“Black Ink” Sonographic Pattern As A Predictor for Invasive Papillary Thyroid Micro carcinoma: A Case Report

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ABSTRACT

Background: This study investigated the utility of the Sonographic pattern "Black Ink" with BRAF mutation testing of thyroid fine-needle aspiration cytology specimens for the risk papillary thyroid microcarcinoma (PTMC).
Case Presentation: We describe a case of a 41-year-old Caucasian woman affected by a ultrasonography “Black Ink” papillary thyroid microcarcinoma (PTMC) of the left lobe of the thyroid gland with very tiny size (Ø 0.4 cm). The characteristics, with the Diagnostic Imaging using Ultrasonography (US), Superb Micro-Vascular Imaging (SMI), fine-needle-aspiration cytology (FNAC) and mutation analysis are here discussed.There are more rare subtypes of thyroid cancer as papillary microcarcinoma "Black Ink" that even if small, are invasive and there is why the need to early diagnosis to avoid their aggressive behavior is needed. Nowadays, focusing on the size, the cut-off for non-occult tiny tumors has dropped to 0.3 cm.This value is of great relevance.
Conclusion: Ultrasonography, FNAC and BRAF molecular study have proven to be the most sensitive diagnostic combination for the early detection of thyroid cancer. Despite the size of this micro-lesion, the Black Ink ultrasonographic pattern associated with malignant cytology at FNAC represents an important biological risk factor and could still be a predictor of the PTMC and risk for neck lymph node metastases.

Keywords**:** Ultrasonography; Superb Micro-Vascular Imaging; Papillary Thyroid Micro carcinoma; Black Ink Sonographic Pattern; $BRAF^{V600E}$

1. INTRODUCTION

The data from the United States National Cancer Institute Surveillance, Epidemiology and End Results Program (SEER) shows that the incidence of thyroid cancer is on the rise [1]. Pathologically, this rise is mainly due to the increase of papillary carcinomas fraction and the greatest increase, based on pathologic staging, accounts for papillary microcarcinoma [2]. According to the latest statistics, nearly 90% of cases of thyroid cancers are papillary carcinomas [3]. Microcarcinoma (papillary carcinoma of any subtype with a diameter of less than 1 cm) bears from 30% to 70% of cervical lymph node metastasis [4 -5].Some of these papillary thyroid microcarcinoma (PTMC) are completely encapsulated, composed of papillary and/or follicular structures histologically characterized by overlapping nuclei with ground glass appearance and invaginations of cytoplasm into the nuclei [6].These tumors tend to behave indolently but some a fraction, even if of small size, clearly infiltrate the thyroid parenchyma and their malignant behavior has been proven by several authors [7-8-9] after initial surgery regional lymph nodes metastasis was observed in 5.4% to 13% of patients [10-11]. As a whole, thyroid cancers are associated to a very favorable prognosis with a 5-year survival rate of 98% [12-13].The purpose of this case report is to detail the Black Ink sonographic pattern and histologic characteristics of lesions that better relate to this neoplastic subgroup with worse biological behavior.

In the B-mode ultrasound imaging appears as a micro-focus of radial shape with markedly hypoechoic echostructure, irregular and infiltrative margins (very suspicious).

Clinical-pathological features of “Black Ink” lesion of the thyroid have also been discussed in a previous study [14-15] and found correspondent to an invasive papillary carcinoma classic variant. The causes of papillary carcinoma are unknown, but there are well known risk factors for the development of thyroid cancer (ionizing radiation, iodine deficiency, autoimmunity, familiarity) [16-17]. Ultrasonography represents the most sensitive imaging method for early diagnosis of thyroid lesion [18-19].

The SMI (Superb Micro-Vascular Imaging) can underline the vessel flow of the newly formed tortuous vessels around tumors [20].

The FNAC (Fine Needle Aspiration Cytology) allows the selection of cases that require surgery with a 92-95% diagnostic accuracy (very high positive predictive value) based mainly on the nuclear features of cancer cells [21]. Mutation status of the cytological specimens can be additionally tested in preoperative and postoperative samples by several procedures and its results are diagnostically and prognostic ally, relevant. Real-time polymerase chain reaction (RTPCR) is very often used, being a solid tool in the landscape of mutation tests which today spread from "Rule out to Rule In" cancer panels [22].

Associations of $BRAF^{V600E}$ mutation with the ACR TI-RADS and clinicopathological characteristics were analyzed [23].

We describe a thyroid ultrasonography “Black Ink” focus which corresponded to a purely invasive PTMC follicular variant of tiny size (0.4 cm) in the left thyroid lobe, identified by combined ultrasonography examination, SMI, FNAC; also mutation status was examined.

2. CASE PRESENTATION

A 41-year-old Caucasian woman with no personal history of thyroid disease presented herself to perform a thyroid ultrasound examination. Routine laboratory test results were normal. BMI result was 31 kg/m2. Bi-dimensional ultrasonography (DUS 2) performed using high frequency 10-14 MHz linear probes and real-time US system (Toshiba Aplio 500, Toshiba Medical System Corp., Otawarashi, Japan and Mindray M9, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) highlighted a highly suspicious micro-focus in the left lobe of the thyroid, radial shape with markedly hypoechoic echostructure, irregular margins, size 0.4 cm .

We labelled these micro-focuses found on US as "Black Ink" images and classified them as TI-RADS 5, a very high risk lesion according to ATA guidelines (Fig. 1) with the following descriptive images: Black Ink sonographic pattern (Fig. 1A), papillary thyroid microcarcinoma (PTMC) in the left lobe of tiny size (Ø 0.4 cm) (Fig. 1B), micro-focus black ink (Fig. 1C), color-scale ultrasonography (Fig. 1D), micro-focus of radial shape with markedly hypoechoic echostructure, irregular margins (Black Ink highly suspicious) (Fig. 1E) and finally B-Mode ultrasound imaging of black ink color map (Fig. 1F).

The SMI, was performed in combination with grayscale sonography for improved detection of thyroid malignancy (Fig. 2) and showed a clear flow through newly formed tortuous vessels specifically at the periphery of the micro-focus (Fig. 3).

