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DIAGNOSTIC VALUE OF MOLECULAR MARKERS IN EVALUATION OF THYROID NODULES

Summary of the Doctoral Thesis

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LIST OF ABBREVIATIONS

CD56 - neural cell adhesion molecule 56

CG - colloid goiter

CI - confidence interval

COX-2 - cyclooxygenase-2

DNS - deoxyribonucleic acid

E-CAD - epithelial cadherin

FC - follicular cancer

FNA - fine-needle aspiration

HBME-1 - Hector Battifora mesothelial antigen-1

HE - haematoxylin–eosin

ICH - immunocytochemistry

IHC - immunohistochemistry

Ki-67 - antigen Ki-67

NPV - negative predictive value

p53 - protein 53

PPV - positive predictive value

PTC - papillary thyroid carcinoma

SD - standard deviation

US - ultrasound

WHO - World Health Organisation

1. INTRODUCTION

Thyroid nodules are very common and are usually discovered during routine medical care. It is estimated that 5% of the general population develops clinically palpable thyroid nodules. With the emergence of ultrasound (US) impalpable thyroid nodules can be detected in 20-67% of general population [1, 2]. Most of the discovered nodules are benign, however, there are approximately 44,000 estimated new cases of thyroid cancer and 1700 estimated deaths in 2010 in the United States [3]. The yearly incidence has increased from 3.6 per 100,000 in 1973 to 8.7 per 100,000 in 2002 - a 2.4× increase and this tendency appears to be continuing [4]. This is due to an increase in detection of small papillary thyroid cancer (PTC), which increased from 2.7 to 7.7 per 100,000 - a 2.9× increase [4].

The yearly incidence of thyroid cancer in Latvia has increased from 2.59 per 100,000 in 1991 to 9.39 per 100,000 in 2011. It is 3.62× increase and most likely that this tendency will be continuing to rise [5, 6].

Thyroid cancer represents ~1-2% of all malignancies [7], in addition papillary thyroid carcinoma (PTC) constitutes about 80% of all thyroid malignancies [8].

Differential diagnosis of thyroid nodules could be difficult due to overlapping morphological features as well as due to inability to evaluate vascular and / or capsular invasion in fine-needle aspiration (FNA) cytological specimens. As a result, up to 85% of patients after thyroid operation have benign morphology [9].

Rarely but nevertheless thyroidectomy carriers significant risks of operative complications, including permanent hypoparathyroidism and damage to the recurrent laryngeal nerve, which can result in chronic aspiration and compromised voice quality. Additionally, thyroidectomy commits the patient to a lifetime of thyroid replacement therapy and a recommendation of longterm surveillance for recurrent disease.

While typically minimally aggressive, thyroid cancer can be lethal, therefore the question rises of how incidentally discovered thyroid nodules should be investigated in cost-effective and safe manner to identify the rare patient with a clinically significant malignancy.

Today, in scientific publications many attempts have been described to find additional criteria to distinguish follicular adenomas from carcinomas, in both surgical and FNA cytological specimens.

A growing number of molecular or immunohistochemical (IHC) markers have been identified and tested with considerable variability in the outcomes of these studies. It is believed that no single IHC marker by itself is completely sensitive and specific enough, therefore the most appropriate panel of markers are wanted and many studies are made in this field. Considering the data in literature, of a large number of investigated IHC markers, only a few have emerged as potentially useful. In fact, several reports on this topic have provided conflicting results. Variances are mainly due to the apparently false-positive staining of some of the markers in normal thyroid or adenomas [10, 11].

To try to resolve the aforementioned issues, we designed this study on a relatively large number of histologically proven thyroid follicular lesions, and tested a panel of commercially available IHC markers: *Hector Battifora mesothelial antigen-1* (HBME-1), *neural cell adhesion molecule 56* (CD56), *epithelial cadherin* (E-CAD), *cyclooxygenase-2* (COX-2), *antigen Ki-67* (Ki-67) and *protein 53* (p53). The most promising markers were further tested on a FNA cytology of the thyroid.

Hector Battifora mesothelial antigen-1 (HBME-1), first described in 1992 by Battifora et al. [12], is a mouse monoclonal antibody directed against an antigen of the microvillous surface of mesothelioma cells [13]. Although this antibody was originally developed as a mesothelioma marker, it was subsequently applied to the diagnosis of malignant thyroid conditions [12, 14].

CD56 antigen, recognised by Leu-19 is a glycoprotein, expressed on about 15% of normal peripheral blood lymphocytes. Its presence in natural killer cells was established in 1983 by Griffin et al. [15] and in 1985 by Hercend et al. [16]. CD56 may also be expressed in activated T cells, large granular lymphocytes, specific endocrine and brain tissue, thus it is believed that CD56 antigen may be involved in cell adhesion. CD56 is a neural cell adhesion molecule that is present on follicular epithelial cells of the normal thyroid [17].

E-Cadherin (E-CAD) is transmembrane glycoprotein and belongs to family of adhesion receptors [18]. E-CAD is present in most epithelial cells and appears to play an important role in epithelial integrity, in cell adhesion and differentiation, as well as in the maintenance of cell polarity and tissue architecture. Cell lines normally expressing E-CAD show an epitheloid morphotype and are not invasive, whereas those lacking E-CAD are fibroblastoid and have a highly invasive growth [19]. The expression of E-CAD in normal thyroid was first described in 1989 by Eidelman et al. [20].

COX-2 gene is an early response gene that is induced rapidly by growth factors, tumour promoters, oncogenes, and carcinogens. Multiple lines of evidence suggest that COX-2 is important in carcinogenesis. Increased COX-2 protein expression levels have been described in malignant thyroid tumours, but not in benign thyroid nodules in 2002 by Specht et al. [21].

Ki-67 antigen is preferentially expressed in the active phases of cell cycle and is recognised with monoclonal antibody MIB1. The antigen is rapidly degraded as the cell enters non-proliferative state. As a marker of cellular proliferation activity, Ki-67 antigen detection has become a mainstay in the morphological assessment of tumours. First study of Ki-67 expression in thyroid

lesions is published in 1991 by Rigaud et al. [22].

p53 is an important tumour suppressor gene as it integrates multiple stress signals and regulates cell response to DNA damage and is capable of inhibiting cell proliferation and transformation [23, 24]. Mutations in the p53 tumour suppressor gene are present in approximately 50% of all human cancers, and they represent the most common genetic changes in malignant cells [25].

1.1. Aim of the study

To evaluate the diagnostic value of molecular markers in management of thyroid nodules.

1.2. Objectives

1. To determine and evaluate diagnostic accuracy of molecular markers (HBME-1, COX-2, E-CAD, CD56, Ki-67 and p53) on histological samples of benign and malignant thyroid lesions.
2. To establish the panel of markers that can be recommended as an adjunct to morphology criteria.
3. To test the panel of markers before operation in thyroid FNA immunocytochemistry.
4. To develop practical recommendations in management of benign and malignant thyroid nodules.

