may be related to mutation promoting in a poor Se environment in the genetic material of Coxsackie B virus's genetic material, resulting in its increased virulence and extensive myocardial damage leading to dilated cardiomyopathy [74]. An Se shortage in neonates and children is a risk factor for decreased motor and cognitive function. However, children's cognitive outcome is also affected by other factors associated, among others, with poor care, inadequate cognitive stimulation, and parasitic infections [75, 76].

Se supplementation in adults and the elderly population

Fluctuations in Se serum concentration are believed to intensify chronic inflammation, increase monocyte migration into the endothelium, and promote monocyte transformation into macrophages and eventually foam cells, thus advancing the development of atherosclerosis and increasing the risk of cardiovascular diseases (CVD) [77]. A recent meta-analysis reported a 50% increase in Se concentration to be associated with 24% reduction in the risk of developing ischaemic heart disease [78]. A similar conclusion was drawn by a study that reported a reduced risk of CVD (RR: 0.66, 95% CI: 0.40–1.09) and mortality (RR: 0.69, 95% CI: 0.57–0.84) in individuals with high Se status [79]. Also, UK and Denmark PRECISE randomised, double-blinded, placebo-controlled, clinical trials explored the effects of Se supplementation on total cholesterol (T-C) and non-HDL cholesterol [80, 81]. In the UK PRECISE clinical study (n = 501), healthy participants (60 to 74 years old) were supplemented with 100, 200, or 300 μg/day Se-enriched yeast tablets or placebo for 6 months [80]. Compared to placebo, Se supplementation of 100 and 200 μg/day significantly decreased T-C and non-HDL cholesterol in the plasma. Supplementation with 300 μg/day for 6 months significantly increased HDL-cholesterol but did not affect either total or non-HDL cholesterol. Conversely, a similar study carried out in a Danish population found no significant difference in total or HDL cholesterol concentrations between placebo and Se supplementation groups after 6 months or 5 years, despite similarities in Se baseline concentrations in the 2 populations (88.6 μg/L in Denmark, 91.2 μg/L in the UK) [81]. Se homeostasis is also implicated in the maintenance of blood pressure. A recent observational study conducted in a group of 2169 Inuit described a potential blood pressure-lowering effect seen with an Se-rich diet [82]. Participants with low Se and high mercury serum levels were more likely to develop CVD, including hypertension (OR = 1.76), stroke (OR = 1.57), and heart attack (OR = 1.26). In contrast, other studies described a positive association between high Se

serum concentration and hypertension [83, 84]. Based on the given research, the cardiovascular benefits of Se supplementation are unclear and should not be recommended. Both Se deficiency and excess have been associated with increased mortality. According to U.S. Third National Health and Nutrition Examination survey analysis (n = 13887), mortality exhibited a U-shaped association with the lowest mortality rate at a serum Se concentration of 135 μg/L and high mortality with either lower or higher Se concentration [4, 85]. In the Danish PRECISE clinical trial, mortality was assessed 10 years after 5-year supplementation with 100, 200, or 300 μg Se/day as Se-enriched yeast or placebo yeast [86]. Supplementation with 300 μg Se/day resulted in an 11.3% (95% CI: 0.0–22.6%) increase in all-cause mortality compared to placebo 10 years after treatment. Conversely, a prospective study found an inverse relationship between Se intake and all-cause CVD mortality in men and women. However, no significant difference in mortality was observed between Se intake and cancer-related mortality in both sexes [87].

Conclusions

According to our literature review, the recommendation of Se supplementation is supported by everyday clinical practice. However, Se supplement seems to be uncontrollably prescribed (no baseline and follow-up Se assessment) and not adjusted to the specific population's profile (children, adults, males, females, pregnant, non-pregnant, AIT, non-AIT thyroid disease, and area of living). According to the authors' best knowledge, no commercially available oral Se preparation has been approved by the U.S. Food and Drug Administration. Thus, available Se formulations and effective doses need to be revised and standardised. The reference ranges for Se concentrations in various geographical areas are lacking, and there is a need to determine thyroid tissue-specific biomarkers for Se functional assessment. Clinical trials have explored many health benefits of Se supplementation; however, its narrow therapeutic index, patients' geographical location, individual Se intake, and specific clinical setting should be considered to evaluate the benefit-risk balance before Se supplementation. Future studies comparing regions with varying natural supplies of Se and iodine and investigating the effects of the interplay of these elements on the course of AIT would be valuable. Among successful Se supplementation candidates may be pregnant females, especially those from iodine- and Se-depleted areas, with risk factors like AIT. Se implementation in this population may prevent the development of selenopenia, PPT, hypertension, and poor neonatal outcomes. However, clear recommendations on indications for treatment, therapy duration, and suitable doses need to be established. Factors determining the response to Se should be elucidated, taking into consideration genetic predisposition and mutual interrelations with other trace elements.

REVIEW

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Metformin in patients with type 2 diabetes mellitus and heart failure: a review

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Abstract

Diabetes mellitus is a major, global problem. Among the numerous complications of diabetes, there is increasing concern over the coexisting heart failure. Metformin is the most frequently used oral antidiabetic drug that is considered to be safe and effective in the management of type 2 diabetes mellitus. Since the publication of the UK Prospective Diabetes Study, it has been suggested that metformin might improve cardiovascular prognoses. Results from available studies have shown that metformin therapy in patients with type 2 diabetes mellitus and heart failure was associated with improved clinical outcomes when compared with other oral antidiabetic agents, insulin, or lifestyle management. However, there have been no randomized controlled trials evaluating the influence of metformin use on clinical outcomes in patients with type 2 diabetes mellitus and heart failure. New evidence from large cardiovascular outcome trials that showed a reduction in heart failure hospitalization for SGLT2 inhibitors caused changes in recommendations on the management of hyperglycaemia. Currently, the European Society of Cardiology recommends sodium-glucose co-transporter 2 inhibitors in patients with type 2 diabetes mellitus and heart failure or at high risk for heart failure, as a first choice in drug naïve patients, or as a second drug if the patient is already on metformin. The aim of our study is to review the current state of knowledge about the position of metformin in the treatment of patients with type 2 diabetes mellitus and heart failure. **(Endokrynol Pol 2021; 72 (2): 163–170)**

Key words: *metformin; type 2 diabetes mellitus; heart failure*

Introduction

Diabetes mellitus is a significant medical, social, and economic problem. Data from the International Diabetes Federation show that diabetes affects 463 million adults worldwide, and it is estimated that one in two people living with diabetes are unaware of their condition [1]. In Poland, the number of people suffering from diabetes amounted to 2.533 million in 2017 [2]. Among the numerous health complications of diabetes mellitus there is increasing concern over a previously undervalued issue: coexisting heart failure (HF).

In 2020, on the 56th annual meeting of the European Association for the Study of Diabetes (EASD), the CAPTURE study on the prevalence of cardiovascular disease (CVD) involving 9823 patients with type 2 diabetes mellitus (T2DM) from 13 countries was presented. Overall, 34.8% of participants had CVD, and 2.4% suffered from HF [3]. In the analysis from the CVD-REAL 2 multinational cohort study on the risk of cardiovascular (CV) events and death in 38,6248 adult patients with T2DM, newly initiated on sodium-glucose co-transporter 2 (SGLT2) inhibitors or dipeptidyl peptidase-4 (DPP-4) inhibitors, HF was present in 7% of patients at baseline [4]. The prevalence of HF in diabetic patients in real-world studies ranges from 5 to 6.8% [5, 6].

Metformin is the most frequently used oral antidiabetic drug that is considered to be safe and effective in the management of T2DM [7]. It acts as a glucose-lowering agent through the decrease of hepatic glucose production, as well as lowering insulin resistance in peripheral tissues. Besides its neutral effect on body weight and its positive impact on lipidogram, metformin's influence on the CV system seems to be cardioprotective, because it has a beneficial impact on the vascular wall and clotting system parameters [8]. Since the publication of UK Prospective Diabetes Study 34 (UKPDS 34), metformin has emerged as a drug that seems to decrease the risk of diabetes-related endpoints, including macrovascular and microvascular complications, in overweight patients with T2DM. The results of UKPDS 34 showed that patients who were allocated metformin had a 32% lower risk ($p = 0.0023$) of developing any diabetes-related endpoint, a 36% lower risk

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(p = 0.011) of all-cause mortality, and a 39% lower risk (p = 0.010) of myocardial infarction (MI), in comparison to management with diet alone [9]. Moreover, in the post-trial 10-year follow-up of intensive glucose control, metformin proved to have a long-term effect on macrovascular outcomes, decreasing the risk of MI by 33% (p = 0.005) [10].

New evidence from large cardiovascular outcome trials (CVOTs), which showed CV benefits from the use of newer glucose-lowering drugs in patients with CVD or at very high/high CV risk, led to changes in the recommendations on the management of hyperglycaemia.

The aim of this article is to review the current state of knowledge about the position of metformin in the treatment of patients with T2DM and HF.

Metformin in patients with type 2 diabetes mellitus and heart failure: a review of research studies

In the analysis of 1833 new users of oral antidiabetic agents with incident HF, metformin use, in monotherapy (n = 208) or in combination with sulfonylureas (n = 852), was compared with sulfonylurea monotherapy (n = 773) (Tab. 1). During the 2.5-year follow-up, all-cause mortality and all-cause hospitalization, both at 1 year and at the end of the follow-up period, were evaluated. Metformin, alone or in combination with sulfonylureas, was associated with reduced 1-year and longer-term all-cause mortality in comparison to sulfonylurea monotherapy. On the other hand, there was no significant association between compared groups and all-cause hospitalization. In the composite outcome analysis fewer deaths and/or hospitalizations occurred in patients on metformin monotherapy and combination therapy when compared with sulfonylurea monotherapy [11].

Sulfonylurea monotherapy was also used as a reference group in the observational study of 10,920 patients treated with metformin, sulfonylureas, and/or insulin, and hospitalized for the first time for HF. Metformin in monotherapy and in combination with sulfonylureas was associated with lower all-cause mortality compared with sulfonylureas in monotherapy. The results were similar in a separate analysis of patients using and not using insulin [12].

In the observational study of 16,417 patients with T2DM, discharged from a hospital with a major discharge diagnosis of HF, the influence of insulin-sensitizing drugs on 1-year all-cause mortality, 1-year all-cause hospitalization, and HF hospitalization was assessed. Individuals treated with thiazolidinediones (n = 2226), metformin (n = 1861), as well as both thiazolidinediones and metformin (n = 261) had a lower risk of death compared with patients not treated with insulin-sensitizing drugs (n = 12069). There was no difference in all-cause hospitalization between insulin-sensitizing agents, although patients receiving metformin had a lower risk of hospitalization for HF, while patients receiving thiazolidinediones had a higher risk of hospitalization for HF when compared with the therapy without an insulin-sensitizing drug [13].

MacDonald et al. designed a case-control study that assessed treatment with metformin in patients newly diagnosed with HF and T2DM, during a median follow-up of 2.8 years. A comparison group consisted of individuals without an antidiabetic drug in their therapy. Metformin monotherapy was associated with lower all-cause mortality when compared with patients not exposed to antidiabetic therapy [14].