FNAC procedure was proposed to the patient and, after that informed consent had been obtained, it was fulfilled under ultrasound guidance (Fig. 4) using a 23 gauge needle by execution of two passages through the lesion. In order to carry out both traditional cytology and Cell Block Procedure part of the material was spread on two slides (fixed by isopropanol spray) then the needle was washed by repeated pumping of 1.5 ml PreserveCyt Solution (70100-002, Hologic, Marlborough, USA). Following fixation cells were centrifuged at 3000 rcf for 20 minutes at room temperature with a swinging bucket rotor, the supernatant was vacuum aspirated and cell

pellet re-suspended with 50 µl of Thromborel S (10446442, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), 50 µl of citrate plasma from a healthy donor was added (previously pretested negative for mutational analysis). Clotting of the mixture was performed for 2 hours at 37°C, subsequently the cell block was paraffin embedded by standard procedure. Cytological examination of the conventional slides resulted inadequate for diagnosis mainly because of very few atypical cells recognizable on the slide (Papanicolaou stain) while Cell Block preparation (3 µm sections Ematoxilin/Eosin stain) allowed clear-cut recognition of malignancy resulting in cytologic diagnostic category TIR5 according to Italian Consensus for the classification and reporting of thyroid cytology corresponding to category VI according to The Bethesda System for Reporting Thyroid Cytopathology (Fig. 5) with a cytologic pattern pointedly very suspicious for papillary carcinoma. Somatic mutation analysis was also then performed on Cell Block Preparation tissue of the lesion collected by scalpel dissection of ten 6 µm tick sections. Paraffin was eliminated washing three times the sample with xylene (06-1304F, Bio-Optical, Milan, Italy). Xylene was eliminated by one wash with ethanol (A9314, Romil Ltd, Cambridge, UK); all washes were performed by continuous vortexing at low speed for 10 minutes followed by centrifugation at 15000 rcf for 5 minutes. After ethanol elimination tissue was air dried for 2 minutes, then DNA was extracted by QI Amp DNA mini kit (51304, Qiagen, Hilden, Germany) applying overnight protease K digestion at 45°C and following manufacturer's instructions. DNA was eluted by 100 µl AE buffer and quantified by Qubit 1X dsDNA HS Assay Kit (Q33230, Thermo Fisher Scientific, Waltham, USA);unless when specified all steps were performed at room temperature. Mutations were screened by Thyroid Cancer Mutation Detection Kit (THDNA-RT64, Entro Gen Inc., Woodland Hills, USA), cycling steps (1x95°C 10 minutes, 40x 95°C 10 seconds 60°C 60 seconds) and DNA amount were set according to manufacturer's instructions on a Line Gene 9660 real time machine (Hangzhou Bioer Co. Ltd, Hangzhou, P.R.China). Results were negative for any tested mutation of BRAF, KRAS, NRAS, HRAS genes. Patient became a candidate for surgery therefore lobectomy with isthmectomy (Fig. 6, 7) and additional Delphian lymph node (DLN) removal after surgical exploration (not a full central compartment prophylactic dissection) was the treatment of choice. This strategy was based on the following criteria: detection of unilateral cancer; maximum size <0.5 cm (small PTMC); non-highlighted of extra thyroid extension (ETE) by detailed sonographic evaluation; absence of clinical proof of lymph node metastasis; negative mutational status of the cytological sample.

Histopathological tissue examination of the thyroidectomy showed that lesion was made of epithelial cells forming only differentiated follicular structures devoid of content or containing hyper eosinophilic colloid which general contours were thorny and irregular, overtly infiltrating thyroid parenchyma. The neoplastic follicles were also found imprisoned in a dense sclerotic matrix or intermingled with normal non neoplastic thyroid follicles and small blood vessels. The neoplasm was solitary and showed no relationship to thyroid capsule (contained in thyroid) no evidence of vascular or lymphatic invasion and no metastasis to the lymph node received (final TNM 8th eds. staging pT1, pN0) (Fig. 8, 9) inasmuch Radioiodine-I^{131} therapy (RIT) was not administered.

There were also no other ultrasonography suspicious lesion in the remaining right lobe ad molecular analysis subsequently on the thyroidectomy specimen was confirmed negative. Patient underwent hormonal and tumor markers evaluation plus, ultra sonographic re-evaluation of the neck after 3 months and showed no sign of recurrence.

3. DISCUSSION

Papillary thyroid carcinoma (PTC) is usually a non-encapsulated tumor mass sharply circumscribed from the adjoining thyroid parenchyma and its margins may be bosselated or scalloped. It is not uncommon for this neoplasm to extend to the thyroid capsule which usually is not violated.

Papillary thyroid microcarcinomas (non-incidental PTMC inferior to 1 cm) can have aggressive features and disease recurrence somewhat, similar to conventional PTC, therefore it would be appropriate then to be managed like any other papillary thyroid malignancy [24] nonetheless many studies have shown that lymph node metastasis is associated with tumor size. Kasai and Sakamoto claim that the size of PTMC is a risk of lymph node metastasis and vascular invasion and have given great importance to the size and the location of the tumor [25]. Tuttle et al., believe that papillary thyroid microcarcinomas whose diameter is >0.5 cm have significantly higher rates of central lymph node metastasis, and are often multifocal (35%) [26]. Hunt and collaborators

suggested that the location of the tumor is closely related to LLN metastasis [27]. Zhang and colleagues found that tumors in the upper third of the thyroid were at greater risk of LLN metastasis, but at lower risk of CLN metastasis [28].The sonographic_"Black Ink" micro-focus identified in the present study was found in the middle third of the left thyroid lobe, presenting a radial shape, markedly hypoechoic echostructure, irregular margins, characteristics that even with a maximum size 0.4 cm still keep a high predictive score/value/index for malignancy ($p < 0.001$) [29].

Anyway it must be also said that hyperplasia and inflammation that can take place around cancer nests, resulting in indistinct borders may render ultrasound somewhat incapable of definitively distinguish the cancer nest from the surrounding tissue.