1.3. Working hypothesis

1. Selected molecular markers (HBME-1, COX-2, E-CAD, CD56, Ki-67 and p53) are useful as an adjunct to morphology criteria of thyroid pathology.
2. Expression of each marker differs and the combination of several

markers raises the diagnostic accuracy.

3. It is possible to develop technology for immunocytochemical markers on FNA samples.
4. Preoperatively clinically and radiologically indeterminate findings of thyroid nodules could be supplemented by immunocytochemical investigation of FNA biopsy samples.

1.4. Scientific and practical diagnostic novelty

From six molecular markers previously not thoroughly analysed and with equivocal published diagnostic value, three of them in our study showed promising results to differentiate between benign and malignant thyroid nodules. Technology for immunocytochemical analysis of thyroid FNA biopsy material was customised and applied. In the similar way biopsy material could be analysed in the other fields. To our knowledge, this is the first time in Latvia when technology of immunocytochemical analysis of FNA biopsy material of thyroid was used.

1.5. Personal contribution

The author was involved in all stages of the study, including the study design, selection of the markers, thyroid operations with further patients management as well as participating in thyroid US investigation and FNA biopsies. The literature review, scientific measurements, statistical analysis and interpretation were performed by the author. The author supervised processes of immunohistochemical visualisation and is the author of the demonstrated microphotographies.

1.6. Ethical concerns

The study was approved by the Committee of Ethics at Pauls Stradins Clinical University hospital, reference Nr. 151209-3L.

1.7. Structure and size of the work

The Promotional Work is written in english in classical structure. It consists of introduction, literature review, materials and methods, results, discussion, conclusions, practical recommendations and references. Promotional work consists of 116 pages including 20 tables and 20 figures. List of literature consists of 258 references.

2. MATERIALS AND METHODS

2.1. The study design

In the first stage of the study one hundred and sixty-three thyroidectomy specimens were selected consecutively from the files of the Institute of Pathology, Pauls Stradins Clinical University hospital, Riga, Latvia between 2006 and 2010. The diagnoses were verified by repeated histological examination.

Immunohistochemical investigation was performed using six immunohistochemical markers: HBME-1, COX-2, E-CAD, CD56, Ki-67 and p53.

Inclusion criteria were:

- Differentiated thyroid cancer originating from follicular epithelial cell including all types of papillary thyroid cancer and follicular cancer;
- Unequivocal morphological findings;
- Diameter of largest nodule not less that 1 cm;
- Enough archival paraffin-embedded tissue material to analyse;
- Period from operation till immunohistochemical analysis not exceeding eight years.

Exclusion criteria were:

- Hürthle cell variant of follicular cancer;
- Undifferentiated and poorly differentiated cancer including anaplastic carcinoma and insular carcinoma;
- Diameter of largest nodule less that 1 cm;
- Medullary thyroid cancer;
- Malignant lymphoma or metastatic cancer
- Deficient archival paraffin-embedded tissue material to analyse;
- Period from operation till immunohistochemical analysis more than eight years.

In the second stage of the study sixty-eight thyroid FNA cases confirmed by subsequent surgical resection specimens, during the period of 2010-2011, were selected from the Institute of Pathology, Pauls Stradins Clinical University Hospital, Riga, Latvia.

Immunocytochemical investigation was performed using most promising markers according to the first stage results (E-CAD, CD56, HBME-1).

Inclusion criteria were:

- Available FNA smear as well as operation material for the same case according to the above mentioned inclusion criteria;
- Period less than one year from FNA biopsy of the thyroid till operation and final morphology.

Exclusion criteria were:

- Unavailable operation material;
- Period more than one year from FNA biopsy of the thyroid till operation and final morphology.

2.2. Materials

In the first stage of the study after the histological verification of diagnosis the study group consisted of 50 malignant and 113 benign thyroid lesions including 36 papillary thyroid cancers (PTC) and 14 follicular cancers (FC) as well as 36 follicular adenomas (FA) and 77 cases of colloid goiter (CG).

In the second stage of the study the study group consisted of 26 malignant and 42 benign thyroid lesions including 25 PTC and 1 FC as well as 22 FA and 20 cases of CG.

2.3. Methods

2.3.1. Tissue processing and general histological report

The tissues of operation materials were fixed in 10% neutral buffered formalin, sampled widely during the grossing of operation material, processed and embedded in paraffin blocks as described by Bancroft et al. [26].

During screening, the archival diagnoses were verified by the examination of these slides. The diagnostics, typing and grading of thyroid pathology were performed according to the WHO classification of tumours [27]. Only cases with unequivocal histological diagnosis were included in the study group.

2.3.2. Immunohistochemistry

For immunophenotypic studies, formalin-fixed, paraffin-embedded tissues were cut in 3-µm-thick sections on electrostatically charged Histobond glass slides and incubated in 60°C for 1 h. Deparaffining and rehydration were carried out by routine treatment in xylene for 4 × 5 min and graded ethanol for 2 × 3 min, 99.9%; 4 × 3 min, 96% and 5 min, 70%. Endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide in methanol for 10 min. All

chemicals were produced by Sigma-Aldrich (Steinheim, Germany).

After rinsing in TBS buffer (pH 7.6, Tris-buffered saline, THAM-HCl 50 mM/L, NaCl 150 mM/L) for 5 min, the slides were subjected to heat-induced antigen retrieval (HIER) treatment in domestic microwave oven for 3 × 5 min at maximum power in basic (TEG, pH 9.0, Tris base 10 mM/L, EGTA 0.5 mM/L) buffer. After HIER, the slides were allowed to cool at room temperature for 20 min in the HIER buffer.

The slides were encircled with Dako pen (Dako, Glostrup, Denmark) and transferred to magnetic immunostaining trays (CellPath plc, Newtown, UK). After the rinse with TBS buffer for 5 min, the incubation with primary antibodies (Table 2.1.) was carried out at room temperature for 60 min. Unbound primary antibodies later were removed by repeated rinses with TBS buffer 2 × 5 min.

2.1. tabula

Characteristics of the antibodies

Target antigen	Monoclonal antibody	Antibody dilution
E-CAD	NCH-38	1:50
CD56	123C3	1:100
HBME-1	HBME-1	1:50
COX-2	CX-294	1:200
p53	DO-7	1:400

All antibodies were produced by Dako, Glostrup, Denmark.

A commercially available polymeric EnVision+ System, bound with horseradish peroxidase (Dako), was used for visualisation. The colour development was obtained with 3,3-diaminobenzidine (Dako) for 10 min. Positive and negative control slides were included in each run.

The evaluation and scoring was performed by the author under

supervision of experienced pathologist. The expression of p53 protein and Ki-67 was evaluated in the nuclei of cancer cells counting positive nuclei among 200 neoplastic cells and expressing the result as the percentage of positive cells. The expression of E-CAD, CD56 and HBME-1 as well as COX-2 were scored semiquantitatively by staining the intensity and percentage of positive cells. The staining intensity was estimated as negative, 0; weakly positive, 1; moderately intensively staining, 2 or intensively positive, 3. The lesion was considered positive for a marker when the expression intensity was at least 1.5.