In the analysis of 6185 ambulatory patients with HF and T2DM, 1561 participants treated with metformin were compared with 4624 patients not treated with metformin. During the follow-up period of 2 years, the risk of death and the risk of hospitalization were assessed. Additionally, the relationship between metformin use and outcomes (time to death, time to HF hospitalization, and time to any hospitalization) was assessed using propensity score-matched analysis that consisted of 29 baseline variables, and compared patients receiving metformin with patients not receiving metformin. Metformin therapy was associated with reduced mortality in comparison to therapy without metformin, in both unadjusted and propensity score-matched analysis. The risk of hospitalization for HF as well as all-cause hospitalization was lower in patients receiving metformin compared with those not receiving metformin in unadjusted analysis; however, in propensity score-matched analysis there was no statistically significant difference between groups [15].

Shah et al. investigated the use of metformin in patients with left ventricular ejection fraction (LVEF) below 40% (mean LVEF 24 ± 7%) and T2DM. Forty-two per cent of patients were in New York Heart Association (NYHA) class III and 45% in NYHA class IV. Ninety-nine patients who were on metformin therapy, as a monotherapy or in combination with other antidiabetic drugs, were compared with 302 patients using oral antidiabetic drugs other than metformin and/or insulin. During the 6-month follow-up period, LVEF significantly improved in patients on metformin therapy compared with patients without metformin in their therapy. However, after adjustment for angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blockers (ARB) and B-blocker therapy the improvement in LVEF was nonsignificant. Patients on metformin therapy had significantly longer survival and lower risk of combined endpoint (all-cause mortality and the need for urgent

Table 1. *Metformin in patients with type 2 diabetes mellitus and heart failure (HF): a review of research studies*

Clinical trial	Population	Comparison groups	Median follow-up	End points	Results adjusted HRs (95% CI) or percentage
Eurich et al. [11]	1833 new users of oral antidiabetic agents with incident heart failure	Metformin monotherapy (n = 208) Combination therapy: sulfonylurea and metformin (n = 852) *vs.* Sulfonylurea monotherapy (n = 773)	2.5 years	1-year all-cause mortality	Metformin monotherapy 0.66 (0.44–0.97) Combination therapy 0.54 (0.42–0.70);
				Long-term all-cause mortality	Metformin monotherapy 0.70 (0.54–0.91) Combination therapy 0.61 (0.52–0.72)
				1-year all-cause hospitalization	Metformin monotherapy 0.84 (0.67–1.04) Combination therapy 0.92 (0.80–1.06)
				Long-term all-cause hospitalization	Metformin monotherapy 0.87 (0.73–1.05) Combination therapy 0.93 (0.83–1.05)
				1-year composite outcome (all-cause hospitalization or all-cause mortality)	Metformin monotherapy 0.79 (0.65–0.98) Combination therapy 0.86 (0.75–0.98);
				Long-term composite outcome (all-cause hospitalization or all-cause mortality)	Metformin monotherapy 0.83 (0.70–0.99) Combination therapy 0.86 (0.77–0.96);
Andersson et al. [12]	10,920 patients hospitalized for the first time for HF with prior diabetes	Metformin monotherapy (n = 688) Metformin plus sulfonylurea (n = 1549) Metformin plus insulin (n = 468) Metformin plus sulfonylurea plus insulin (n = 247) Sulfonylurea plus insulin (n = 635) insulin (n = 3718) *vs.* Sulfonylurea monotherapy (n = 3615)	844 days	Long-term all-cause mortality	Metformin monotherapy 0.85 (0.75–0.98) Metformin plus sulfonylurea 0.89 (0.82–0.96) Metformin plus insulin 0.96 (0.82–1.13) Metformin plus sulfonylurea plus insulin 0.94 (0.77–1.15) Sulfonylurea plus insulin 0.97 (0.86–1.08) Insulin 1.14 (1.06–1.20) Separate analysis of patients not using insulin, compared with the use of sulfonylureas as monotherapy: • metformin monotherapy 0.89 (0.78–1.02) • sulfonylurea plus metformin 0.9 (0.83–0.93) Separate analysis of patients using insulin, compared with patients receiving sulfonylureas plus insulin: • metformin plus insulin 0.96 (0.79–1.15) • metformin plus sulfonylurea plus insulin 0.96 (0.77–1.19) Insulin in monotherapy 1.17 (1.04–1.3)

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Table 1. *Metformin in patients with type 2 diabetes mellitus and heart failure (HF): a review of research studies*

Clinical trial	Population	Comparison groups	Median follow-up	End points	Results adjusted HRs (95% CI) or percentage
Masoudi et al. [13]	16,417 patients with diabetes discharged after hospitalization with the principal discharge diagnosis of heart failure.	Metformin in therapy (n = 1861) Thiazolidinedione in therapy (n = 2226) Combination therapy: thiazolidinediones and metformin (n = 261) *vs.* no insulin sensitizer in therapy (n = 12069)	1 year	1-year all-cause mortality	Metformin in therapy 0.86 (0.78–0.97) Thiazolidinediones in therapy 0.87 (0.80–0.94); Combination therapy: thiazolidinediones and metformin 0.76 (0.58–0.99)
				1-year all-cause hospitalization	Metformin in therapy 0.94 (0.89–1.01) thiazolidinediones in therapy 1.04 (0.99–1.10) Combination therapy: thiazolidinediones and metformin 0.82 (0.69–0.96)
				1-year HF hospitalization	Metformin in therapy 0.92 (0.92–0.99) Thiazolidinediones in therapy 1.06 (1.00–1.09) Combination therapy: thiazolidinediones and metformin 0.85 (0.71–1.01)
MacDonald et al. [14]	1633 case subjects (patients newly diagnosed with diabetes and heart failure who had died; 1633 1:1-matched control subjects (patients newly diagnosed with diabetes and heart failure who had not died)	Metformin monotherapy (n = 376) Sulfonylurea monotherapy (n = 753) Thiazolidinedione monotherapy (n = 9) Insulin monotherapy (n = 230) Combination therapy with insulin (n = 122) Combination oral therapy without insulin (n = 470) *vs.* no antidiabetic drug therapy (n = 1306)	2.8 years	Long-term all-cause mortality	Metformin monotherapy 0.65 (0.48–0.87) Sulfonylurea monotherapy 0.84 (0.67–1.06) Thiazolidinedione monotherapy 1.08 (0.23–5.07) Insulin monotherapy 1.24 (0.85–1.80) Combination therapy with insulin 0.72 (0.44–1.17) Combination oral therapy without insulin 0.74 (0.56–0.99)
Aguilar et al. [15]	6185 ambulatory patients with HF and diabetes; 2874 propensity score--matched patients	Metformin in therapy (n = 1561) *vs.* no metformin in therapy (n = 4624)	2 years	2-year all-cause mortality	0.76 (0.63–0.92)
				2-year all-cause hospitalization	0.94 (0.83–1.07)
				2-year HF hospitalization	0.93 (0.74–1.18)
Shah et al. [16]	401 patients with diabetes and advanced, systolic HF	Metformin in therapy (n = 99) *vs.* no metformin in therapy (n = 302)	2 years	1-year all-cause mortality	0.63 (0.21–1.89)
				2-year all-cause mortality	0.79 (0.36–1.71)
				1-year all-cause mortality/urgent heart transplant	0.85 (0.38–1.92)
				2-year all-cause mortality/urgent heart transplant	0.8 (0.42–1.53)

→

Table 1. *Metformin in patients with type 2 diabetes mellitus and heart failure (HF): a review of research studies*

Clinical trial	Population	Comparison groups	Median follow-up	End points	Results adjusted HRs (95% CI) or percentage
Wróbel et al. [17]	2398 diabetic patients with acute coronary syndrome with no history of cardiovascular disease prior to the reported episode, treated with percutaneous coronary intervention	Metformin in therapy (n = 1199) *vs.* no metformin in therapy (n = 1199)	8 years	EF at discharge < 40%	12% of patients on metformin therapy *vs.* 17% of patients without metformin in therapy
Fácila et al. [18]	835 patients with diabetes discharged with a principal diagnosis of decompensated heart failure	Metformin in therapy (n = 560) *vs.* no metformin in therapy (n = 276)	2.4 years	Long-term all-cause mortality	0.68 (0.53–0.87)

EF — ejection fraction; HR — hazard ratio; CI — confidence interval

heart transplant) compared with patients without metformin in their therapy. In multivariate analysis there was no significant difference in survival between the analysed groups [16].

In the analysis from the PL-ASC registry (Polish Registry of Acute Coronary Syndromes), diabetic patients after acute coronary syndrome treated with percutaneous coronary intervention with no history of prior CVD were assessed. Patients treated with metformin were compared with patients not treated with metformin, before admission to a hospital. LVEF at discharge from the hospital was evaluated. The number of patients with LVEF below 40% was significantly lower in patients on metformin therapy in comparison to patients receiving antidiabetic drugs other than metformin (12% *vs.* 17%, $p < 0.001$) [17].

The impact of metformin use on patients with T2DM discharged from a hospital with a major diagnosis of acute HF was assessed in 835 participants. During a mean follow-up period of 2.4 years, long-term all-cause mortality was significantly lower in patients treated with metformin in comparison to patients without metformin in their therapy. In the multivariate analysis, metformin use was also significantly associated with lower all-cause mortality rates [18].

Metformin in patients with type 2 diabetes mellitus and heart failure: a review of guidelines

In the position statement from the Heart Failure Association (HFA) of the European Society of Cardiology (ESC) on type 2 diabetes mellitus and heart failure, metformin was presented as an antidiabetic drug that might be safe in heart failure and could be recommended as a first-line therapy for patients with T2DM and HF, who have preserved or moderately reduced renal function. However, the document emphasizes the lack of randomized controlled trials of metformin use in patients with T2DM and HF [19].

According to the 2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD, metformin is safe at all stages of HF, reducing the risk of death and HF hospitalization in comparison to insulin and sulfonylureas. In the previous guidelines (2013) metformin was considered as a first-line therapy in patients with T2DM, independently of the patient's cardiovascular profile [20]. However, there have been changes in the recommendations due to the recent publication of several CVOTs that indicate CV benefit from the use of glucagon-like peptide-1 (GLP-1) receptor agonists and SGLT2 inhibitors in patients with T2DM and CVD or those at very high/high CV risk. As a consequence,

the current guidelines suggests that the choice of antidiabetic drug in patients with T2DM should depend on the presence of CVD and CV risk. Thus, patients with T2DM and prevalent CVD or with very high/high CV risk should receive drugs with proven CV benefit, GLP-1 receptor agonists, or SGLT2 inhibitors, as a first choice in drug-naïve patients or as a second drug if the patient is already on metformin. On the other hand, in patients with T2DM without CVD or at moderate CV risk, metformin should be recommended as first-line therapy, especially in overweight individuals. In regard to the selection of glucose-lowering agents in patients with T2DM and HF or at high risk for HF, metformin and SGLT2 inhibitors are the first-line therapy. SGLT2 inhibitors are recommended for the treatment of patients with T2DM and HF (class I of recommendation) because they seem to reduce HF-related endpoints, while metformin should be considered in these patients (class IIa of recommendation), which is based on observational studies and everyday clinical practice [21].