Folkman first reported a hypothesis that tumors would be unable to grow beyond a microscopic size of 1 to 2 mm^3 without continuous recruitment of new capillary blood vessels [30].The SMI allows detection of microvascularization at the periphery of the micro-focus with accuracy [31] and showed 91.2% specificity and 75.9% sensitivity, which were superior to Power Doppler Flow Imaging (PDFI), at 82.3% and 41.8%, respectively (P <.01) [32] therefore peripheral vascularization was independent risk factor for thyroid malignancy and can be useful for detecting thyroid carcinoma. We are also convinced that it is very important to discuss the value of the tumor size a predictor of lymph node involvement and to predict the stage and the aggressiveness and prognosis of microcarcinomas in order to choose the right treatment as this is still controversial.

Lim et al., (2009) [33] indicated it to be 0.7 cm, Zhang et al., (2012) [34] indicated it in 0.6 cm, and Chang et al., (2015) [35] suggested 0.5 cm. Wang argue the cut-off value of tumor size to predict the risk of CLNM in papillary thyroid microcarcinoma is 0.575 cm (area under the curve 0.721) according to the ROC curves [36].

Nowadays, on the wake of the thorough focal point on the size and the echo structural characteristics of "Black Ink" US findings, focusing on the percentage of biological risk factors due to very small tumors, the cut-off could drop to 0.3 cm [15]. This value is of great importance because the dimensions of PTMC in ultrasound images are fundamental data to predict the stage and the aggressiveness and prognosis of microcarcinomas and can help us choose the right treatment. Noguchi et al., they state in their study that the important goal is to determine the measure of appropriate surgery for the small thyroid carcinomas [37].

The diagnosis of tumors less than 0.5 cm in diameter requires very careful cytopathological evaluation. If an accurate diagnosis can be made before surgery, physicians will then be able to select and propose to the patient a reasonable surgical approach, nonetheless they will still debate on whether or not to perform prophylactic central lymph node dissection. We also suggest this shall be carefully tailored for each patient according to multiple risk factors and ultrasonography clinical data instead that on the sole malignancy on cytology report [38].

Based on nuclear crowding and presence of prominent nuclear grooves and pseudoinclusions FNAC was classified TIR5 according to the Italian consensus [39] corresponding to Category VI according to the Bethesda Classification [40]. Despite the tiny size of some lesions, the diagnostic image can be strongly suspicious for papillary carcinoma and the FNAC procedure can still accurately detect malignancy.

Mutation analysis can easily reliably be performed on fine needle aspiration biopsy specimens [41] for indeterminate cases and preoperative information of BRAF mutation status can be very valuable in leading the management of this cancer at various stages, helping determine the extent of initial surgical treatment, the need for radioiodine ablation, and the level of vigilance in the subsequent follow-up of the patient. Importantly this mutation in thyroid cancer is associated with a markedly increased rate of recurrence and even mortality of PTC [42].

Associations of $BRAF^{V600E}$ mutation with the ACR TI-RADS and ultrasound and clinic pathological characteristics were analyzed in solitary PTMC proved pathologically in a recent study by Shangguan et al., [43] which showed that the ultrasound features of the irregular margin in the solitary PTC and PTMC, $BRAF^{V600E}$mutation was associated with ACR TI-RADS point scores, which was positively correlated to risk of $BRAF^{V600E}$ mutation.

We can state that the BRAF mutation has emerged as a promising prognostic factor in the risk stratification of PTMC [44].

Lee et al., provide further evidence supporting the prognostic potential of BRAF mutation in PTMC [45].

Lin and colleagues suggested that pre-operative screening for BRAF mutation using fine needle aspirate biopsy could potentially guide the initial treatment of PTMC, with positive BRAF status requiring more aggressive therapy.

BRAF mutations are associated with high risk features, including extra-thyroid cancer extension (ETE) and multifocality, and are also predictive of an increased risk of lateral compartment nodal disease [46].

Yokozawa and Ahuia have respectively documented that 15,9% of cancers less than 1 centimeter show an extra thyroid invasiveness and that occult metastasis of thyroid cancer to the lymph nodes is up 20% of cases [47-48].

Nevertheless, some authors suggest that a subset of PTMCs with aggressive behavior requiring therapeutic management similar to larger tumors exist (PTCs) [49-50]. According to Hay two are the important parameters to keep in mind in the possible onset of a recurrence: multifocality (number of focus) and the type of surgical treatment (total thyroidectomy versus lobectomy) [51]. In our case

there was no significant family history, the patient was in good general health with no weight loss. A strategy using ultrasound to identify the appropriate candidate for lobectomy has been implemented [52]. Eligibility for lobectomy was based on the following data: unilateral single focus in one lobe of the gland; tumor size <0.5 cm; clinical silence for lymph node metastasis and a detailed ultrasound preoperative assessment of non-extra thyroidal extension (ETE). The patient underwent lobectomy, isthmectomy and removed a Delphian lymph node (DLN) size (Ø 0.2 cm). DLN metastasis it was not detected on histological examination. The collected data suggest that the main factor behind this therapeutic decision was the surgeon's recommendation. These findings indicates that surgeons do not capture the shared decision making process in the medical record, which is a potential area for quality improvement. Recently published guidelines from the American Thyroid Association (ATA) recommend less aggressive approaches like thyroid lobectomy and active surveillance, which creates a significant model shift in the treatment of these patients [53]. In addition, the patient was informed that to be compliant with the American Thyroid Association guidelines for differentiated thyroid cancer, the majority of patients undergoing thyroid lobectomy may require thyroid hormone supplementation to maintain a thyroid-stimulating hormone level <2 mlU / L [54]. Cut surface showed a whitish, solid, equatorial nodule of 0.4x0.3x0.3 cm in the left lobe with indistinct borders. On histological examination the lesion was classified as a follicular variant of PTMC, the neoplasm being made of well-formed papillae intermingled with normal thyroid parenchyma. Lymph node showed no involvement by tumor cells, and final pathological stage was pT1a, pN0 according to TNM 8th ed. [55-56-57].