2.3.3. Fine needle aspiration and immunocytochemistry

US followed by FNA was performed by one experienced radiologist using GE Voluson E8 ultrasound machine and 11L-D linear transducer at Pauls Stradins Clinical University Hospital, Institute of Diagnostic Radiology, Riga, Latvia.

In case of a single thyroid nodule, only those nodules above 1 cm were further evaluated by FNA. In a multinodular gland the dominant nodule was evaluated or the nodule with most suspicious US findings. US characteristics associated with a higher risk of malignancy are as described previously: marked hypoechogenicity compared to normal thyroid parenchyma, microcalcifications, irregular or microlobulated margins, increased intranodular vascularity and a taller than wide shape [28].

A minimum of 2 passes were employed. Needle placement was documented by taking pictures. Aspirated material was placed, smeared on Histobond adhesive glass slides and air dried.

For immunophenotypic studies the cell smears were air dried and fixed in 96% ethanol for 10 min. Further immunophenotypic technology was done as described in section 2.3.2.

2.3.4. Methods of statistical analysis

The statistical evaluation of the data was carried out using the Statistical Package for Social Sciences (SPSS® version 18.0) and Microsoft Excel 2011 (Mac). In the present study descriptive statistics was used as well as 95% confidence interval for single proportion and for mean as described by Altman et al. [29]. The confidence interval calculations were made by Confidence Interval Analysis (CIA) software. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated.

3. RESULTS

3.1. The results of immunohistochemical staining

In the first stage of the study 50 malignant and 113 benign thyroidectomy specimens were analysed including 36 papillary thyroid cancers and 14 follicular cancers as well as 36 follicular adenomas and 77 cases of colloid goiters. The mean age of the patients was 57 ± 14.52 (24 - 85 years). There were 20 male patients and 143 females.

Of 36 papillary thyroid cancers 26 were stage I tumours and 10 were stage II. In follicular cancers group 10 were stage I tumours and 4 were stage II.

3.1.1. Expression of E-CAD

The mean expression of E-CAD in FA was 2.2 (95% CI = 1.92-2.48) which is significantly higher than in the tissues surrounding FA - 0.63 (95% CI = 0.39-0.87). FC also was characterised by high expression of E-CAD - 2.1 (95% CI = 1.64-2.56). Unfortunately, there was no possibility to analyse expression of E-CAD in thyroid tissue surrounding FC due to its widespread

invasion.

In contrast to FA and FC, PTC showed lowest expression of E-CAD - 0.55 (95% CI = 0.34-0.75).

In CG expression of E-CAD was 1.39 (95% CI = 1.23-1.54) which is lower that FA or FC, but higher than in PTC.

Results of the expression of E-CAD in different thyroid lesions are summarised in Table 3.1.

Table 3.1.

Descriptive statistics of E-CAD expression in different thyroid lesions

Target structure	E-CAD expression intensity				
	N	Mean ± SD	95% CI	Min	Max
Follicular adenoma	36	2.20 ± 0.82	1.92 - 2.48	0.15	3
Thyroid tissue surrounding follicular adenoma	32	0.63 ± 0.71	0.39 - 0.87	0	1.5
Papillary thyroid cancer	36	0.55 ± 0.61	0.34 - 0.75	0	2.4
Thyroid tissue surrounding papillary thyroid cancer	34	1.60 ± 0.85	1.30 - 1.90	0.3	3
Colloid goiter	77	1.39 ± 0.69	1.23 - 1.54	0	3
Follicular cancer	14	2.10 ± 0.80	1.64 - 2.56	0	3

Abbreviation in the Table: N, number of measurements; SD, standard deviation; CI, confidence interval for a mean; Min, minimal observed value; Max, maximal observed value.

Taking into account the presented results, the sensitivity, specificity, PPV and NPV of E-CAD were calculated. E-CAD shows high sensitivity and specificity for separating FA from the tissue surrounding FA with values 0.88 and 0.87, respectively. PPV and NPV were 0.88 and 0.87.

The sensitivity and specificity were 0.91 and 0.89 for FA compared to PTC. For separating PTC from tissue surrounding PTC E-CAD shows 0.70

sensitivity and 0.86 specificity.

The expression frequency of E-CAD was found in 32/36 (88.8%; 95% CI = 74.7-96.0%) cases of FA. In contrast, thyroid tissue surrounding FA showed very low E-CAD expression frequency - 4/32 (12%; 95% CI = 5.0-28.0%). Notable is the fact that expression of E-CAD in case of PTC was found only in 3/36 cases (8.3%; 95% CI = 2.8-21.8%). More frequently E-CAD expression was present in thyroid tissue surrounding PTC, namely, 20/34 (58.8%; 95% CI = 42.2-73.6%).

Results of the expression frequency of E-CAD in different thyroid lesions are summarised in Table 3.2. Expression of E-CAD in FA and PTC is showed in figure 3.1.A,B.

Table 3.2.

Frequency of E-CAD expression in different thyroid lesions

Target structure	E-CAD	
	Expression n (%)	95% CI
Follicular adenoma (n=36)	32 (88.8%)	74.7 - 96.0
Thyroid tissue surrounding follicular adenoma (n=32)	4 (12.5%)	5.0 - 28.0
Papillary thyroid cancer (n=36)	3 (8.3%)	2.8 - 21.8
Thyroid tissue surrounding papillary thyroid cancer (n=34)	20 (58.8%)	42.2 - 73.6
Colloid goiter (n=77)	25 (32.4%)	23.0 - 43.5
Follicular cancer (n=14)	13 (92.8%)	68.5 - 98.7

Abbreviation in the Table: n, absolute number; CI, confidence interval of a proportion.

3.1.2. Expression of CD56

The mean expression of CD56 in FA was 2.2 (95% CI = 1.88-2.51) which is significantly higher than in the tissue surrounding FA - mean value 0.95

(95% CI = 0.67-1.22). FC was characterised by highest expression of CD56 - mean value 2.3 (95% CI = 1.72-2.87).

In PTC expression of CD56 was significantly lover than in the tissue surrounding PTC, namely, 0.2 (95% CI = 0.13-0.26) and 1.02 (95% CI = 0.85-1.18), respectively.

In CG expression of CD56 was 0.85 (95% CI = 0.70-0.99) which is lower that FA or FC, but higher than in PTC.

Results of the expression of CD56 in different thyroid lesions are summarised in Table 3.3.

Table 3.3.