New evidence from CVOTs has also implied an important change in the 2018 Consensus Report by the American Diabetes Association (ADA) and the EASD on the Management of Hyperglycaemia in Type 2 Diabetes. Since SGLT2 inhibitors have been proven to reduce hospitalization for HF in patients with atherosclerotic cardiovascular disease in comparison to placebo, and they are recommended in patients with T2DM and coexisting HF or at risk for HF, as a part of glucose-lowering treatment. Nevertheless, metformin remains the preferred initial medication for the management of T2DM unless it is contraindicated or not tolerated. Subsequently, if HbA_{1c} is above the target and HF predominates, a SGLT2 inhibitor with evidence of reducing HF progression should be added to the therapy [22].

Furthermore, in 2019 the Consensus Report on the Management of Hyperglycaemia in Type 2 Diabetes was updated by the ADA and the EASD, as a consequence of new research findings. The updated report suggests considering an initial combination therapy, composed of metformin and SGLT2 inhibitor, in new-onset diabetes mellitus if HF coexists, independently of baseline HbA_{1c} or individualized HbA_{1c} target, due to reduced hospitalization for HF, major adverse cardiovascular events, and cardiovascular death, especially in patients with reduced left ventricular ejection fraction (LVEF < 45%) [23].

Discussion

The impact of metformin therapy on clinical outcomes in patients with T2DM and coexisting HF has been assessed in large observational studies. In this review, 8 observational studies were included (Table 1). Despite an extensive literature search, no randomized controlled trials evaluating the effects of metformin therapy in patients with T2DM and HF were identified. Indeed, the execution of a randomized controlled trial could be difficult because of the common use of metformin in patients with HF [24, 25]. For many years metformin was contraindicated in patients with HF due to the risk of metformin-associated lactic acidosis. However, the incidence of lactic acidosis in clinical practice has proved to be very low and the United States Food and Drug Administration has removed congestive HF from the boxed warning section of metformin [26]. Nevertheless, hypoxic states such as the setting of acute congestive HF remain contraindications for metformin use, especially when hypoperfusion and hypoxaemia coexist [27]. At the same time, in the Polish Summary of Product Characteristics, HF is consistently one of the main contraindications of metformin, although it should be assumed that this refers to acute states [28].

Data from available studies have shown that metformin use, assessed in monotherapy or in combination with other oral antidiabetic drugs and/or insulin, in patients with T2DM and HF, was associated with improved clinical outcomes when compared with other oral antidiabetic agents, insulin, or lifestyle management. Based on this result, metformin is considered to be a safe drug in heart failure and remains the first-line treatment in patients with heart failure and diabetes, which is supported by clinical experience [22]. However, the safety of metformin in the results of observational studies might be a consequence of its cautious use in patients with HF due to its contraindications. Additionally, when metformin is compared to other antidiabetic agents, there is a probability that the outcomes are the consequence of a harm effect of the comparator agent. This is the reason why there is a need for randomized controlled trials to show not only the safety of metformin but also its beneficial effect on the onset and the course of heart failure.

SGLT2 inhibitors have emerged as antidiabetic drugs that significantly reduce HF hospitalization and CV death in patients with T2DM and high CV risk, when compared with placebo in large CVOTs [29, 30]. Moreover, trials on dapagliflozin and empagliflozin showed that SGLT2 inhibitors reduce HF hospitalization and CV death in patients with HF, not only when diabetes coexists but also in patients without diabetes [31]. Therefore, the position of metformin in the treatment of patients with T2DM and HF has changed. Currently, ESC Guidelines recommend SGLT2 inhibitors in patients with T2DM and HF or at high risk for HF, as a first choice in drug-naïve patients or as a second drug if the patient is already on metformin [21]. However,

this statement is not confirmed by any randomized controlled clinical trial, and it has not been proven that starting glucose-lowering treatment with SGLT2 inhibitors, instead of metformin, is beneficial. In CVOTs, SGLT2 inhibitors were added to the standard therapy in which more than 70% of patients had already received metformin at baseline. Additionally, hospitalization due to HF was a secondary endpoint in each trial; thus, groups of patients with HF were not thoroughly characterized at baseline and were relatively small. Finally, there are no clinical trials on SGLT2 inhibitors that show a reduction of CV risk in patients with $HbA_{1c} < 7\%$, because all of the participants of CVOTs had $HbA_{1c} > 7\%$ at baseline [22].

Conclusions

According to the Summary of Product Characteristics, metformin is contraindicated in patients with HF. However, metformin is the most frequently used oral antidiabetic drug in T2DM; thus, a great number of patients with HF receive metformin despite the contraindications. The analysis of data from observational studies and meta-analyses shows that metformin has a favourable effect in patients with T2DM and HF. Modification of the Summary of Product Characteristics should be considered after performing randomized controlled trials on metformin treatment in patients with T2DM and HF.

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest, expert testimony, or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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Selected thyreology problems during the COVID-19 pandemic. Hypothyroidism and hyperthyroidism — did anything change?

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Abstract
On March 11, 2020, the World Health Organisation (WHO) observed the scale of epidemic risk and declared the state of the COVID-19 pandemic. Most countries, including Poland, implemented national and local emergency management plans to deal with the imminent threat of SARS-CoV-2 infection, one of the most serious in this century, according to many experts. In the era of pandemic, during which an epidemiological regime and social distancing are constantly recommended, and routine medical care and planned surgical procedures have been postponed or significantly reduced, patients and their physicians have to struggle on a daily basis with difficult access to diagnostic and therapeutic procedures. This is a great challenge for both groups. The aim of this study is to assess the current state of knowledge about thyreological diseases during the COVID-19 pandemic and to provide indications for the introduced therapeutic changes on the basis of recent scientific literature published up to December 2020 and searches of the PubMed, Google Scholar, EMBASE, and Web of Science databases, which searched for keywords related to SARS-CoV-2 and its influence on thyreology problems. The main focus was on diagnostic and therapeutic differences in the era of the COVID-19 pandemic, bearing in mind the most common endocrinopathies, i.e. hypothyroidism and hyperthyroidism, as well as advantages and disadvantages and possibilities of using telemedicine in the common practice of a specialist physician. **(Endokrynol Pol 2021; 72 (2): 171–178)**

Key words: *COVID-19 pandemic; COVID-19; SARS-CoV-2; TSH; hypothyroidism; hyperthyroidism; orbitopathy; levothyroxine; antithyroid therapy; ATD*

Introduction

On March 11, 2020, the World Health Organisation (WHO) observed the scale of epidemic risk, which at that time comprised more than 118,000 cases in 114 countries, including 4291 fatalities, and declared the state of the COVID-19 pandemic [1]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in Wuhan, China, which had a huge impact on China and the world. The disease caused by SARS-CoV-2 was named as coronavirus disease 2019 (COVID-19) [2].

Most countries, including Poland, implemented national and local emergency management plans to deal with the imminent threat of SARS-CoV-2 infection, one of the most serious in this century, according to many experts. From March 4, 2020, when the first coronavirus case was registered in Poland, until mid-December this year, more than 1,300,000 confirmed SARS-CoV-2 infections were reported, including more than 28 thousand fatalities [3, 4]. In the era of the pandemic, during which an epidemiological regime and social distancing are constantly recommended, and the routine medical care and planned surgical procedures have been postponed or significantly reduced, patients and their physicians have to struggle on a daily basis with difficult access to diagnostic and therapeutic procedures. This is a great challenge for both groups [5, 6].

The aim of this study is to assess the current state of knowledge about thyreological diseases during the COVID-19 pandemic and to provide indications for the introduced therapeutic changes.

How to treat hypothyroidism effectively

Hypothyroidism is one of the most common endocrinopathies. All these symptoms adversely affect the course of infection with the SARS-CoV-2 virus. The

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subclinical form represents between 3% and 15% of all diseases in the world, while explicit hypothyroidism occurs in 0.3–0.8% of the population [7–9]. It is characterised by a deficiency of peripheral thyroid hormones: thyroxine (FT4) and triiodothyronine (FT3), and although it generally seems quite easy to diagnose and treat, it can even be fatal in severe cases if the appropriate therapy is not implemented. Clinical symptoms vary individually, depending on age, gender, or cause. Most adults show signs of reduced metabolism, such as generalised fatigue, progressive drowsiness, cold intolerance, weight gain, gastrointestinal disorders, as well as reduced tone of voice or memory and concentration disorders. Dry, peeling, thick, and cold skin, hair, eyebrows, and eyelashes loss, and generalised swelling of the eyelids, face, or whole body occur. Moreover, it is possible to observe bradycardia, tachyarrhythmia, or diastolic hypertension. All these symptoms adversely affect the course of infection with the SARS-CoV-2 virus. As a result, among others, the comfort of life is deteriorated, occupational performance is reduced, and the risk of cardiovascular disease is increased. In the case of hypothyroidism, we also observe lipid and carbohydrate disorders [10-15]. The standard method of treatment is hormone replacement therapy with levothyroxine (L-T4), which is carried out chronically, until the end of life, in an endocrinology clinic or general practice [4,9].

Both the American Thyroid Association (ATA) and European societies — the European Thyroid Association (ETA) and the British Thyroid Association (BTA), state in their latest reports that so far no patients with hypothyroidism have been reported to be at greater risk of COVID-19 infection or of a more serious clinical course of the infection, especially if they are pharmacologically balanced. They emphasise that autoimmune thyroid disease does not cause immunosuppression, including that of the p/virus [16–18]. Additionally, BTA states that there is also no evidence of more frequent infections in patients with poorly controlled thyroid disease. Although there are currently no clear reports that the SARS-CoV-2 virus can in any way affect the function of the thyroid gland, in patients with unbalanced, explicit hypothyroidism, in case they become ill, it can be expected that the clinical course of disease may be more complex, and this is due to different systemic relationships. Therefore, it is important that patients do not interrupt L-T4 substitution treatment during the pandemic [17, 18].

The aim of hypothyroidism treatment is to relieve symptoms of the disease, normalise the TSH concentration, and avoid underdosing or overdosing of L-T4. As important as normalisation of the patient's clinical status is biochemical equalisation. Determination of the thyroid-stimulating hormone (TSH) level is the most sensitive test in hypothyroidism of primary origin, and the sensitivity of measurement is estimated to be above 95%, while specificity is about 90%. The TSH is excreted by pulsation, with low pulse amplitude and long T1/2. However, its daily variations are very small and are not significant in routine diagnostics. It is recommended that the TSH determination is carried out in the morning, preferably after a good night's sleep, but it can be performed also during the day. It is necessary during fasting, because a meal inducing somatostatin secretion may inhibit TSH secretion in this mechanism [19–21]. On the other hand, the change of the TSH level after taking L-T4 dose (half-life = 7–8 days) occurs with some delay; thus, it is not important whether the TSH determination will be carried out after the morning drug administration or on a completely empty stomach [14, 19]. However, it is important for the determination of FT4, and therefore in this case it is recommended that the test be performed before taking the morning L-T4 dose. In fact, the reference values of TSH levels, for the whole population, range from 0.25–0.4 mIU/L to 4.2–4.9 mIU/L, depending on the laboratory method used; however, the target TSH levels should vary depending on the age and clinical status of the patient, as shown in Table 1. Therefore, individualisation of the treatment target for hypothyroidism is an important part of the therapy in the present day. Moreover, medicinal products containing L-T4 are characterised by a narrow therapeutic range. For this reason, in choosing an appropriate dose of L-T4, a personalised approach

Table 1. ***Target thyroid-stimulating hormone (TSH) concentration depending on the age and clinical status of the patient according to Biondi et al. [19]***

Age [years]/Clinical status	Serum TSH concentration [mU/L]
Planned pregnancy	Lower norm range < 1.2
Pregnancy I trimester	< 2.5
Pregnancy II and III trimester	< 2.5 or < 3.0
Children with congenital hypothyroidism	< 5.0, optimally 0.5–2.0 with FT4 concentration in the upper normal range at 1 year of age
Young adults	1.0–2.5
Middle age	1.0–3.0
≤ 65	> 4.5
60–70	> 6.0
70–80	> 7.0–8.0
Central hypothyroidism	FT4 in the upper half of the norm
Thyroid cancer	According to the principles of thyroid cancer stratification

FT4 — free thyroxine

to each patient should be adopted to avoid under- or overdosing.

