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MESTRADO INTEGRADO EM MEDICINA

2022/2023

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The role of 5-hydroxymethylcytosine as a potential epigenetic biomarker in a large series of thyroid neoplasm

Março, 2023

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Mestrado Integrado em Medicina

Área: Ciências Médicas e da Saúde > Ciências da Saúde

Tipologia: Dissertação

Trabalho efetuado sob a Orientação de:

Professor Doutor Valdemar de Jesus Conde Máximo

Trabalho organizado de acordo com as normas da revista:
Endocrine Pathology

Março, 2023

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Ciências Médicas e da Saúde > Ciências da Saúde

TÍTULO DISSERTAÇÃO

The role of 5-hydroxymethylcytosine as a potential epigenetic biomarker in a large series of thyroid neoplasm

ORIENTADOR

Professor Doutor Valdemar de Jesus Conde Máximo

COORIENTADOR (se aplicável)

ASSINALE APENAS UMA DAS OPÇÕES:

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTE TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.	☐
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Faculdade de Medicina da Universidade do Porto, 23/03/2023

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Dedico este trabalho à minha irmã, pais e avós por todo o apoio e amor incondicional

Agradecimentos

Agradeço aos meus orientadores, Professor Doutor Valdemar Máximo e Professora Doutora Sule Canberk por me terem ajudado a encontrar soluções criativas para os desafios enfrentados durante esta investigação. Agradeço também à Professora Doutora Paula Soares pelo apoio durante todo o projeto. A sabedoria e generosidade deste grupo foram determinantes e sinto-me honrado por ter feito parte de uma equipa que trabalha na fronteira do conhecimento.

The role of 5-hydroxymethylcytosine as a potential epigenetic biomarker in a large series of thyroid neoplasm

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Abstract

Cytosine modifications at the 5-carbon position play an important role in the regulation of gene expression, and its deregulation is considered a hallmark of cancer. Recent studies demonstrate that 5-hydroxymethylcytosine (5-hmC) generated through 5-methylcytosine (5-mC) oxidation is significantly depleted in several human cancers. Although its role in tumour progression is still unknown, the 5-hmC loss has been proposed as a marker of tumour malignancy. Concerning thyroid tumours, the literature is scarce, and the studies are sparse in a number of cases and diversity of histotypes, not allowing clear conclusions. In this work, we evaluated the levels of 5-hmC, by immunohistochemistry, in a retrospective series of 318 thyroid tumours, including benign, low-risk, and malignant tumours, classified according to the 4th edition of WHO, and correlated its expression with demographic and clinicopathological features of the patients and tumours, aiming to verify whether 5-hmC levels can be used as a diagnostic, prognostic or therapeutic marker.

Our data show a significant association between loss of expression of 5-hmC and extrathyroidal extension, invasive/infiltrative capsule status, lymphovascular invasion, bilaterality, multifocality, tumour malignancy, and an unprecedented link with oncocytic morphology. Additionally, in a subgroup of 183 papillary thyroid carcinoma (PTC) cases, we also observed a statistically significant loss of 5-hmC in cases with *TERT promoter* mutations and distant metastasis. Our study evidences an important role for 5-hmC in thyroid tumourigenesis and indicates that 5-hmC levels have the potential to be used as a diagnostic and prognostic marker, however, more studies are needed to fully verify its potential.

Keywords: Thyroid tumours, Hürthle cell tumour, Oncocytic cell tumours, Epigenetics, 5-hmC.

Introduction

In the last decades, the role of epigenetic modifications on gene transcription and in oncogenic pathway activation has been enlightened in cancer research. “DNA methylation,” “histone modification,” and “RNA silencing” are considered as main mechanisms of epigenetic regulation of gene expression [1, 2]. Of those, “DNA methylation”, particularly at cytosine bases (C) have been fueled by the discovery of how these DNA changes might affect chromatin status and interfere with gene transcription. The cytosine residues modifications are orchestrated by enzymes that transfer the methyl group (‘writers’), enzymes that modify or revert the methyl group modification (‘erasers’), and enzymes that mediate the interactions of proteins or protein complexes with the modification (‘readers’). While the malfunction of these “writers” during cell replication results in passive DNA demethylation, the alterations catalyzed by the “erasers” represent an active mechanism of DNA demethylation. All these modifications cause a dynamic DNA methylome, leading to an altered gene expression pattern without affecting the core DNA sequence [3].