Descriptive statistics of CD56 expression in different thyroid lesions

Target structure	CD56 expression intensity				
	N	Mean ± SD	95% CI	Min	Max
Follicular adenoma	36	2.20 ± 0.92	1.88 - 2.51	0.6	3
Thyroid tissue surrounding follicular adenoma	32	0.95 ± 0.77	0.67 - 1.22	0	3
Papillary thyroid cancer	36	0.20 ± 0.19	0.13 - 0.26	0	0.6
Thyroid tissue surrounding papillary thyroid cancer	34	1.02 ± 0.47	0.85 - 1.18	0.6	2.4
Colloid goiter	77	0.85 ± 0.63	0.70 - 0.99	0	3
Follicular cancer	14	2.30 ± 1.00	1.72 - 2.87	0.4	3

Abbreviation in the Table: N, number of measurements; SD, standard deviation; CI, confidence interval for a mean; Min, minimal observed value; Max, maximal observed value.

Taking into account the presented results, the sensitivity, specificity, PPV and NPV of CD56 were calculated. CD56 sensitivity, specificity for separating FA from the tissue surrounding FA were 0.88 and 0.82 as well as

PPV and NPV were 0.83 and 0.87 respectively. Comparing FA and PTC the sensitivity and specificity were 1.0 and 0.85, respectively. For separating PTC from tissue surrounding PTC CD56 shows 0.53 sensitivity and 1.0 specificity.

The expression frequency of CD56 was found in 28/36 (78.0%; 95% CI = 62.0-88.2%) cases of FA. In contrast, thyroid tissue surrounding FA showed comparatively low CD56 expression frequency - 4/32 (12.5%; 95% CI = 5.0-28.0%). In case of PTC there where no CD56 expression observed and only one case in thyroid tissue surrounding papillary thyroid cancer. Interestingly in case of FC 13/14 cases CD56 expression was obtained (92.8%; 95% TI = 68.5-99.0%).

Results of the expression frequency of CD56 in different thyroid lesions are summarised in Table 3.4. Intense expression of CD56 in FA and absence of expression in PTC is showed in figure 3.1.D, E.

Table 3.4.

Frequency of CD56 expression in different thyroid lesions

Target structure	CD56	
	Expression n (%)	95% CI
Follicular adenoma (n=36)	28 (78.0%)	62.0 - 88.2
Thyroid tissue surrounding follicular adenoma (n=32)	4 (12.5%)	5.0 - 28.0
Papillary thyroid cancer (n=36)	0 (0%)	0 - 9.6
Thyroid tissue surrounding papillary thyroid cancer (n=34)	1 (3.0%)	0.5 - 15.0
Colloid goiter (n=77)	10 (13.0%)	7.2 - 22.2
Follicular cancer (n=14)	13 (92.8%)	68.5 - 99.0

Abbreviation in the Table: n, absolute number; CI, confidence interval of a proportion.

3.1.3. Expression of HBME-1

The expression of HBME-1 was absent in benign thyroid lesions including FA, CG and pericancerous tissue but was notably high in PTC with the average intensity 2.80 (95% CI = 2.68-2.91) and 4/14 cases of FC with the average intensity 0.90 (95% CI = 0.10-1.70).

Results of the expression of HBME-1in different thyroid lesions are summarised in Table 3.5.

Table 3.5.

Descriptive statistics of HBME-1 expression in different thyroid lesions

Target structure	HBME-1 expression intensity				
	N	Mean ± SD	95% CI	Min	Max
Follicular adenoma	36	0.09 ± 0.37	0 - 0.22	0	1.8
Thyroid tissue surrounding follicular adenoma	32	0.001 ± 0.003	0 - 0.002	0	0.03
Papillary thyroid cancer	36	2.80 ± 0.33	2.68 - 2.91	2.1	3
Thyroid tissue surrounding papillary thyroid cancer	34	0.006 ± 0.03	0 - 0.016	0	0.15
Colloid goiter	77	0	0	0	0.15
Follicular cancer	14	0.90 ± 1.40	0.10 - 1.70	0	2.85

Abbreviation in the Table: N, number of measurements; SD, standard deviation; CI, confidence interval for a mean; Min, minimal observed value; Max, maximal observed value.

Taking into account the presented results, the sensitivity, specificity, PPV and NPV of HBME-1 were calculated. Sensitivity, specificity, PPV and NPV for separating PTC from the tissue surrounding PTC using HBME-1 is 1.00. To differentiate FA from PTC sensitivity and specificity is 1.00 and 0.97. Besides PPV and NPV is 0.97 and 1.00 respectively. HBME-1 has an extremely high value in the differential diagnostics of PTC showing high ability to

discriminate between PTC and FA or benign tissues.

The expression frequency of HBME-1 was found only in 1/36 (3%; 95% CI = 0.5-14.1%) cases of FA and in none of thyroid tissue surrounding FA as well as CG. While in PTC expression was present in 36/36 (100%; 95% CI = 90.3 - 100%) but there where no expression in thyroid tissue surrounding PTC. FC showed expression of HBME-1 in 4/14 cases (28.5%; 95% CI = 11.7 - 54.6%).

Results of the expression frequency of HBME-1 in different thyroid lesions are summarised in Table 3.6. Expression of HBME-1 in PTC and FC is showed in figure 3.1.C, F.

Table 3.6.

Frequency of HBME-1 expression in different thyroid lesions

Target structure	HBME-1	
	Expression n (%)	95% CI
Follicular adenoma (n=36)	1 (3.0%)	0.5 - 14.1
Thyroid tissue surrounding follicular adenoma (n=32)	0 (0%)	0 - 10.7
Papillary thyroid cancer (n=36)	36 (100%)	90.3 - 100.0
Thyroid tissue surrounding papillary thyroid cancer (n=34)	0 (0%)	0 - 10.1
Colloid goiter (n=77)	0 (0%)	0 - 4.7
Follicular cancer (n=14)	4 (28.5%)	11.7 - 54.6

Abbreviation in the Table: n, absolute number; CI, confidence interval of a proportion.

3.1.4. Expression of COX-2

The expression of COX-2 was low in all lesions and did no show any statistical significant differences between groups. Results of the expression of COX-2 are summarised in Table 3.7.

Table 3.7.

Descriptive statistics of COX-2 expression in different thyroid lesions

Target structure	COX-2 percentage of positive cells				
	N	Mean ± SD	95% CI	Min	Max
Follicular adenoma	36	0.25 ± 0.41	0.11 - 0.39	0	0.53
Thyroid tissue surrounding follicular adenoma	32	0.12 ± 0.24	0.03 - 0.20	0	0.31
Papillary thyroid cancer	36	0.21 ± 0.38	0.08 - 0.34	0	0.51
Thyroid tissue surrounding papillary thyroid cancer	34	0.20 ± 0.32	0.09 - 0.31	0	0.47
Colloid goiter	77	0.34 ± 0.42	0.24 - 0.43	0	0.72
Follicular cancer	14	0.34 ± 0.67	0 - 0.73	0	0.85

Abbreviation in the Table: N, number of measurements; SD, standard deviation; CI, confidence interval for a mean; Min, minimal observed value; Max, maximal observed value.