Despite the tiny size of this micro-focus 0.4 cm, “Black Ink” patterned lesions are likely inclined to be histopathological diagnosed as papillary thyroid invasive cancers (entailing potential aggressive behavior) [58-59]. On the very morphological point of view by microscopic inspection the pathologist could receive the impression to be looking at a newborn tumor that would possibly grow in a diffuse fashion once it will become bigger, raising the hypothesis that our tumor could be a very early form of “Diffuse Follicular Variant of Papillary Thyroid Carcinoma”, an uncommon tumor (1-2% of PTC), typically found in young female patients, which usually does not tend to form a mass but grows more as a network of tumor-sclerotic strands throughout the gland parenchyma and possesses sensibly higher intrinsic metastatic potential and aggressive behavior [60].

Being by nature, an infiltrating type, PTMC is classified in the dimensional group of tiny papillary tumors (Yamamoto et al., 1990) [61]. It is very important that the dimensions should be cautiously interpreted in the current growing subgroup of PTMC to verify which diameter would be more representative of the risk of malignant tumors of the nodules [62] supported by the recent demonstration in two Italian studies that the BRAF mutation in PTMC was associated with aggressive clinic-pathological features such as extra thyroid extension, lymph node metastasis and advanced TNM stages in PTMC and that could represent a new dimension in the risk stratification of PTMC and the most appropriate measure of surgical and medical treatments [63-64].

4. CONCLUSION

We have described a case of a 41-year-old woman with a micro-focus Ø 0.4 cm, Black Ink sonographic characteristics, submitted to FNA for cytology and molecular study, treated with lobectomy with a subsequent histological report of invasive papillary thyroid microcarcinoma follicular variant. Ultrasonography, FNAC and BRAF molecular study have proven to be the most sensitive diagnostic combination for the early detection of thyroid cancer. Nowadays, focusing on biological risk factors for very small tumors, with the cut-off of ultrasonography recognizable tumors has even dropped to 0.3 cm. This value is of great importance because the dimensions of PTMC in ultrasound images are still fundamental data to predict the stage and the aggressiveness of tumors and they can also become an advantageous tool for the prognosis of micro carcinomas and play their role in helping physicians wisely choose a tailored treatment.

Deep collaboration between pathologist, sonographer and surgeon is crucial point in dealing with ultrasonography “Black Ink” patterned lesion especially when subsequent FNAC shows TIR5 cytology because the management of the postoperative specimen can be tricky and even well-trained pathologist could find it difficult to ascertain and preserve such tiny tumors for complete pathological examination.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHOR'S CONTRIBUTION

ET has prepared the manuscript, conceived imaging, developed the concept and wrote most of the manuscript. BA and AA have developed and contributed to the manuscript, cytology and histology study and mutation analysis. SL has prepared the cytological and histological sections. MA and ED helped with the clinic and surgery. CDB helped to the manuscript. GT developed the ideas, contributed and supervised the manuscript.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for the publication of this paper and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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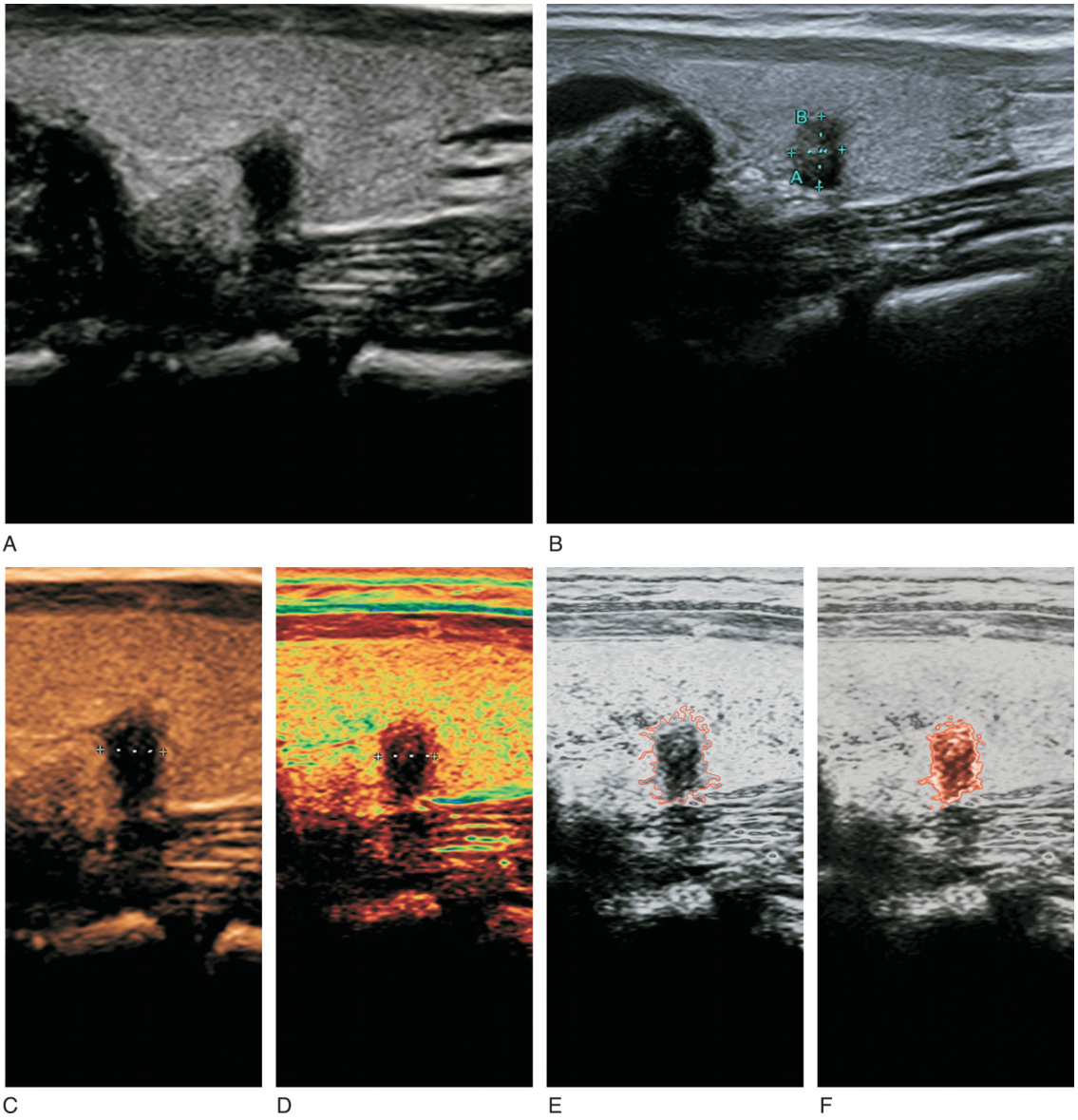

Fig. 1. Black Ink Sonographic Pattern in 41-year-old Caucasian woman patient

A. Black Ink Sonographic Pattern. **B**. Papillary Thyroid Microcarcinoma (PTMC) left lobe, tiny size (Ø 0.4 cm). **C.** Micro-focus Black Ink. **D.** Color-Scale Ultrasonography. **E.** Micro-focus of radial shape with markedly hypoechoic echostructure, irregular margins (Black Ink Highly Suspicious). **F.** B-Mode Ultrasound Imaging of Black Ink Color Map.