For several years in the United States and in some European countries, and recently also in Poland, a new preparation containing a liquid form of LT-4 produced as an oral solution of L-T4 sodium in purified water and glycerol has been available. Many scientific papers published so far have pointed out that the liquid L-T4 was more effective than the previously commonly used pill form of L-T4, especially in patients with absorption disorders as a result of taking other drugs, diseases of the digestive system, or undergoing bariatric surgery. Better pharmacokinetics of liquid L-T4 in patients without malabsorption syndrome have also been confirmed: patients undergoing replacement or suppressive therapy, who changed their tablet to liquid form in an equivalent dose, achieved better hormone control and required less frequent TSH measurements. The drug also proved to be effective and easy to use in patients fed through an enteral probe. Interestingly, liquid L-T4 seems to be equally effective when taken just before or during the first meal of the day. The analysis of usefulness of the drug in particular groups of patients, including newborns, pregnant women, and the elderly, confirmed the high value and safety of L-T4 liquid therapy. However, it should be emphasised that in the population of examined newborns, a higher incidence of TSH suppression was observed with an equivalent dose of liquid L-T4 compared to tablet therapy. Therefore, special attention should be paid to this group of patients in order to avoid an excessive drug dose [22–30].

Also, in view of this group of patients, who, despite an appropriate dose of L-T4 tablet form, did not achieve satisfactory effects of biochemical equalisation, the change to liquid form of the preparation should be considered. It is also important to remember that in spite of proper biochemical equilibrium in about 10% of patients, unfortunately, no satisfactory clinical equilibrium, i.e. improvement in quality of life (QOL), is achieved in the subjective assessment of a patient [14, 31]. So far, despite the fact that the brain is considered a target organ for thyroid hormones, it has not been proven that there is a reference concentration for TSH that would improve the patient's mood or cognitive function. Samuels MH et al. have shown that despite the objective lack of benefit, patients who received higher doses of L-T4, with lower TSH values, reported an improvement in mental function, which is undoubtedly very important in the era of the COVID-19 pandemic, when the sanitary regime and social distancing play key roles in the fight against SARS-CoV-2 infections [32].

However, it should not be forgotten that preparations of dried thyroid, FT3, or complex FT4 with FT3 are also available on the market. Many randomised studies indicate that therapy with a mixture of synthetic doses of liothyronine (L-T3) and L-T4 in different proportions, although non-physiological, is safe and seems to be effective in controlling the symptoms of hypothyroidism. Patients report faster improvement of mood as well as cessation of depressive disorders. In particular, this is observed in patients with a genetic defect in deiodinase activity. On the other hand, this type of therapy may induce side effects, such as a higher risk of drug overdose and the appearance of symptoms of hyperthyroidism, including cardiac arrhythmias and hypertension. In addition, it is impossible to maintain the most physiologically similar level of free thyroid hormones, thus making it difficult to individualise the dose for each patient, and it may also adversely affect the course of SARS-CoV-2 virus infection [19, 32–35].

A preparation containing FT3 alone in the form of synthetic L-T3 sodium is also available on the market. However, L-T3 has a short half-life and is rapidly absorbed, resulting in large non-physiological variations in its serum concentration. In addition, twice-a-day supply of the drug is required. Although dried thyroid preparations, FT3, or complex FT4 with FT3 are also available on the market, a number of scientific societies, including the ETA, ATA, and the American Association of Clinical Endocrinologists (AACE), do not recommend their use. In the lack of available studies confirming higher efficacy of combined therapy with FT3 and FT4, it seems that the synthetic sodium of levorotatory thyroxine in oral supply, whether in tablet or liquid form, should now be the standard treatment for hypothyroidism, regardless of its cause [32–35].

In light of the epidemiological recommendations during the COVID-19 pandemic, including social distancing, as well as limitations in access to personal visits to specialist clinics and additional examinations, it is recommended that patients with recently diagnosed hypothyroidism, despite the lack of knowledge of the exact diagnosis of this disease, should implement an appropriate L-T4 substitution therapy as part of online telephone advice or videoconferencing [5, 6], bearing in mind that full diagnostics should be supplemented as soon as possible as the availability of laboratory and imaging tests is restored.

It should be emphasised that L-T4 substitution treatment in full due dose should be implemented in pregnant women, newborns, and young adults with a short history of disease, while in elderly patients over 50–60 years of age and those at risk of cardiovascular diseases the therapy should be started with a small dose with a plan to increase it in the following days and weeks of treatment. The most stable absorption of L-T4 in tablet form occurs during fasting from 30–60 minutes before breakfast or at least 3–4 hours after the last meal. Given

the limited access to specialist outpatient clinics, and thus less control over the enforcement of therapeutic recommendations, even in non-cooperative patients, a full weekly substitution dose can be administered once a week [19, 36]. In addition, it is important to note that the e-prescription formula allows for the prescription of a 12-month treatment, although the patient receives the drug in 2 doses every 6 months.

In the times before the outbreak of pandemic, it was recommended to assess the serum TSH levels 6–8 weeks after the inclusion of treatment and every 6–12 months after the equalisation. During pregnancy, monthly TSH monitoring with fT4 was recommended [17, 19]. However, in the current epidemiological situation, if regular monitoring of TSH levels is not possible and the patient taking the previously recommended dose of L-T4 feels well, it is possible to extend the time of TSH control. If, on the other hand, there is a feeling of malaise or a significant change in body weight, TSH determination seems to be necessary. A special group of patients included in the current recommendations are pregnant women. During pregnancy, the dose of L-T4 should be increased, even if monitoring of thyroid function becomes difficult, with the recommendation to follow the principle "Stay at home to protect yourself and your baby" [5, 18].

In the period of difficult access to general practitioners and specialists, as well as laboratory tests, it is not recommended to discontinue the existing therapy. In the absence of a personal visit, a video conference or telephone advice should be preferred. If possible, a long-term therapeutic plan should be scheduled, informing the patient about the possibilities of safe purchase of drugs and the principle of not collecting excessive medical devices during a pandemic [17].

Selected aspects of hyperthyroidism diagnostics and treatment

Hyperthyroidism is a much greater thyreological challenge compared to hypothyroidism. It is estimated that this endocrinopathy occurs in Europe in about 0.8% of the population, while in the United States of America the rate is 1.3%. The number of cases increases with age and more often affects women. It should be emphasised that from the moment of diagnosis, it requires an in-depth examination by an experienced endocrinologist. Both recently diagnosed and already treated, but poorly controlled, hyperthyroidism is a risk of severe SARS-CoV-2 infection. It is also worth highlighting that changes in the cardiovascular system, including cardiac arrhythmias, thrombotic events, and exacerbation of circulatory or coronary insufficiency, which constitute a serious threat to the health and life of patients, may also increase the risk of severe course of COVID-19 infection. Therefore, a proper diagnosis should be made as soon as possible, and appropriate therapy should be undertaken [37–39].

In the times before the outbreak of the pandemic, when access to a specialist and laboratory tests was much easier, it was not very difficult to make a proper diagnosis of hyperthyroidism. The clinical symptoms vary according to age, gender, incidence of coexisting diseases, and duration of the disease and its cause. In most patients with explicitly defined thyrotoxicosis, elevated concentrations of free thyroid hormones FT3 and/or FT4 and TSH suppression are observed [37, 40, 41].

There is no evidence to date that patients with poorly controlled hyperthyroidism are at greater risk of systemic viral infection. However, it cannot be excluded that these patients have a higher risk of complications, including the development of thyroid crisis, caused by any infection, including SARS-CoV-2 [6]. Therefore, in such a situation, patients with recently diagnosed hyperthyroidism should be quickly diagnosed and covered with appropriate treatment.

The diagnostic and aetiological process will certainly be helpful: medical history (pregnancy, iodine contamination, use of antiarrhythmic medicinal products including amiodarone, exposure to iodine-contrasting agents and genetic burden), physical examination, hormonal tests (TSH, FT4, FT3), immunological determinations: thyrotropin receptor antibodies (TRAbs), thyroid peroxidase antibodies (TPOAbs), thyroglobulin antibodies (TgAbs), human chorionic gonadotropin (hCG), and imaging. On the basis of an individual analysis of each case of hyperthyroidism, the physician should be able to decide on the best way to carry out the medical service in a specialist clinic, taking into account the current possibilities of telemedicine (teleconferencing, videoconferencing), make a personal visit, and even consider referring the patient directly to the hospital for hospitalisation. However, it is important to remember that it may be difficult or impossible to objectify the patient's ailments with a clinical examination during a distance visit. Therefore, any suspicion of thyrotoxicosis based on the clinical symptoms observed by the patient should be confirmed by laboratory tests [5, 42, 43]. However, always, regardless of the availability of tests in every patient in whom we have confirmed or suspected hyperthyroidism, the severity of symptoms should be assessed, as well as the risk of exacerbation of coexisting diseases. In all cases of life- and/or health threatening conditions, the patient should be urgently referred to hospital [5].

Beginning antithyroid therapy (ATD) (methimazoles, MMI) and propylthiouracil (PTU), and its de-

rivative carbimazole, it is necessary to remember that the use of this group of medications requires special attention. Approximately 13% of patients, especially in the first 3 months of treatment, experience side effects, and their frequency depends on the dose of thyrostatic medicine used. The most frequent reports are itchy generalised or localised rash on the torso, joint pain, fever with symptoms suggestive of pharyngitis, and less frequently nausea, abdominal pain, or melaena with accompanying dark urine [44–46]. However, the greatest risk during oral ATD is agranulocytosis, as this is a life-threatening condition and is an urgent indication for hospitalisation, although it is observed in less than 0.5% of patients. Neutropaenia is usually characterised by fever and sore throat, sometimes accompanied by oral ulceration, which in the era of COVID-19 pandemic may suggest a mild form of SARS-CoV-2 infection (fever, dry cough, fatigue, flu-like symptoms). These symptoms may be difficult to distinguish, both for the patients and their physicians; thus, in the case of a justified suspicion of neutropaenia, the patient should be advised to immediately discontinue ATD and perform an urgent peripheral blood morphology. It is worth noting that in COVID-19 infection lymphopaenia and thrombocytopaenia are observed, but they are not contraindications for anti-thyroid therapy [5, 6, 17]. The absolute contraindications are agranulocytosis, toxic liver damage, and vasculitis [44]. Therefore, prior to the inclusion of ATD the blood count should be examined with a smear and the transaminase activity should be determined. If, due to epidemiological reasons and limited access to primary healthcare resources, it is not possible to perform these tests, it is recommended that ATD treatment be discontinued and the patient be observed. If symptoms disappear within a week, treatment may be continued, and if they become more severe during discontinuation or relapse after re-admission, the patient should be advised to contact his/her endocrinologist or primary care physician immediately [5,1 7].