3.1.5. Expression of Ki-67

The expression of Ki-67 was generally low, not reaching 5%. However, there were statistically significant differences between PTC with expression 2.36 (95% CI = 2.07-2.64) and the surrounding tissues with expression 0.99 (95% CI = 0.83-1.14). In FC expression was 3.62 (95% CI = 3.00-4.25) which is significantly higher than in FA 1.07 (95% CI = 0.85-1.29) or CG 0.69 (95% CI = 0.56-0.82).

Overall proliferative activity was significantly higher in cancers. Results of the expression of Ki-67 are summarised in Table 3.8.

Table 3.8.

Descriptive statistics of Ki-67 expression in different thyroid lesions

Target structure	Ki-67 percentage of positive cells				
	N	Mean ± SD	95% CI	Min	Max
Follicular adenoma	36	1.07 ± 0.65	0.85 - 1.29	0.1	1.7
Thyroid tissue surrounding follicular adenoma	32	0.75 ± 0.52	0.56 - 0.93	0	1.2
Papillary thyroid cancer	36	2.36 ± 0.85	2.07 - 2.64	0.2	4.7
Thyroid tissue surrounding papillary thyroid cancer	34	0.99 ± 0.45	0.83 - 1.14	0	1.3
Colloid goiter	77	0.69 ± 0.57	0.56 - 0.82	0	1.0
Follicular cancer	14	3.62 ± 1.10	3.00 - 4.25	0.4	4.9

Abbreviation in the Table: N, number of measurements; SD, standard deviation; CI, confidence interval for a mean; Min, minimal observed value; Max, maximal observed value.

3.1.6. Expression of p53

No expression of p53 was found in any of the groups.

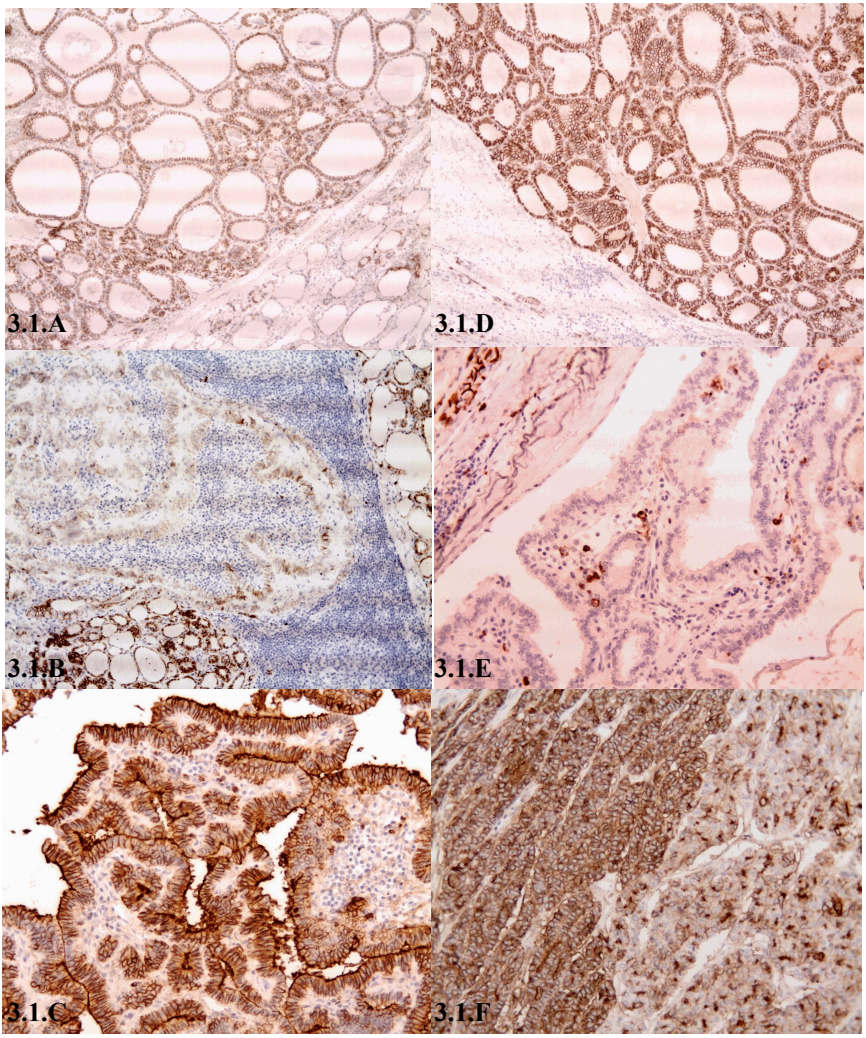

Figure 3.1.A, intense membranous expression of E-Cadherin in follicular adenoma. Immunoperoxidase, magnification 50×. **3.1.B**, weak membranous expression of E-Cadherin in papillary thyroid cancer. Note intense expression in the adjacent benign thyroid tissue. Immunoperoxidase, magnification 50×. **3.1.C**, intense membranous expression of HBME-1 in papillary thyroid cancer. Immunoperoxidase, magnification 100×. **3.1.D**, intense membranous expression of CD56 in follicular adenoma. Immunoperoxidase, magnification 50×. **3.1.E**, absence of CD56 expression in papillary thyroid cancer. Immunoperoxidase, magnification 100×. **3.1.F**, intense heterogeneous expression of HBME-1 in follicular cancer. Immunoperoxidase, magnification ×100.

3.2. The results of immunocytochemical staining

In the second stage of the study we picked three of six markers (E-CAD, CD56 and HBME-1) analysed in the first stage who showed most promising and acceptable results. Twenty-six malignant and 42 benign thyroid FNA cases confirmed by subsequent surgical resection were included. Study group consisted of 25 papillary thyroid cancers, 1 follicular cancer as well as 22 follicular adenomas and 20 cases of colloid goiters.

The mean age of the patients was 54 ± 13.96 (22 - 77 years). There were 6 male patients and 62 females. Of all cancers 24 were stage I tumours and 2 were stage II.

3.2.1. Expression of E-CAD in FNA material

The expression by immunocytochemistry of E-CAD was found in 16/22 (72.7%; 95% CI = 52.0-87.0%) cases of FA. In contrast, PTC showed very low E-CAD expression 2/25 (8%; 95% CI = 2.2-25.0%). In case of CG expression of E-CAD was 2/20 (10%; 95% CI = 2.8-30.1%). No expression was found in FC.

Results of the immunocytochemical staining of E-CAD are summarised in Table 3.9. Immunocytochemical expression of E-CAD showed in figure 3.2.A.

Table 3.9.

Frequency of E-CAD expression in FNA material of different thyroid lesions

Target structure	E-CAD	
	Expression n (%)	95% CI
Follicular adenoma (n=22)	16 (72.7%)	50.0 - 88.0
Colloid goiter (n=20)	2 (10%)	1.75 - 33.1

Target structure	E-CAD	
Papillary thyroid cancer (n=25)	2 (8%)	1.40 - 27.5
Follicular cancer (n=1)	0 (0%)	0 - 95.0

Abbreviation in the Table: n, absolute number; CI, confidence interval of a proportion.