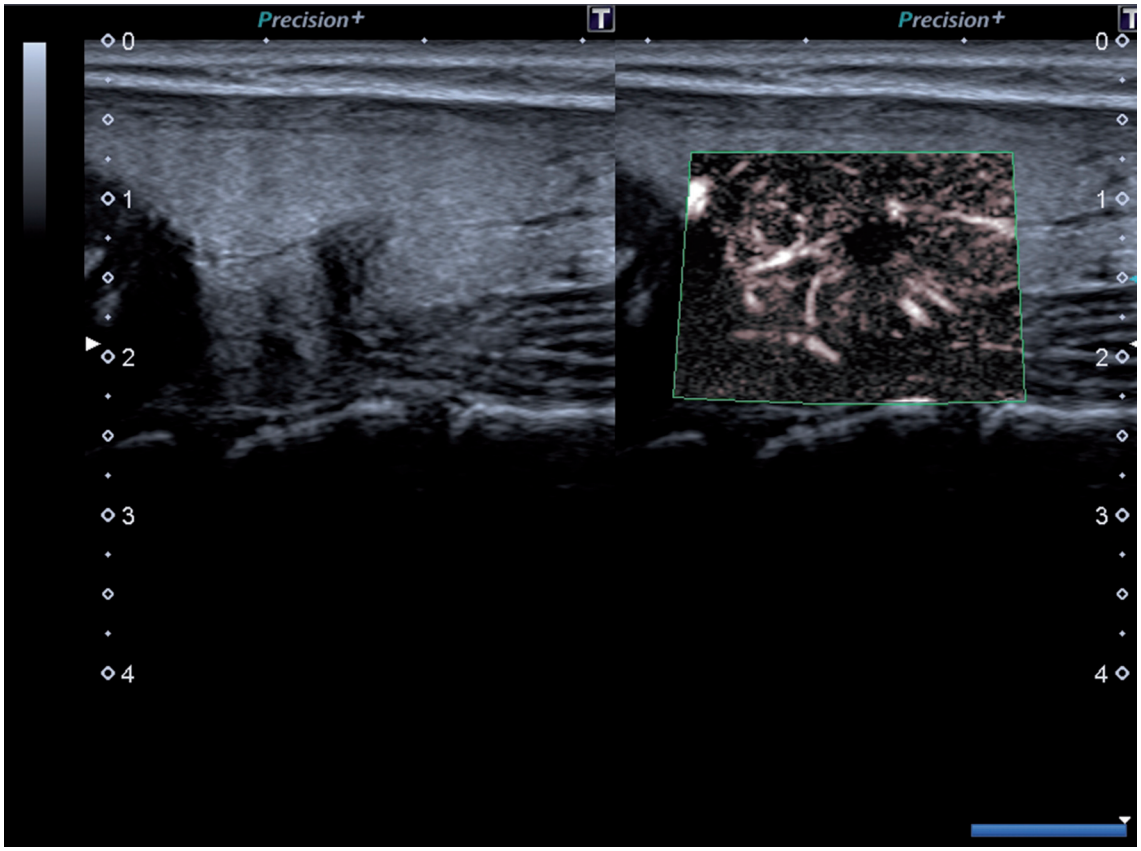

Fig. 2: SMI, Superb Micro-Vascular Imaging.