In the era of the SARS-CoV-2 pandemic, the "Block-Replace Regimen (BRR)" combination therapy scheme may be considered, given the reduced availability of laboratory tests. It allows for less frequent control of thyroid function and prevents enlargement and the occurrence of hypothyroidism phase. However, it is important to remember that it brings a higher risk of side effects. The exact scheme of combination treatment of BRR is shown in Figure 1 [5, 43].

In selected cases where, despite the use of oral ATD therapy or due to its side effects, it has not possible to obtain the disease control, it is recommended to try to

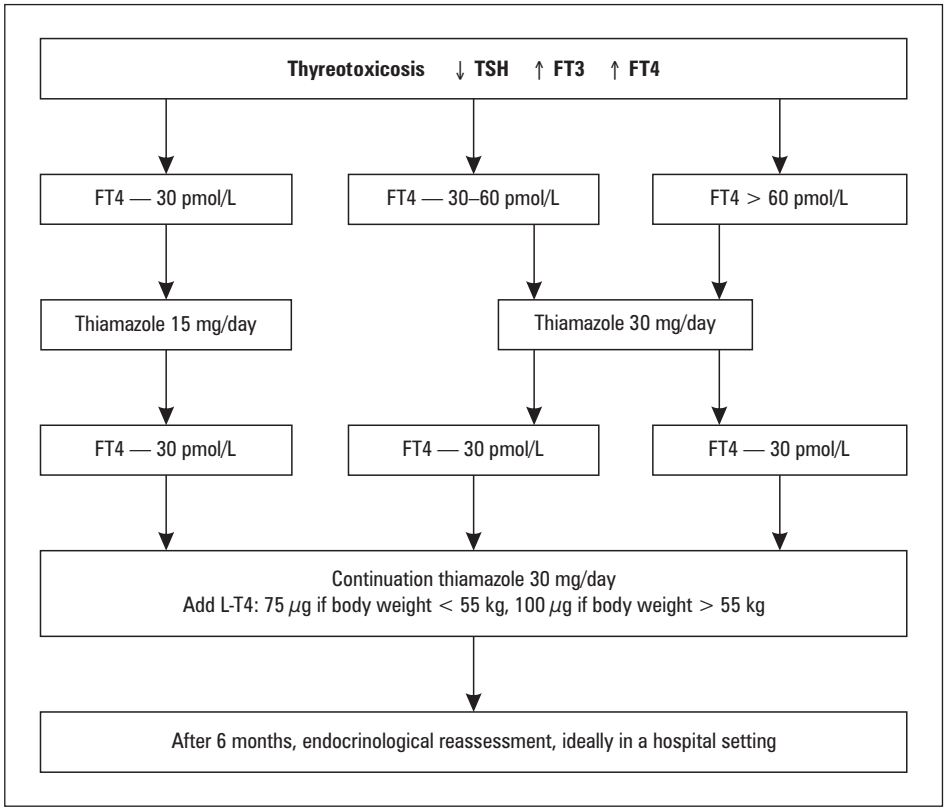

Figure 1. *Combination therapy "Block-Replace Regimen (BRR)" in the treatment of hyperthyroidism in adult patients during the COVID-19 pandemic, according to Boelaert et al. [5]*

refer the patient to urgent surgery or to use radioactive iodine therapy [46].

Graves' disease is one of the main symptoms of hyperthyroidism, and one of its non-thyroidal complications is a syndrome of ophthalmic symptoms caused by immunological inflammation of muscles, as well as adipose tissue and connective tissue of orbits — so-called thyroid-associated orbitopathy. In anti-inflammatory therapy, weekly intravenous glucocorticosteroids (GKS) are usually used [47]. Unfortunately, due to the immunosuppressive effect of GKS, the patients treated by us are more exposed to SARS-CoV-2 infection, and this also applies to the therapy with modern drugs such as mycophenolate, azathioprine, or antibody-class drugs: rituximab, teprotumumab, and tocilizumab [48]. Therefore, together with the patient, we should try to minimise the risk of infection by adapting to the safety rules recommended by the Ministry of Health, as well as the European Endocrine Society, which advises 12-week isolation for these patients [49]. On the other hand, it is important to remember that immunosuppressive treatment should absolutely not be discontinued. The treating physician, considering the benefits and risks in a specific epidemiological situation, should be able to finally decide on the continuation of treatment. One option, if it is not possible to administer GKS IV, is to replace the therapy with an oral form, for the patient to receive treatment at home. Additionally, in preventing the progression of orbitopathy, it is recommended that smoking be avoided, both actively and passively, and/or to enhance the effects with selenium preparations [50]. In the paper Xia J et al. it was indicated that conjunctivitis may be one of the symptoms of COVID-19 infection, because the presence of SARS-CoV-2 mRNA was detected in tears. Therefore, patients with orbitopathy in whom COVID-19 has been confirmed represent a significant risk of infection, especially when soft tissues of the orbit are also occupied. It is necessary to make these patients aware of the recommended precautions to prevent infection with the SARS-CoV-2 virus. The WHO recommends keeping a safe distance from other people, i.e. social distancing, covering the nose and mouth, as well as washing and disinfecting hands thoroughly and regularly [51].

An equally important group of patients with hyperthyroidism are pregnant women. This particular group of patients should be particularly careful and should, as much as possible, implement all personal protective equipment to protect against SARS-CoV-2 infection [52]. During the COVID-19 pandemic, it is recommended that the therapy of hyperthyroidism in pregnant women should be carried out according to the current standards of management. In the first trimester of pregnancy (up to 16 weeks), the PTU and then MMI should be treated with the lowest possible dose of ATD [53]. When starting MMI therapy, it is important to remember to inform the patient about possible teratogenic effects of the drug on the foetus [54]. It is important to point out that BRR therapy is contraindicated in pregnant women.

Conclusions

Many international scientific societies, including the International Thyroid Federation, emphasise that there is currently no evidence that patients with thyroid diseases are more likely to be infected with SARS-CoV-2. There is also no evidence that people with poorly controlled thyroid disease are more likely to be infected. However, it is important to remember that the pandemic and resulting epidemiological regime may cause difficult access to basic and specialist health care. In order to prevent the spread of the SARS-CoV-2 virus, it is recommended that the need for personal visits to a specialist physician be minimised, with contacting a telemedicine provider being preferred. Direct visits should be dedicated only to selected groups of patients requiring personal contact with the physician.

Due to the possibility of difficult access to specialised laboratory and imaging tests, it is recommended that patients with recently diagnosed hypothyroidism or hyperthyroidism start treatment as soon as possible, with the possibility of postponing additional tests to establish the full aetiology of the disease. In the case of patients already treated, it is important to emphasise the necessity of continuing the existing treatment. It is recommended that the physician providing treatment be contacted on a regular basis via telecommunication. Physicians, including endocrinology specialists, should educate their patients in the use of virtual advice and various social platforms, and thus limit personal visits during the pandemic.

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Graves' disease and exophthalmos — a mask for meningioma

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Key words: *Graves' orbitopathy; meningioma; exophthalmos*

Graves' orbitopathy can rarely be a mask of other conditions. In the described case, visual disturbances turned out to be related not to the underlying disease, but to the neurological one — meningioma.

A 45-year-old patient with a long story of nodular goitre was admitted to the department due to thyrotoxicosis in the course of Graves-Basedow disease. She reported nervousness, emotional lability, muscle weakness, breathlessness, palpitations, insomnia, irregular bowel movements with a tendency for loose stools, hypertension (max. 190/120 mm Hg), and muscle and stomach pain. Additionally, deterioration in visual acuity and double vision with extreme right, left, and upward gaze were presented. Symptoms appeared 2 weeks after the removal of the uterus due to fibroids (about 3 months before hospitalisation) and gradually increased. On admission, biochemical symptoms of thyrotoxicosis were observed (Tab. 1). The neck ultrasound showed the thyroid gland with a volume of approx. 50 mL, with hypervascularity and heterogeneous hypoechogenicity, with no obvious focal changes. Intravenous and then an oral thyreostatic, beta-blocker, and steroid were included in the treatment causing an improvement of the clinical condition and a decrease in the level of thyroid hormones.

The patient was hospitalised again due to recurrence of hyperthyroidism. Severe symptoms of thyrotoxicosis and persistent visual acuity disturbances in the left eye, double vision with extreme right, left, and upward gaze developed. In the treatment of thyrotoxicosis, initially intravenous then orally, thyreostatics were used, with positive effect. Due to large fluctuations in the value of thyroid hormones, L-thyroxine and thyreostatics were included in the treatment. Additionally, due to the persistent asymmetric exophthalmos and the unstable course of treatment, diagnostics was extended to imaging tests. CT examination (Fig. 1) of the orbits was performed: in the left orbit, adjacent to the left optic nerve near the optic nerve canal, a soft tissue lesion of $18 \times 7 \times 9$ mm was found, which modelled the course of the optic nerve and was located in its direct contact. The uneven outline of the orbital walls was also visible at this location. Diagnostics was extended to head and orbital MRI (Fig. 2, 3). A pathological mass was confirmed at the apex of the left eye socket, showing a connection with the intracranial infiltrate, with a thickening of the adjacent bone structures. The morphology of the signal of infiltrative changes, along with sclerotisation of bone structures and calcifications in the intracranial infiltration, suggested a lesion of the meningioma overgrowing the bone. Left frontal craniotomy and resection of the left optic nerve tumour were performed after a neurosurgical consultation and the stabilisation of the general condition and normalisation of thyroid hormones. Histopathological examination revealed small peripheral nerve trunks and micro-foci of meningioma infiltration among small hyphae of adipose tissue. After the meningioma removal, the patient was qualified for radical Graves' disease treatment with the use of radioactive iodine in an elective mode, with positive effect.