3.2.2. Expression of CD56 in FNA material

The expression by immunocytochemistry of CD56 was found in 12/22 (54.5%; 95% CI = 34.6-73.0%) cases of FA. In contrast, PTC showed very low CD56 expression 1/25 (4%; 95% CI = 0.7-20.0%). In case of CG expression of CD56 was 1/20 (5%; 95% CI = 0.9-23.6%) No expression was found in FC. Results of the immunocytochemical staining of CD56 are summarised in Table 3.10. Immunocytochemical expression of CD56 showed in figure 3.2.B.

Table 3.10.

Frequency of CD56 expression in FNA material of different thyroid lesions

Target structure	CD56	
	Expression n (%)	95% CI
Follicular adenoma (n=22)	12 (54.5%)	34.6 - 73.0
Colloid goiter (n=20)	1 (5%)	0.9 - 23.6
Papillary thyroid cancer (n=25)	1 (4%)	0.7 - 20.0
Follicular cancer (n=1)	0 (0%)	0 - 79.3

Abbreviation in the Table: n, absolute number; CI, confidence interval of a proportion.

3.2.3. Expression of HBME-1 in FNA material

The expression by immunocytochemistry of HBME-1 in PTC was significantly higher than in another thyroid lesions included in the study 24/25

(96%; 95% CI = 80.4-99.3%). HBME-1 expression was absent in FA and CG as well as in the only case of FC. Results of the immunocytochemical staining of HBME-1 are summarised in Table 3.11.

Table 3.11.

Frequency of HBME-1 expression in FNA material of different thyroid lesions

Target structure	HBME-1	
	Expression n (%)	95% CI
Follicular adenoma (n=22)	0 (0%)	0 - 15.0
Colloid goiter (n=20)	0 (0%)	0 - 16.1
Papillary thyroid cancer (n=25)	24 (96%)	80.4 - 99.3
Follicular cancer (n=1)	0 (0%)	0 - 80.0

Abbreviation in the Table: n, absolute number; CI, confidence interval of a proportion.

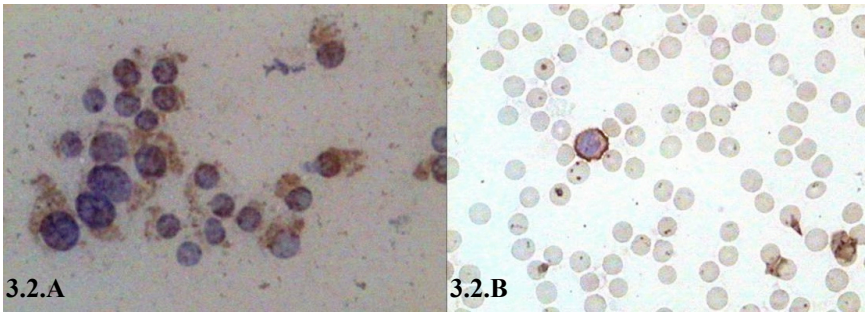

Figure 3.2.A, cytoplasmic expression of E-CAD in a group of thyroid epithelial cells. Immunoperoxidase, anti-E-CAD, magnification × 400. **3.2.B**, intense membranous expression of CD56 in a single epithelial cell despite the rich presence of red blood cells. Immunoperoxidase, anti-CD56, magnification × 400.

4. DISCUSSION

Thyroid nodules are fairly common findings in clinical practice affecting approximately 40% of the population between 30 and 60 years old in the United States, besides thyroid cancer is the most common endocrine malignancy. Luckily most of these nodules are benign tumors or hyperplastic lesions and only a minority are malignant or suspicious tumours that require surgery. Therefore it is important to identify these benign lesions for proper management and to realise best possible benefit for the patients. For postoperative management of patients with thyroid nodules, accurate diagnosis is very critical as well. Errors in any of the patients management stage can lead to significant psycho-social problems and unnecessary surgery increasing healthcare cost [13].

The local importance of the problem is emphasised by the growing incidence of thyroid cancer in Latvia [30].

According to data provided by Central Statistical Bureau of Latvia there is a 3.3× increase in thyroid cancer incidence comparing year 1990 (n=59) with 2011 (n=195). According to data of year 2011 incidence of thyroid cancer in women is 5× more than in man [30].

The decision whether to observe the nodules or to refer patient to surgical operation, is based on clinical information, thyroid US, scintigraphy, and FNA diagnosis. Although thyroid cancer constitutes one of the curable cancers, the differential diagnosis can often be ambiguous.

First FNA of the thyroid in Latvia was performed in 1991 by Peteris Prieditis. In early years there were only some cases of thyroid FNA no exceeding 20-30 per year [31]. Nowadays FNA of the thyroid are performed in several places, namely, in Pauls Stradins Clinical University hospital, Clinic Teika, Riga Eastern Hospital Latvian Oncology Centre, and much less in Latvian cites Valmiera and Liepaja. Most of all thyroid FNA are performed in

Clinic Teika - 1200 per year (data of year 2011), then follows Riga Eastern Hospital Latvian Oncology Centre - 700 per year and 400 per year in Pauls Stradins Clinical University hospital [32].

In fact, the present consensus is that thyroid FNA biopsy is the procedure of choice for evaluation of nodules therefore cytologic interpretation can play a very important role in further clinical management of the patient. There are well-known limitations in the role of thyroid FNA, most importantly its inability to differentiate benign from malignant follicular neoplasms, since the final diagnosis rests on the histologic identification of capsular and / or vascular invasion, not available for cytology. Therefore these patients usually undergo surgical resection, while only about 10% of them will after all have malignant tumors [9].

According to aforementioned reasons, during the last several years researchers have focused on finding molecular or IHC markers that could help in the distinction between benign and malignant thyroid nodules.

According to our data, **HBME-1** had a high expression level in papillary thyroid cancer and 4/14 follicular cancer on tissue samples. High HBME-1 expression was present also on FNA samples. There was no HBME-1 expression in benign lesions of surgical tissue samples as well as in FNA material. This agrees with the study by Nasr et al. [14] on thyroidectomy specimens showing positive expression of HBME-1 in 49/51 (96%) of papillary thyroid cancer, whereas normal thyroid tissue was consistently negative. Overall, HBME-1 in his study showed 96% sensitivity and 93% specificity for PTC.

Although Miettinen's study analysing operation material showed HBME-1 expression in 145/145 papillary thyroid cancer and 27/27 follicular cancer. In contrast, 33% cases of nodular goitres and papillary hyperplasia either showed no reactivity or were focally positive [33]. Notable is the fact that all cases of FC were with high expression. In our study only 4/14 cases of FC had

high expression level. This difference could be explainable with different antibody clone. However we believe that the invariably high expression suggests the possibility of technological failure.

A study by Saleh et al. [34] performed ICH staining of HBME-1 on cell block sections of thyroid FNA. They concluded that HBME-1 also had a high immunoexpression level in malignant tumours 24/27 (88.8%) compared to benign lesions 12/44 (27.3%). This agree with our study as well with the previous studies showing a high rate of immunoexpression of HBME-1 in malignant thyroid tumours.