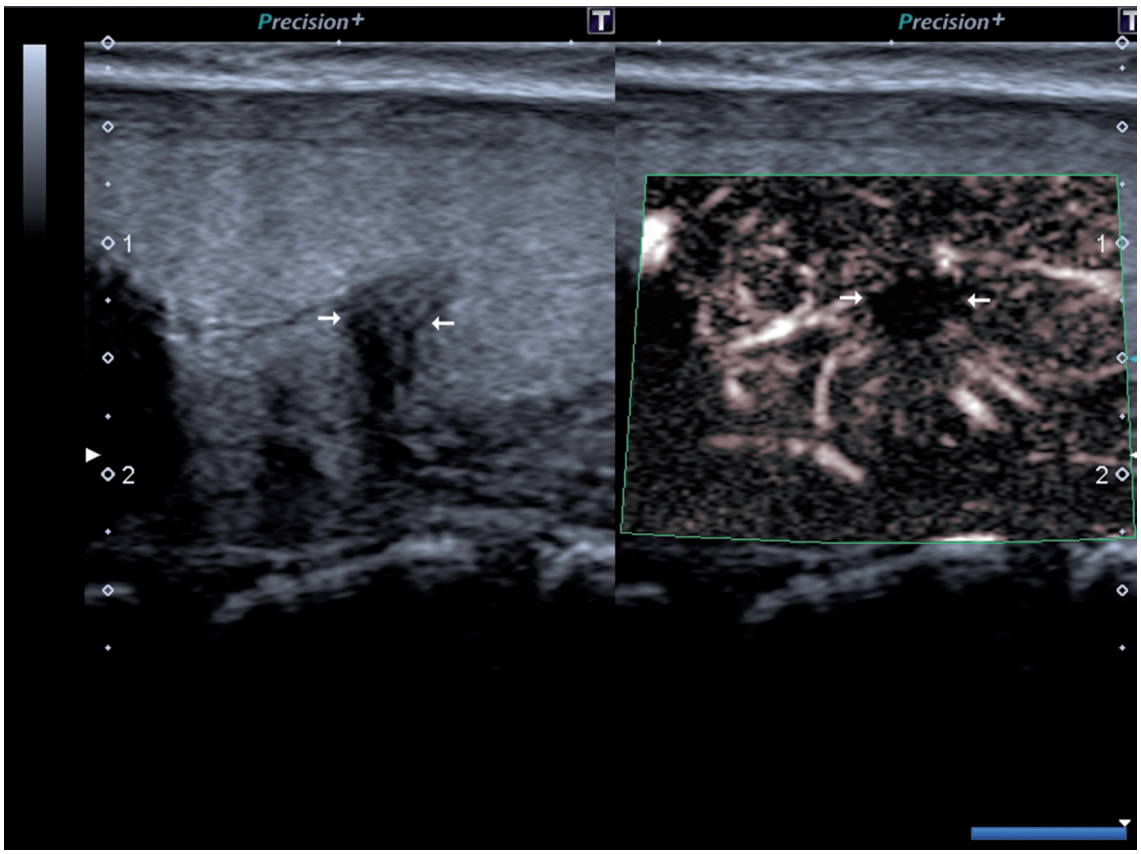

Fig. 3: SMI Test, shows a clear flow through newly formed tortuous vessels at the periphery of the specific micro-focus.