Table 1. *Hormonal assessment*

Hormone (range)	16 VI 2017	21 VII 2017	07 VIII 2017	24 VIII 2017	28 IX 2017
TSH [0.270–4.200 uIU/mL]	< 0.005	< 0.005	0.137	< 0.005	0.066
FT3 [3.1–6.8 pmol/L]	17.47	22.85	3.55	13.43	7.62
FT4 [12.0–22.0 pmol/L]	39.04	35.77	3.6	21.25	17.43

TSH — thyroid-stimulating hormone; FT3 — free triiodothyronine; FT4 — free thyroxine

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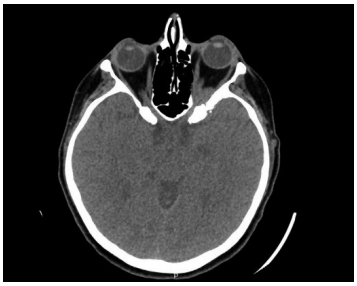

Figure 1. *Computer tomography imaging of the orbit — first obtained imaging result of meningioma (located on the left orbit). Courtesy of Department of Diagnostic Imaging, University Hospital in Krakow*

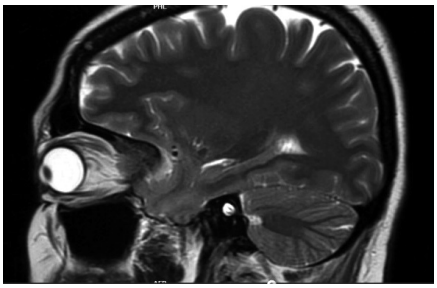

Figure 2. *Magnetic resonance imaging of head (sagittal T2-weighted FRFSE sequence) showing meningioma extending to the orbit. Courtesy of the Department of Diagnostic Imaging, University Hospital in Krakow*

Meningiomas are benign slow-growing tumours that arise from arachnoid cap cells. They are the second most common brain tumour in the adult population. The female–male ratio is 1.8:1, and the usual age at occurrence is between 30 and 50 years [1]. The symptoms and clinical signs of meningiomas depend on the location of the tumour. Early diagnosis is important because total removal of the tumour may be feasible and the vision of the patient can be preserved [2].

Graves' orbitopathy is the main extrathyroidal manifestation of Graves' disease. It is most often the result of autoimmune reactions with tissue components in the orbit [3–5].

In the literature, cases describing the coincidence of both diseases are extremely rare. In the described case there was suspicion that visual disturbances and left eye exophthalmos were associated with a complication of the underlying disease — orbitopathy. Due to the asymmetry of exophthalmos and the unstable course of treatment, it was decided to extend the diagnostics to imaging tests (they are often deferred in the management in patients with a combination of typical symptoms: thyroid gland dysfunction, eyelid retraction, symmetric proptosis, and restrictive strabismus [6]). The accelerated diagnosis allowed the presence of another

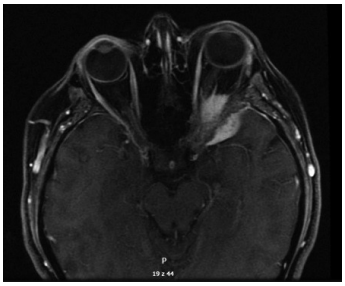

Figure 3. *Magnetic resonance imaging of orbit (axial Lava Flex sequence) showing meningioma extending to the orbit and intracranial infiltrate with a thickening of the adjacent bone structure. Courtesy of the Department of Diagnostic Imaging, University Hospital in Krakow*

cause of the symptoms to be shown — meningioma. The implemented procedure made it possible to control the ailments quickly and prevent exacerbation. The applied radioiodine treatment also allowed the underlying disease to be controlled and the level of hormones to be stabilised. No other complications related to thyroid disease were observed.

This case illustrates the importance of careful attention of treatment and monitoring, despite the most typical course of disease, and if necessary to accelerate and extend the treatment with additional tests.

Author's statement

K.M. is the first author.

Conflict of interest

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Severe hypophosphataemic osteomalacia related to low-dose adefovir dipivoxil therapy in a hepatitis B virus patient

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Key words: *hypophosphataemic osteomalacia; adefovir dipivoxil; chronic hepatitis B*

A 47-year-old man presented in September 2011 with a 2-year history of bone pain without antecedent trauma. He began to complain of mild pain in the lumbosacral region and did not pay attention to it in August 2009. However, he reported generalised bone pain involving the sternum, several ribs, left ankle, and large joints, and progressive weakness in his muscles, especially in his leg muscles, in May 2011. A diagnosis of ankylosing spondylitis was made in some hospitals. Although given conventional therapy, including nonsteroidal anti-inflammatory drugs, sulfasalazine, methotrexate, and the supplementation of calcium and vitamin D, the patient did not show considerable improvement in bone pain. He was laid up due to long illness.

The patient's past medical history was uncomplicated. He had a history of chronic hepatitis B infection and cirrhosis, receiving adefovir dipivoxil at 10 mg daily for 49 months. He was without hypertension or diabetes mellitus and previously had not taken any other medication known to harm the skeletal system. On physical examination, he presented severe tenderness over the sternum, the ribs, both shoulder joint areas, sacroiliac joint, knee joint, and left ankle. The remainder of the clinical examination was approximately normal.

Laboratory examination on admission revealed severe hypophosphataemia (0.42 mmol/L), mild hypocalcaemia (2.05 mmol/L), and an increased serum creatinine (131 umol/L). Alkaline phosphatase was elevated (245 U/L), but parathyroid hormone and vitamin D were within normal range. ALT and AST were 17 IU/L and 19 IU/ L, respectively. However, in his serum hepatitis B virus (HBV) DNA was detected (1.354e + 003 copies/mL; normal range, ≤ 5.000e + 002 copies/mL). A routine blood test demonstrated a decreased haemoglobin level (98 g/L) and normal leukocyte and platelet count. Bone marrow biopsy was performed, and nothing abnormal was found. Vitamin B12, folate, and haemolytic test were normal, and iron was slightly elevated. Urinalysis indicated positive albumin and glucose. A 24-hour urinary study showed hyperphosphaturia. Ultrasound examination showed a diffused hepatic and renal lesion. No splenomegaly was found. Dual-energy X-ray absorptiometry revealed a decreased hip joint bone mineral density of 0.724 g/cm^2 (T-score, −1.7; Z-score, −1.2). A whole-body ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP) bone scan showed widespread foci in the ribs bilaterally, both shoulder joints, the sacroiliac joints, the right femoral head, the knees, and the left ankle (Fig. 1). The patient refused the recommendation of kidney biopsy.

A diagnosis of hypophosphataemic osteomalacia secondary to low-dose adefovir dipivoxil therapy was made. Entecavir 0.5 mg daily was given to replace adefovir dipivoxil. Both laboratory parameters and bony pain improved dramatically after oral phosphate and

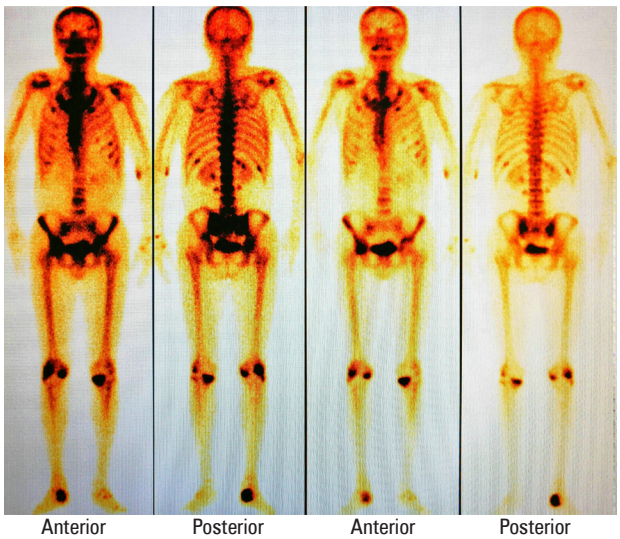

Figure 1. *A whole-body ^{99m}Tc-methylene diphosphonate (99mTc--MDP) bone scan shows multiple foci of increased radiotracer uptake in the ribs bilaterally, both shoulder joints, the sacroiliac joints, the right femoral head, the knees, and the left ankle*

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Table 1. *Laboratory data*

Laboratory test	2 months earlier	At admission	8 weeks after ADV withdrawal	Reference range
Serum				
White blood cells	4.2	7.7	8.5	(4–10)×109/L
Haemoglobin	87	98	126	(110–160) g/L
Platelets	150	203	287	(100–300)×109/L
ALT	22	17	17	(0–40) U/L
AST	24	17	19	(0–40) U/L
Alkaline phosphatase	154	241	175	(35–150) U/L
Albumin	36.6	36.5	40.1	(35–55) g/L
Blood urea nitrogen	4.61	4.17	3.78	(2.3–7.2) umol/L
Creatinine	116	131	89	(44–110) umol/L
Glucose	4.84	4.15	4.79	(3.9–6.1) mmol/L
Chloride	115.5	108.7	109	(101–111) mmol/L
Sodium	144.7	142.6	139	(135–145) mmol/L
Calcium	1.94	2.05	2.37	(2.1–2.8) mmol/L
Phosphate	0.26	0.42	0.92	(0.8–1.4) mmol/L
Potassium	2.88	3.87	4.03	(3.5–5.5) mmol/L
Parathyroid hormone	NA	26.97	NA	(6.0–80) pg/mL
1,25-(OH)2 vitamin D3	NA	31.58	NA	(26–65) ng/L

ADV — adefovir dipivoxil; NA — not available; ALT — alanine aminotransferase; AST — aspartate transaminase

vitamin D supplementation (Tab. 1). One month after cessation of adefovir dipivoxil, bony pain involving the ribs and the sacroiliac joints disappeared. After 7 weeks, the patient could walk without restraint. So far, he has been given a follow-up for 9 years, and reported no bone pain. Serum creatinine, phosphorus, and liver function are normal, and virus replication is at a low level.

Adefovir dipivoxil, an oral nucleotide analogue, has been widely used for the treatment of hepatitis B. It does not produce significant hepatotoxicity, due to minimal hepatic metabolism. However, it displays a dose-dependent nephrotoxicity [1]. Adefovir dipivoxil at a daily dose of 30 mg can cause a mild-to-moderate degree of nephrotoxicity, while no evidence of renal dysfunction was seen at a dose of 10 mg per day after follow-up for 16 months in 2 randomised controlled trials [2]. However, subsequent reports have indicated a high prevalence of nephrotoxicity associated with prolonged usage of adefovir dipivoxil at a lower dose. The excessive accumulation of adefovir dipivoxil in the proximal renal tubules inhibits mitochondrial DNA replication, and can lead to the disorders in phosphorus homeostasis, but it can also disturb reabsorption of protein, glucose, uric acid, and other ions (Fanconi syndrome). Sustained loss of phosphate disturbs normal bone mineralisation and eventually results in osteomalacia [3]. After the cessation of adefovir dipivoxil, nephrotoxicity is partly reversible in some patients [4].

Herein, we report a typical case of hypophosphataemic osteomalacia in a hepatitis B patient following treatment with low-dose adefovir dipivoxil for 49 months. Clinicians should be aware of serious adverse reactions during the use of adefovir dipivoxil at the lower dose of 10 mg daily. Liver function, renal function, and serum electrolytes should be tested closely so as to recognise this complication of drugs and provide appropriate intervention as soon as possible. At the same time, it is important to distinguish hypophosphataemic osteomalacia from ankylosing spondylitis and multiple myeloma. The correct diagnosis can reduce patients' pain and improve their quality of life.

Authors' contribution

Xiushuai Dong drafted the manuscript. Xi Chen, Yaoyao Tian, and Jinghua Wang critically revised the manuscript. All authors contributed to the intellectual content and approved the final version.