Analysing our data papillary thyroid cancer demonstrated a significant reduction in **E-CAD** expression when compared to FA or thyroid tissue surrounding papillary thyroid cancer. In contrast, follicular cancer showed no significant differences in E-CAD expression compared to follicular adenoma and the expression was generally very high. As mentioned before, there was only one case of follicular cancer to analyse in second stage of the study in FNA material. The low and heterogenous expression of E-CAD in papillary thyroid cancer and intensive in follicular adenoma or follicular cancer confirms the results of Brabant et al. [35] where in 9 of 16 patients with papillary thyroid cancer the level of E-CAD was clearly reduced, which was particularly evident when compared to normal tissue located near the tumour.

One of the few studies published so far of E-CAD expression in FNA material of the thyroid are made by Pazaitou-Panayiotou et al. [36]. E-CAD expression was analysed in 83 FNA cases diagnosed as papillary thyroid cancer and results showed positive E-CAD in 5 out of 83 (6%) cases, and 79 (93.97%) cases showed loss of expression. All controls retained their normal expression. These findings are like results of our study. The author concludes that loss of expression of E-CAD may provide an objective diagnostic tool, and its use may be extremely useful in the diagnosis of papillary thyroid cancer, especially in doubtful cases.

So far there are very few studies published concerning **CD56** expression in thyroid neoplasms. Notable is the fact that there are no study published about CD56 expression in such a number of FC cases and only one study exists were CD56 expression is analysed in thyroid FNA material.

Similar results to our finding was published by Migita et al. [37] where CD56 expression intensity was analysed in thyroidectomy specimens. Comparatively confident results of the study showed less than 10% cell expression of CD56 in papillary thyroid cancer whereas colloid goitre and follicular adenoma showed approximately 70% of the cells positive results. These results are similar to our study except for papillary thyroid cancer where it was remarkably decreased.

Study by Zeromski et al. [17] analysed CD56 expression in benign samples of human thyroid collected during surgery. According to study results CD56 expression showed positive results on all benign thyroid tissue examined (simple goiter, Grave's disease, Hashimoto's thyroiditis). Author is speculating that CD56 is involved in the morphogenesis, the growth and the function of thyroid gland in both, normal and pathological conditions, by promoting homotypic cell adhesion [17].

Most recent study done by Kim et al. [38] who analysed CD56 staining on 72 papillary thyroid cancers. Results revealed similar findings to previously published data moreover similar to our study that non-neoplastic areas of all 72 cases showed positive CD56 expression along the plasma membrane; however staining was reduced or absent in 65 papillary thyroid cancers (90.3%). In this study only papillary thyroid cancers were included, besides the CD56 staining did not reveal any significant relationship with clinicopathologic features ($p>0.05$).

Contrary to our work results of **COX-2** expression in thyroid lesions are published by Specht et al. [21]. Author analysed IHC expression of COX-2

in 28 different thyroid specimens. Immunohistochemical analysis of representative cases of thyroid cancer (12 papillary thyroid cancers, 1 follicular cancer) revealed that COX-2 expression was multifocal and moderate to strong in intensity in the majority of cases. Staining for COX-2 according to results was negligible in normal tissue. In tumour tissue, expression of COX-2 was localised to tumour cells, but not to surrounding stromal cells or infiltrating inflammatory cells. These results are in great contradiction to our results where the difference in COX-2 expression between colloid goiter, follicular adenoma, papillary thyroid cancer and follicular cancer was not remarkable and not statistically significant. Furthermore it is well known that technological variations exceed the biological differences regarding COX-2 analysis by IHC [39].

Very similar finding and conclusions with our study was published by Kim et al. [40] where COX-2 expression was studied immunohistochemically in 19 papillary thyroid cancers, 8 follicular cancers, 14 follicular adenomas, and 8 colloid goiters. COX-2 staining was not present in any of the colloid goiters. In contrast, COX-2 staining was observed in all of papillary thyroid cancers. Moreover, 7 of 8 follicular cancers and 11 of 14 follicular adenomas showed COX-2 staining. Kim et al. concludes that COX-2 is not useful as a marker of malignancy since its expression was evident in follicular adenomas and in papillary thyroid cancer and follicular cancer as well. Wherewith author speculates that the enzyme could be involved in the early process of thyroid tumorogenesis. In our study we didn't find such a differences in expression intensities between groups, but overall usefulness of the marker is also found to be very dubious.

Analysing **Ki-67** very confident results, which are in common with findings of our study, are published by Mehrotra et al. [41]. Ki-67 protein expression were assessed by IHC in formalin-fixed, paraffin-embedded thyroid tissues from 128 patients with histologic diagnoses of papillary thyroid cancer

(n = 38), follicular cancer (n = 22), follicular adenoma (n = 33), and colloid goiter (n = 35). As a result Ki-67 labeling was higher in follicular cancer and papillary thyroid cancer than in follicular adenoma or colloid goiter. The Ki-67 discriminated between follicular cancer and follicular adenoma ($P < 0.0001$). However, Ki-67 overlapped widely between the four histologic groups, and the expression of these proteins was also noted to be heterogenous within these lesions. Mehrotra et al. concluded that Ki-67 cannot currently be reliably applied as preoperative markers to distinguish benign from malignant thyroid lesions.

Similar results to our study is published by Liang et al. [42] who analysed more that 10 IHC markers on thyroid tissue samples from 119 patients including 71 papillary thyroid cancers, 26 follicular cancers and 48 follicular adenomas. According to the results Ki-67 expression was not significantly different between the groups and immunopositivity for Ki-67 among the carcinomas was highly variable, ranging from + to +++. Author speculates that these results may be related to antibody selection, inability of the antibody to recognise a protein with altered configuration, or antigen retrieval methodology. As conclusion it makes it unlikely that Ki-67 alone will be of value and practical use in differentiating benign from malignant differentiated thyroid tumours.

p53 according to our study was not expressed in any of the groups. This could be associated with lack of undifferentiated cancer morphologies included as well as lack of lymph node metastasis included. Important factors include possible differences in primary antibody due to different manufacturers.

Confident results are published by Morita et al. [43] who analysed p53 expression in 68 patients in whom thyroidectomy with lymph node dissection had been performed due to papillary thyroid cancer. Results of IHC staining showed overexpression of p53 protein in papillary thyroid cancer, and revealed a statistically significant correlation between overexpression of p53 and large tumour size, presence of lymph node metastasis, and the mean number of lymph

node metastases. So far this is the only study analysing such a number of lymph nodes (n=196).

Differential diagnosis of thyroid nodules and treatment options still remains actual from medical and socio-economic point of view. Possibly this topic will be actual for a very long time. Nowadays recent trends in molecular diagnostics and radiological examinations plays a crucial role in the choice of management of nodular thyroid.