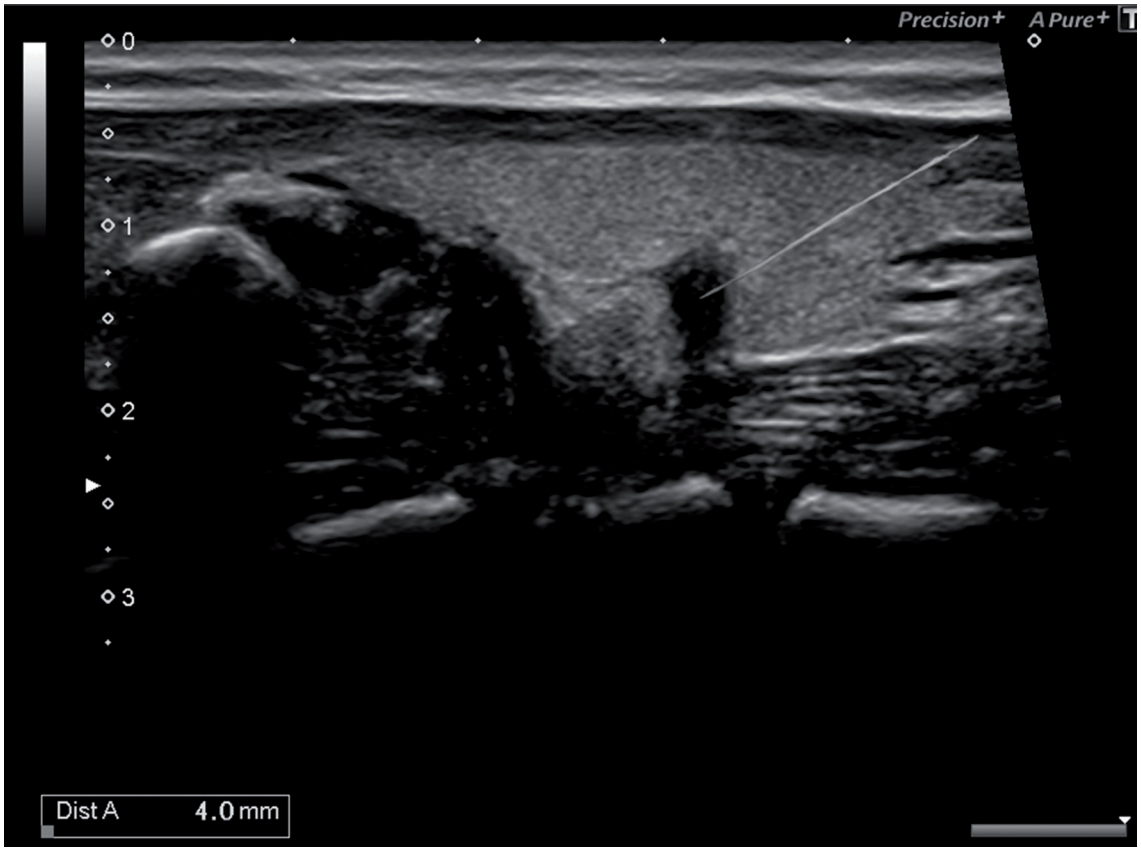

Fig. 4: FNAC (Fine Needle Aspiration Cytology) Showed under guide

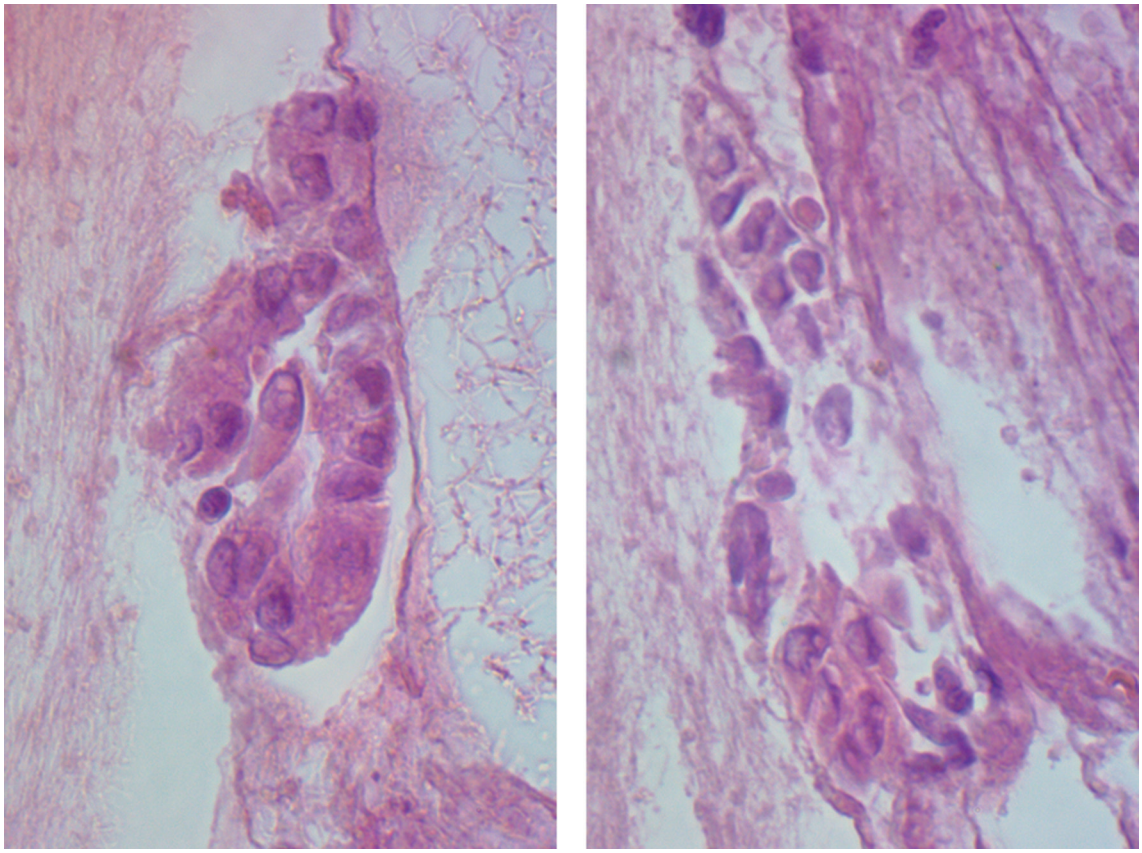

Fig. 5: Cytologic diagnosis (Cell Block Preparation, E/E stain, magnification 630x) showed follicular structures of epithelial cells with very atipical nuclei, prominent nucleoli and deep invaginations (pseudoinclusions) of the nuclear membrane, features of malignancy (TIR5) in thyroid FNA.

Fig. 6: Gross examination of lobectomy and isthmectomy with Delphian lymph node (DLN), size (Ø 0.2 cm), fresh tissue.

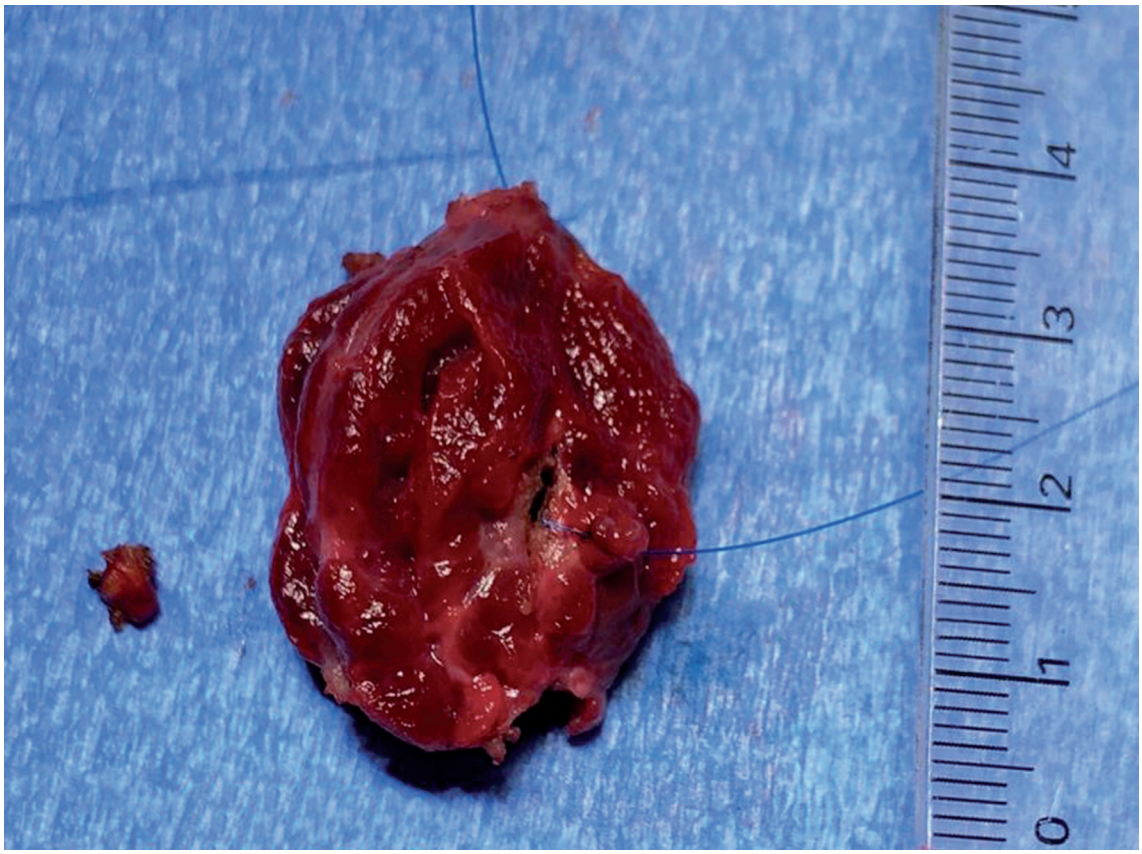

Fig. 7: Fresh tissue cut section of thyroidectomy, showing Black Ink microfocus (PTMC): Position Area Left Lobe.