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Conflict of interest

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Efficacy of multimodal anticancer therapy in the course of pancreatic neuroendocrine carcinoma

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Key words: *neuroendocrine tumours; PRRT; pNEC; somatostatin analogue*

Neuroendocrine neoplasms (NENs) are a heterogeneous group, comprising well-differentiated neuroendocrine tumours (NETs) and poorly differentiated neuroendocrine carcinomas (NECs). NETs and NECs show major differences regarding histology, genetics, biological behaviour, and clinical manifestations [1]. The malignancy of NECs is high, which explains the poor prognosis. NECs are relatively rare; therefore, few data on long-term management are available. The present study shows the 10-year follow-up of a patient diagnosed with a disseminated pancreatic neuroendocrine carcinoma (pNEC) in young age.

In 2008, a 28-year-old Caucasian male with a one-year history of abdominal pains and with no concomitant diseases was admitted to the hospital due to acute pancreatitis. On CT examination, an 11-mm cyst in the tail of the pancreas was described. Due to persistent pain in the epigastrium, accompanied by nausea and vomiting, magnetic resonance imaging was performed revealing a pancreatic tail tumour 54 × 42 mm, suspected to be adenocarcinoma infiltrating splenic vessels and adipose tissue. In addition, metastatic lymph nodes and 2 liver metastases in segments IV and VIIII were described. A liver biopsy confirmed NEN with chromogranin expression. The pancreatic biopsy was non-diagnostic. Somatostatin receptor imaging (SRI) showed tracer uptake in the primary lesion and hepatic metastases. Subtotal left-sided pancreatectomy was performed in April 2009 (histopathologically pNEC, pT4N2M1, Ki-67 50%, lymph node metastases 17/20).

One month later, thermoablation of only one available liver metastasis was performed. Due to very good SSTR expression in the metastatic lesions and the oncologist's suggestion, the patient was qualified for Peptide Receptor Radionuclide Therapy (PRRT). In June 2009 the patient received 740 MGq of [^{90}Y] Y-DOTA-TATE without complication with concomitant chemotherapy (gemcitabine-erlotinib) from June 2009 to August 2011, giving stabilisation of the disease in consecutive follow-up CT scans.

In February 2013, SRI revealed an increased tracer uptake in the retropharyngeal lymph node on the right side. The repeated biopsies of retropharyngeal lymph node were negative.

In June 2016 [^{18}F]F-FDG-PET/CT detected metabolically active infiltration to cervical lymph nodes invading adherent soft tissues.

In September 2016, due to further progression of disease, a paraspinal lump at the base of the skull was removed (histopathologically a metastasis of pancreatic NET, Ki67 3%).

Post-operative SRI in November 2016 showed new metastatic lesions in both liver lobes, which disqualified the patient from radical treatment and surgical interventions. The retropharyngeal lymph node was still present. Treatment with a long-acting somatostatin analogue was initiated in December 2016. Since Jan 2017, the patient has additionally received second-line chemotherapy (capecitabine + temozolomide). So far, 42 cycles of systemic treatment have been administered, with good tolerance of treatment. Since then, no disease progression has been reported.

pNECs are rare tumours, with an incidence of 10–20% according to previous WHO classification (2010), which did not discriminate between pNECs and NET G3. Curative surgery is the first line of treatment, but it is difficult to achieve satisfactory results with this approach alone [1]. Moreover, pNEC patients often present initially advanced, disseminated stages of disease and are not eligible for surgical treatment [2]. PRRT has proved

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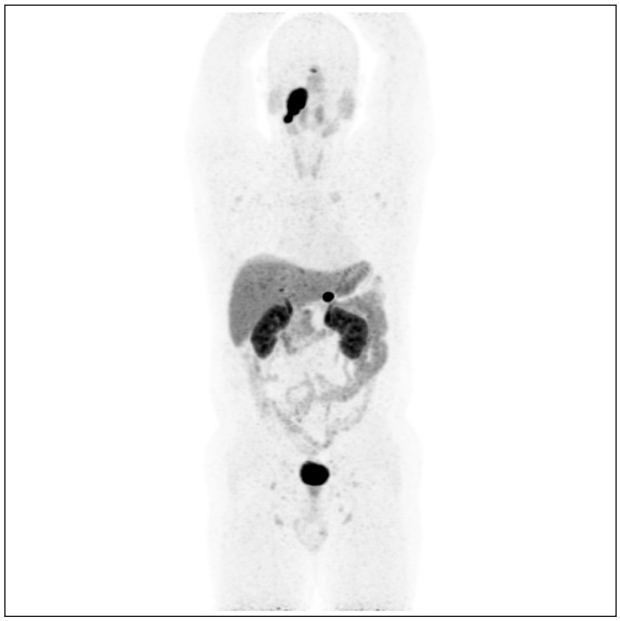

Figure 1. *Very good expression of SSTR in cervical lymph nodes with infiltration of the levator veli palatini, sphincter muscle, the posterior wall of the nasopharynx, and pharyngeal tonsils seen in [^{68}Ga]Ga-DOTA-TATE PET/CT in 2016 before onset of CAPTEM chemotherapy*

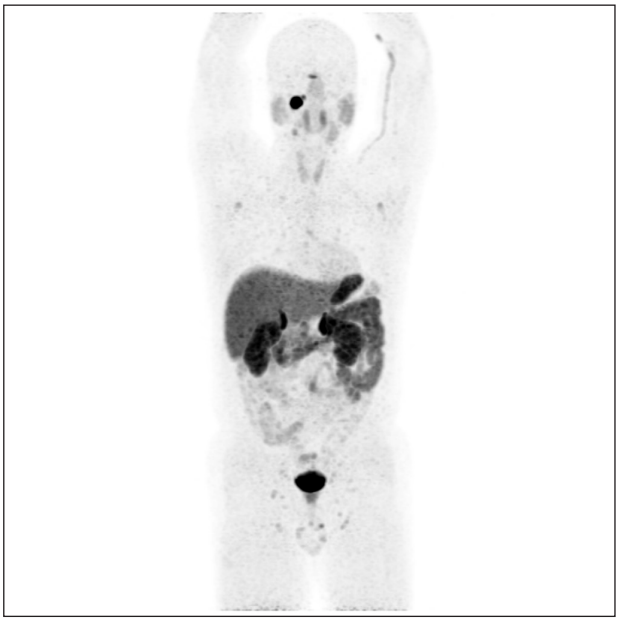

Figure 2. *Very good expression of SSTR in cervical lymph nodes with infiltration of the levator veli palatini, sphincter muscle, the posterior wall of the nasopharynx, and pharyngeal tonsils seen in [^{68}Ga]Ga-DOTA-TATE PET/CT in 2020 after 12 years of multimodality treatment*

to be an effective and well-tolerated treatment modality in patients with pNETs, possibly also in stage G3 [3]. Other treatment patterns are based on multi-agent chemotherapy used in small cell lung carcinoma [1, 4]. Recommended in pNETs, G3 regimens have changed over the years. Current therapeutic options include streptozotocin and 5-fluorouracil or capecitabine with temozolomide. In NECs, platinum-based combinations are widely used as first-line treatment. Rogowski et al. reported that the combination of capecitabine and temozolomide is an effective treatment for patients with NET G3 with high Ki-67 index [5]. This regimen was used in the presented case as a second-line treatment. Survival of patients with advanced stages of disease is estimated in months. In the presented case radical treatment was not possible due to the stage of disease. Despite being NEC (according to WHO 2010 classification), the primary tumour had a very good SSTR expression. Montanier reported a case of pNEC with liver metastases with low mitotic index and a very high Ki-67 index (45–70%) treated with PRRT([^{177}Lu] Lu-DOTATATE), which allowed for 3 years of complete remission [6]. In the presented case the progression-free period equalled 4 years, but the patient received chemotherapy for half of this time. The retropharyngeal lymph node metastasis was identified as a distant metastasis of pancreatic NET, but it was significantly different from the primary lesion. The long course of the disease and good SSTR expression may raise doubts about whether in our case NEC could be NET G3 according to the current WHO classification (2019). To our knowledge, no such case was previously described in the literature.

The use of PRRT with subsequent chemotherapy and treatment with long-acting somatostatin analogue proved to be effective in the presented case, confirming the benefit from multimodal anticancer therapy. There is a need to use all diagnostic methods to select the optimal therapy in patients with pNECs, to provide them with better care and the longest possible survival.

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Myocardial infarction caused by Graves' disease

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Key words: *coronary artery spasm; thyrotoxicosis; myocardial infarction; Graves' disease*

Coronary artery spasm is a rare cause of myocardial infarction (0.5% of cases). It can also be complicated by dysrhythmia or sudden cardiac death. We present a case of a 41-year-old woman with non-ST elevation myocardial infarction caused by coronary artery spasm associated with hyperthyroidism. The coronarography showed a spasm of the left cardiac artery, which was completely relieved by administration of intracoronary nitrate. In the course of the further research the patient was diagnosed with Graves' disease.

A 41-year-old female patient with a history of arterial hypertension and smoking was admitted to the Cardiology Department with severe chest pain remittent for 3 days at rest. ECG showed a sinus tachycardia and features of cardiac muscle ischaemia — ST segment depression on anterior, inferior, and lateral leads and elevation in augmented vector right (aVR) (Fig. 1). The laboratory tests showed elevated markers of myocardial injury — high-sensitivity cardiac troponin I (hs-cTnI: 150 ng/L; ref. range < 16 ng/L). A coronary angiography was urgently performed. It visualised a spasm of the trunk of the left coronary artery constricting the vessel by 70%. No significant stenosis was found in the other arteries (Fig. 2 and 3). After the procedure the patient was transferred to the Cardiac Intensive Care Unit. The chest pain completely subsided. A physical examination revealed higher body temperature, persistent tachycardia, symmetrical exophthalmia, and tremor. In addition, the patient lost 20 kg of body weight within 6 months. Until hospitalisation the patient had no history of thyroid disease. The laboratory tests revealed features of hyperthyroidism — undetectable concentration of thyroid-stimulating hormone (TSH: 0.0 uIU/mL; ref. range 0.35–4.0 uIU/mL) and significantly elevated concentrations of free thyroid hormones (fT3: 20.84 pg/mL; ref. range 1.71–3.71 pg/mL, fT4: 2.76 ng/dL; ref. range 0.71–48 ng/dL). Immunoassays showed elevated concentrations of antibodies against TSH receptor (TRAb: 13.73 U/L; ref. range 0–1.5U/L), antibodies against thyreoglobulin (32.24 IU/mL; ref. range < 4.1 IU/mL), and an abnormally high concentration of anti-thyroid peroxidase antibodies (ATPO > 1000.0 IU/mL; ref. range 0–5.61 IU/mL). USG showed parenchymatous goitre without nodule with decreased echogenicity. Echocardiography performed after the coronarography showed enlarged left ventricle of 6.3 cm (ref. range < 5.5 cm) and hyperdynamic circulation features. Left ventricular ejection fraction was 70%. No segmental contractility disorders were observed. Based on the tests results, Graves' disease was diagnosed. The patient was successfully treated with diltiazem, nitroglycerin, and thiamazole.