DNA methylation and demethylation at the fifth carbon position of cytosine (namely at CpG sites) is one of the frequent epigenetic changes that occur cyclically and require the enzymatic activity of multiple proteins. The addition of a methyl group at the 5-carbon position of cytosine (5-methylcytosine, 5-mC), is orchestrated by DNA methyltransferases (DNMTs) and is normally associated with closed chromatin and repressed gene expression [4]. During the DNA demethylation process, 5-mC can be hydroxylated to 5-hydroxymethylcytosine (5-hmC) by the Ten-Eleven Translocation (TET) family of enzymes [4]. Recently, 5-hmC was shown to regulate many cellular and developmental processes, including the pluripotency of embryonic stem cells, neuron development, and tumourigenesis in mammals [5].

Alterations of global 5-hmC levels begin to be described as an epigenetic marker of cancer and the loss of 5-hmC has already been reported in some cancer models such as melanoma, brain tumours, hematologic malignancies, bile duct, lung, and ovarian cancer, and hepatocellular carcinoma cancer acting as a promoter of neoplastic transformation and progression of neoplastic cells [6-12].

Regarding thyroid tumours, the role of 5-hmC remains to be clarified. There are few studies, and some data begin to emerge, based on relatively small and unrepresentative series, with regard to the tumour pattern diversity [13-16]. In a cohort, only including papillary thyroid carcinomas (PTCs) (n=88) and multinodular goiters (MNGs) (n=20) cases, Tong et al,

described the decreased level of 5-hmC in PTC tumour samples from patients with lymph node metastasis, when compared to PTC tumours samples from patients without lymph node metastasis [13]. Seok et al, showed a significant reduction of 5-hmC expression in anaplastic thyroid carcinoma (ATC) samples in comparison with PTC and follicular thyroid carcinoma (FTC) samples, in a cohort of 24 cases [14]. The same group later focused on follicular patterned thyroid tumours, in a series with 40 cases, and reported decreased 5-hmC expression in the follicular variant of PTC (FV-PTC) with >1% of papillary structure together with frequent regional lymph node involvement and *BRAF V600E* mutation, when comparing with other follicular patterned neoplasms without any papillary structures [15]. Lastly, Oishi et al, analyzed the global 5-hmC level in 85 thyroid carcinomas, through ELISA and IHC, and suggested that the loss of 5-hmC is an epigenetic hallmark of *TERT* promoter-mutated PTCs and ATCs [16].

To better understand the diagnostic and prognostic role of the 5-hmC in thyroid tumourigenesis, our group collected a multi-institutional comprehensive data set. A total of 318 cases representing a wide variety of benign, uncertain malignant potential, and malignant thyroid tumours were analyzed, by immunohistochemistry, for 5-hmC expression. The 5-hmC levels were compared, among the different tumour groups, taking into account their clinical, demographic and genomic characteristics, to be able to validate patterns of 5-hmC expression and its potential use in the clinical management of thyroid neoplasms.

Materials and methods

Patient Tissue Samples

A total of 318 patients from six tertiary centers were included in the study (Centro Hospitalar de Vila Nova de Gaia e Espinho (n=183), Hospital São João (n=51), Trakya University (n=27), IPATIMUP (n=25), Çukurova University (n=18), Acibadem University (n=14)) (Table 1). The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases. The histology of all tumour samples was reviewed independently by an endocrine pathologist (S.C.), and the thyroid tumour classification was performed according to the 4th edition of WHO Classification of Endocrine and Neuroendocrine Tumors [17]. From the 318 patients, 318 tumours were evaluated, and the following clinicopathological characteristics were collected: age, sex, size, focality, laterality, oncocytic morphology, biological behaviour, extrathyroidal extension, capsule status, and

lymphovascular invasion. This work was approved by the Ethics Committee for Health (CES) of the Hospital Center of São João/Faculty of Medicine of the University of Porto (CES 66–19). All the procedures described in this study were done in accordance with national ethical standards (Law n° 12/2005) and Helsinki declaration.