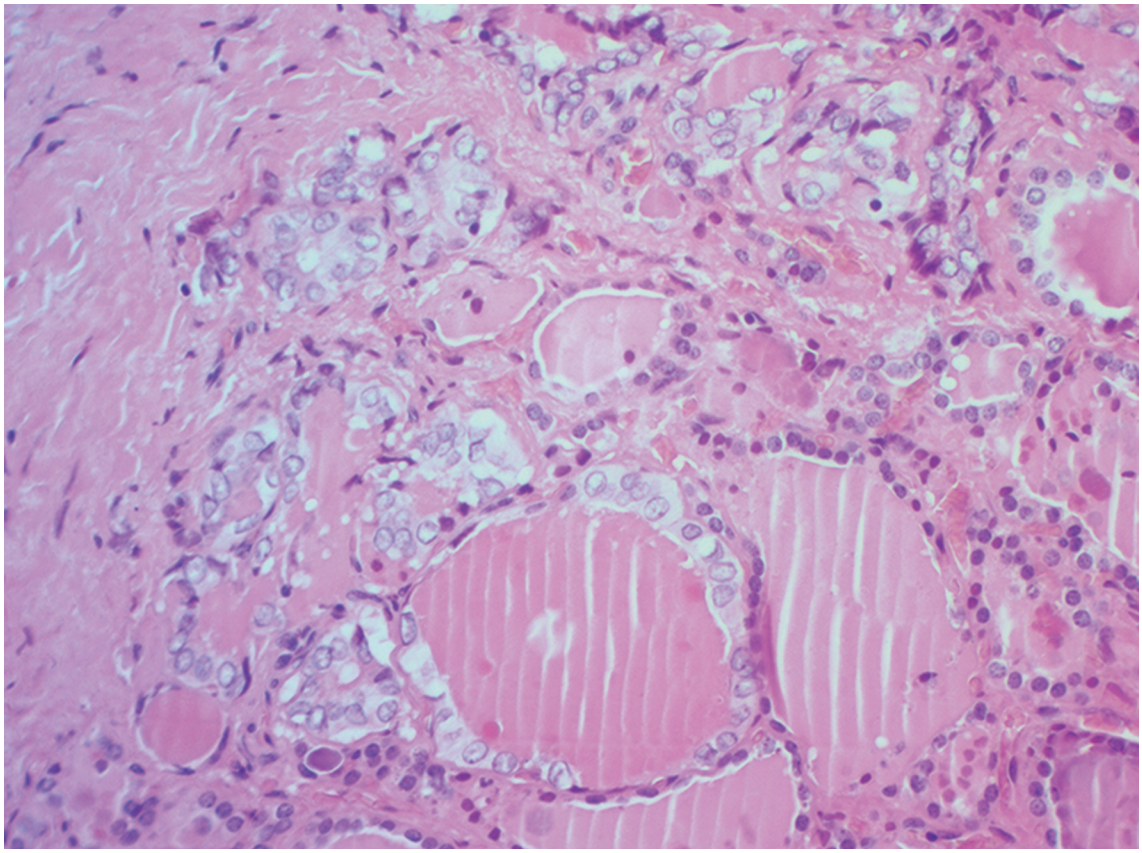

Fig. 8: High power histologic picture (E/E stain magnification 630x) of the micro focus shows irregular nuclear membranes, crowded vesicular nuclei with ground glass appearence and pseudoinclusions. The carcinoma follicles retain darker colloid than normal tissue with scalloping and clefting. Tumor glands are enriched in capillaries and mixed between sclerotic stroma (left) and non neoplastic follicles (right).

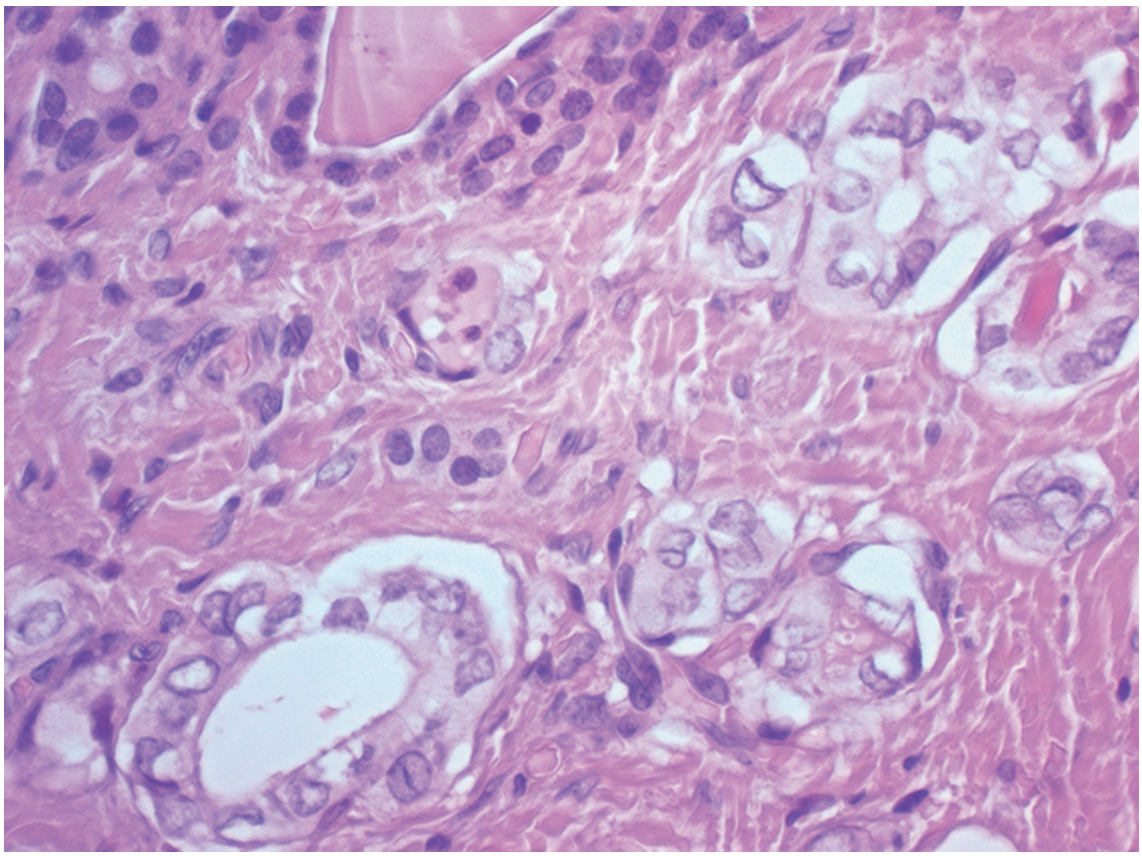

Fig. 9: High power histologic picture (E/E stain magnification 630x) of the micro focus shows atipical tumor follicles in dense eosinophilic stroma (bottom) directly facing non neoplastic glands (up).