5. CONCLUSIONS

1. As significant immunophenotypic differences are found in nodular thyroid diseases, immunohistochemistry can have valuable diagnostic implications
2. The panel consisting of three immunohistochemical markers, HBME-1, E-CAD and CD56, can reliably distinguish papillary thyroid carcinoma from follicular tumours (follicular adenoma and follicular cancer).
3. Our results indicate that intense expression of HBME-1 is found in malignant lesions only. It is also the most sensitive and specific single marker in papillary thyroid cancer.
4. CD56 and E-CAD can assist in decision making about benign and malignant nature of the aspirated material. Loss of expression seems to agree with the presence of papillary thyroid cancer and distinguishes it from follicular tumours. Both of them are characterised by high expression of CD56 and E-CAD.
5. Although proliferation activity significantly differs between benign and malignant thyroid lesions, the practical use of Ki-67 marker is difficult due to generally low values.
6. The lack of p53 expression in our study may be due to lower level of malignancy, as our study group did not include undifferentiated tumours.

7. According to our study, the difference in COX-2 expression between groups was not remarkable and not significant.
8. Developed FNA technologies reveals different frequencies of E-CAD, HBME-1 and CD56 expression in follicular adenoma and papillary thyroid cancer.

6. PRACTICAL RECOMMENDATIONS

1. The established technologies for immunohistochemical analysis of histological material should be introduced for practical diagnostic use.
2. For evaluation of FNA smears we recommend the use of created immunocytochemical visualisation technique.
3. We recommend the established panel of molecular markers (HBME-1, E-CAD and CD56) as an additional criteria to diagnose cancer in preoperative FNA when US characteristics associated with higher risk of malignancy are not conclusive.
4. Taking into account the results of marker expression in FNA, it is possible to evaluate the indications for thyroid operation and its extend more appropriate.

7. LIST OF PUBLICATIONS ON THE STUDY THEME

1. Ozolins A., Narbuts Z., Strumfa I., Volanska G., Gardovskis J. *DIAGNOSTIC UTILITY OF IMMUNOHISTOCHEMICAL PANEL IN VARIOUS THYROID PATHOLOGIES.* Langenbeck`s Archives of Surgery, 2010 p. 885-891.
2. Ozolins A., Narbuts Z., Strumfa I., Prieditis P., Gardovskis J. *DIAGNOSIS AND MANAGEMENT OF THE THYROID NODULES*. Acta Chirurgica Latviensis, 2010, p. 86-91.
3. Ozolins A., Narbuts Z., Strumfa I., Volanska G., Prieditis P., Stepanovs K.,

Gardovskis J. *IMMUNOCYTOCHEMISTRY AS AN ADJUNCT TO FINE-NEEDLE ASPIRATION OF THYROID IN DISTINCTION BETWEEN BENIGN AND MALIGNANT THYROID NEOPLASMS.* Acta Chirurgica Latviensis, 2011, p. 39-43.

4. Ozolins A., Narbuts Z., Strumfa I., Volanska G., Stepanovs, K., Gardovskis J. *IMMUNOHISTOCHEMICAL EXPRESSION OF HBME-1, E-CADHERIN AND CD56 IN THE DIFFERENTIAL DIAGNOSIS OF THYROID NODULES.* Medicina (Kaunas). 2012; accepted for publication.

8. REPORTS ON THE STUDY THEME

1. Thesis and oral presentation - Ozolins A., Narbuts Z., Strumfa I., Volanska G.,Gardovskis J. *DIAGNOSTIC UTILITY OF IMMUNOHISTOCHEMICAL PANEL IN VARIOUS THYROID PATHOLOGIES.* Riga Stradins University, Scientific conference 2010., p. 245.
2. Thesis and poster presentation - Ozolins A., Narbuts Z., Strumfa I., Volanska G., Gardovskis J. *IMMUNOHISTOCHEMICAL PANEL IN THE DIAGNOSTICS OF THYROID CANCER.* Book of abstracts 5th Baltic Congress of Oncology. May 14-15, Riga, Latvia 2010, p. 8.
3. Thesis and oral presentation - Ozolins A., Narbuts Z., Strumfa I., Volanska G.,Gardovskis J. *DIAGNOSTIC UTILITY OF IMMUNOHISTOCHEMICAL PANEL IN VARIOUS THYROID PATHOLOGIES.* Book of abstracts European Society of Endocrine Surgeons 4th Biennial congress. May 13-15, Vienna, Austria 2010.
4. Thesis and poster presentation - Ozolins A., Narbuts Z., Strumfa I., Prieditis P., Stepanovs K., Gardovskis J. *THE IMMUNOCYTOCHEMISTRY AS AN ADJUNCT TO FINE NEEDLE ASPIRATION CYTOLOGY OF THYROID NODULES. FIRST EXPERIENCE IN LATVIA.* Riga Stradins University, Scientific conference 2011., p. 286.

5. Thesis and poster presentation - Ozolins A., Narbuts Z., Strumfa I., Gardovskis J. *IMPACT OF IMMUNOHISTOCHEMICAL VISUALISATION TECHNOLOGY ON THE DIAGNOSTICS OF THYROID PATHOLOGY.* Riga Stradins University, Scientific conference 2011., p. 288.
6. Thesis and poster presentation - Ozolins A., Narbuts Z., Strumfa I., Volanska G., Gardovskis J. *DIAGNOSTIC SIGNIFICANCE OF HBME-1, CD56 AND E-CAD EXPRESSION FOR VARIOUS THYROID PATHOLOGIES.* Riga Stradins University, Scientific conference 2011., p. 289.
7. Thesis and poster presentation - Artūrs Ozoliņš, Zenons Narbuts, Ilze Štrumfa, Guna Volanska, Kaspars Stepanovs, Jānis Gardovskis. *IMUNOCITOĶĪMISKO MARĶIERU NOZĪME VAIROGDZIEDZERU MEZGLU TIEVĀS ADATAS PUNKCIJAS BIOPSIJAS IZVĒRTĒŠANĀ.* Riga Stradins University, Scientific conference 2012., p. 275.
8. Thesis and oral presentation - Ozolins A., Narbuts Z., Strumfa I., Volanska G., Stepanovs K., Gardovskis J. *IMMUNOCYTOCHEMICAL PANEL AS AN ADJUNCT TO FINE-NEEDLE ASPIRATION OF THE THYROID IN VARIOUS THYROID PATHOLOGIES.* European Society of Endocrine Surgeons 5th Biennial congress. May 24-26, Gothenburg, Sweden 2012.
9. Thesis and oral presentation - Ozolins A., Narbuts Z., Strumfa I., Volanska G., Stepanovs K., Gardovskis J. *IMMUNOHISTOCHEMICAL EXPRESSION OF HBME-1, E-CADHERIN, AND CD56 IN THE DIFFERENTIAL DIAGNOSIS OF THYROID NODULES.* 7th Congress of Baltic Association of Surgeons. September 27-29, Riga, Latvia 2012.

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