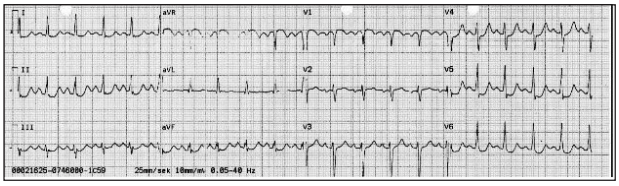

Figure 1. *Electrocardiogram of the patient*

Coronary artery spasm, especially when caused by thyrotoxicosis, is a rare cause of acute coronary syndrome. This case highlights the importance of considering hyperthyroidism in the differential diagnosis of chest pain with normal coronary arteries. Thyroid function testing should be routinely performed on a patient with coronary artery spasm, especially for young female patients without typical risk factors of coronary heart disease. According to the reports, vasospastic angina may be involved in up to 20% of patients with thyrotoxicosis. The exacts mechanisms of artery spasm connected with thyrotoxicosis are unclear. In vitro studies have proven a trend of enhanced vasoconstric-

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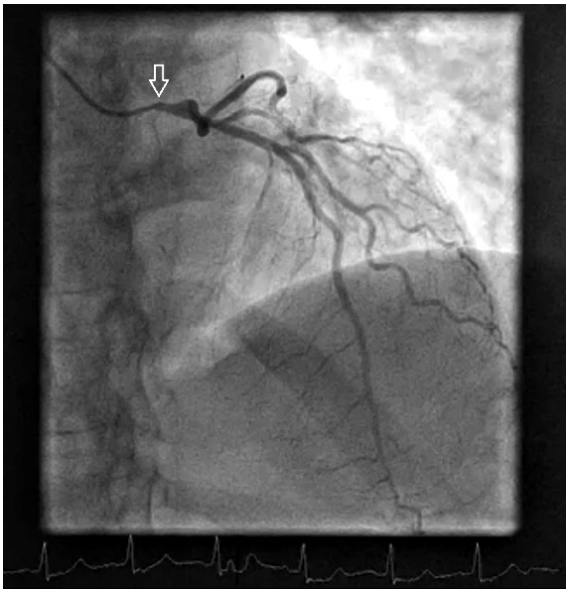

Figure 2. *Coronary angiogram of the patient, left coronary injection significant narrowing of the trunk of the left coronal artery*

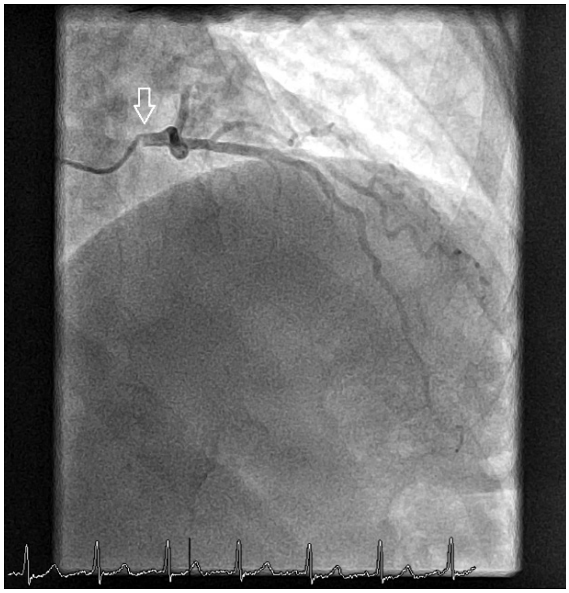

Figure 3. *Coronary angiogram of the patient after administration of intra coronary nitroglycerin*

tion in response to catecholamines and sympathetic α-adrenergic receptor stimulation. The thyrotoxicosis is associated with increased sensitivity and numbers of adrenergic receptors. According to the ESC guidelines on vasospastic angina treatment, calcium channel blockers of the non-dihydropyridine class and long-acting nitroglycerin should be used. In the case of artery spasm in the progress of thyrotoxicosis, it is most important to achieve euthyroidism.

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A rare cause of chronic diarrhoea: a diagnosis to keep in mind

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Key words: *gastrinoma; lymph node; diarrhoea; EUS*

A 61-year-old male patient, who had undergone surgery for a perforated small bowel ulcer two weeks earlier, reported for an outpatient visit to the gastroenterologist (TS). The patient presented with diarrhoea, weight loss, and weakness.

He had suffered from watery diarrhoea, with 20 to 30 bowel movements daily, for five years. Despite having visited many different specialists, his problems remained unresolved. Common causes of diarrhoea were excluded, including bacterial infection, parasites, colorectal cancer, inflammatory bowel diseases, and celiac disease. Colonoscopy with biopsy (2×), computed tomography (CT) of the abdomen (1×), and ultrasonography of the abdomen (3×) were performed and revealed no abnormality. Meanwhile, the patient lost 30 kg and was so weak that he could not even walk. His medical history also included severe reflux oesophagitis and duodenal peptic ulcer, with good response to typical treatment 4 years before. His family history was unremarkable.

Clinical examination revealed mild–severe general status, cachexia, paleness, and tachycardia (110 bpm). Standard laboratory tests showed mild anaemia, hypoproteinaemia, and dyselectrolitaemia. Results of other blood tests were within normal ranges.

According to the clinical presentation, gastrinoma was suspected, and high doses of oral esomeprazole (160 mg daily) were administered. Rapid improvement of symptoms with cessation of diarrhoea was achieved. Gastroscopy revealed thickening of gastric folds (Fig. 1). Positron emission tomography (PET/CT) with [^{68}Ga] Ga-DOTATATE demonstrated a 10-mm soft tissue lesion with increased somatostatin receptor expression located close to the duodenum (Fig. 2). This lesion was visualized as an abnormal lymph node (12.5 mm) in the hepatoduodenal ligament during endoscopic ultrasound (Fig. 3). The patient also had a slightly increased level of gastrin (190 pg/mL; normal level 100 pg/mL).

The patient underwent surgical treatment, and the abnormal lymph node was removed. Histopathological examination revealed typical morphological and immunohistochemical features of a well-differentiated neuroendocrine tumour. The patient has remained symptom free during the 4.5 years since surgery, with a diagnosis of primary lymph node gastrinoma or occult primary gastrinoma with lymph node metastases.

Gastrinoma is a gastrin-producing neuroendocrine tumour that triggers hypersecretion of hydrochloric acid [1]. Most of these tumours arise in the so-called "gastrinoma triangle", delineated by the second and third portions of the duodenum, the head and neck of the pancreas, and the junction of the cystic and common bile ducts [1]. These tumours can also rarely occur in the stomach, lymph nodes, liver, bile duct, ovary, and heart [1]. The existence of true primary lymph node gastrinoma is still an area of debate [2]. What is of special importance is that with

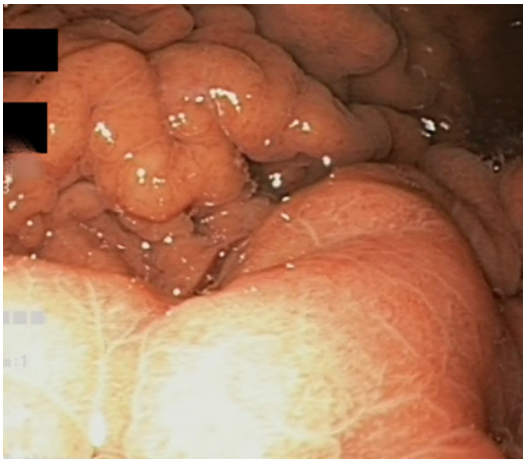

Figure 1. *Endoscopy showing thickening of the gastric folds (an endoscopic feature of gastrinoma)*

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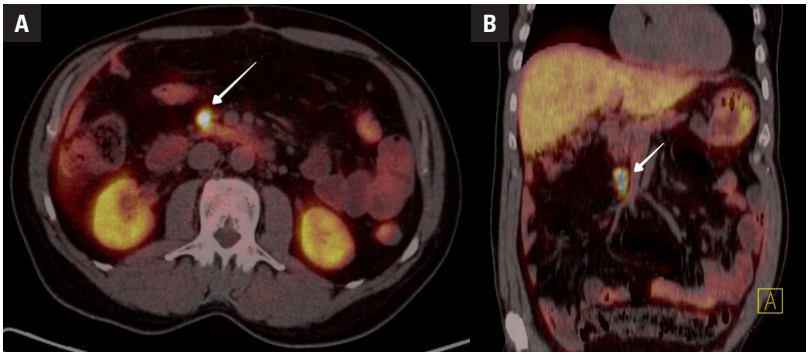

Figure 2. *[^{68}Ga]Ga-DOTATATE PET/CT showing abnormal uptake near the pancreatic head and duodenal wall*

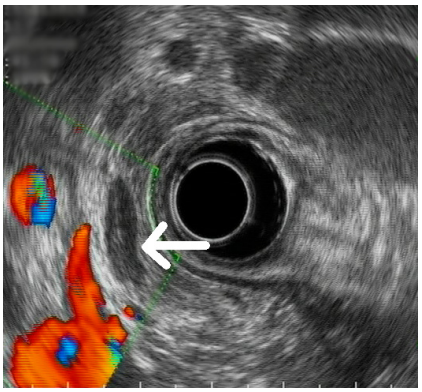

Figure 3. *Endoscopic ultrasound showing 12.5-mm lymph node in the hepato-duodenal ligament*

suspicion of primary lymph node gastrinoma, the possibility of a metastatic lymph node of a gastrin-producing tumour in an occult location (microgastrinoma) should be considered in the differential diagnosis [3]. The diagnosis is thus rather one of exclusion after long-term observation [2] and an indication for careful exploration of the surgical field, including careful assessment of the gastrinoma triangle and intraoperative sonography, transillumination, and duodenotomy [2, 3].

Typical clinical manifestations of gastrinoma include multiple peptic ulcers that are resistant to treatment and in atypical locations, chronic diarrhoea, oesophagitis, and weight loss [1]. The symptoms are often confused with those of other conditions such as indigestion, dyspepsia, or irritable bowel syndrome [4]. Moreover, the use of proton pump inhibitors typically leads to rapid symptom improvement, representing an effective method of symptomatic treatment that carries the risk of masking the clinical course and delaying proper diagnosis for years [1, 4].

From a practical point of view, gastrinoma biochemical diagnostics is based on gastrin serum level, which typically exceeds 10 times the normal concentration, although slightly elevated levels do not exclude the diagnosis [1]. CT, magnetic resonance, endoscopic ultrasound, and PET/CT with ^{68}Ga-DOTA-conjugated somatostatin receptor targeting peptides are the modalities used for location diagnostics of the tumour [1,5]. In our case, taking into account the typical clinical course, despite only slightly elevated levels of gastrin, the attending physician referred the patient for [^{68}Ga]Ga-DOTATATE PET/CT, which has a sensitivity, specificity, and diagnostic accuracy in gastro-entero-pancreatic neuroendocrine tumours reaching 90% [5]. This imaging showed the exact location of the tumour and paved the way for a correct diagnosis and effective treatment.

Our patient had typical clinical manifestations of gastrinoma, yet despite many gastroenterological appointments, the diagnosis was not established for a long time. During this delay, the patient experienced malabsorption and cachexia. For this reason, we believe that it is important to remind clinicians that one cause of diarrhoea is a gastrin-producing tumour. We emphasize that the gastrinoma location can be atypical and that normal or near-normal levels of serum gastrin do not exclude the diagnosis.

Conflict of interest

None declared.

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