Immunohistochemistry (IHC)

Four-micrometer-thick sections from a representative block of each tumour were deparaffinized and rehydrated in a series of decreasing concentrations of ethanol. Deparaffinized sections were subject to heat-induced antigen retrieval in 1 mM pH 9.0 ethylenediaminetetraacetic acid buffer (LabVision Corporation, Fremont, CA, USA). Endogenous peroxidase activity was blocked with UltraVision Hydrogen PeroxideBlock and nonspecific bind was blocked using UltraVision Block reagent from UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA) for 10 min. The sections were then incubated in a humidified chamber with the following primary antibody, according to the manufacturer's specifications: a rabbit monoclonal antibody against 5-hydroxymethylcytosine (cat. no. 39791, 39792; Active motif, Carlsbad, US) at 1:10,000 dilution. All sections were counterstained with Mayer's hematoxylin. Positive controls from previously tested glioma samples were used in every run. To assess the specificity of the immunostaining, tumour sections omitting the primary antibody were used as negative controls.

Evaluation of immunohistochemical staining

The immunoreactivity was present at the nucleus of cancer cells and was semi-quantitatively evaluated for each tumour sample. Immunostaining evaluation (by S.C. and V.M.) was based on the intensity and distribution of the staining, without the knowledge of any clinical information of the cases. The 5-hmC staining intensity was scored as absent (0), weak (1), moderate (2), or strong (3) as shown in Table 2. Weak staining was not visible on a low-power view (×4 objective), while moderate and strong staining was readily visible on low-power magnification. The extent of nuclear staining was classified as negative, <25%, 26–50%, 51–75%, and 76–100% (Table 2). H staining score (H-score) was applied to evaluate the immune expression of 5-hmC. To calculate the H-score (range 0-300) the following formula was used: H-score= 0 x (% of cells staining 0) + 1 x (% of cells staining 1) + 2 x (% of cells staining 2) + 3 x (% of cells staining 3) [18]. For statistical purposes, the score was used either as a continuous variable or divided in a binary fashion: 0-150; 151-300. (Table 2, Figure 1).

DNA Extraction

DNA from FFPE tissues was retrieved from 10 µm sections after H&E guided careful microdissection. DNA extraction was performed using the GRS Genomic DNA Kit BroadRange (GRiSP Research Solutions, Porto, Portugal) following the manufacturer's instructions. Quantitative and qualitative analysis of all samples was then performed by spectrophotometry using Nanodrop N-1000 Spectrophotometer for microvolume UV-Vis measurements (Thermo Scientific, Waltham, MA, USA).-

PCR and Sanger Sequencing Analysis

Genetic characterization of the series of tumors was previously performed regarding *BRAF, RAS (NRAS, HRAS, and KRAS),* and *TERTp* for the most frequent regions mutated in PTC, namely, *BRAF* codon 600, *NRAS* codon 61, *HRAS* and *KRAS* codons 12, 13, and 61, and *TERT* p-124, and −146 regions [19]. Briefly, 25–50 ng of genomic DNA was amplified using the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Amplicons amplification was confirmed in 1–2% agarose gel electrophoresis (GRS Agarose LE, GRiSP, Oporto, Portugal), purified and sequenced by Sanger sequencing using the ABI Prism Big Dye Terminator kit v3.1 Cycle Sequencing (Fisher Scientific Applied Biosystems®, Portsmouth, NH, USA). After precipitation, fragments were analyzed by capillary electrophoresis using the Applied Biosystems 3130/3130 ×l Genetic Analyzers (Foster city, CA, USA). For all cases in which mutations were detected, the presence of the mutation was validated by performing a new and independent analysis [19].

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics version 26 (IBM, New York, NY, USA). Results were expressed in absolute frequency, percentage, mean ± standard deviation (Std), and median ± interquartile range (IR) ÷ 2. Univariate analysis and correlation were performed using a chi-squared test, Fisher´s exact test, and Pearson correlation test. Independent-sample t-test; Mann–Whitney test; One way ANOVA and Tukey HSD tests were applied whenever possible. Statistical significance was accepted with a two-tailed p-value < 0.05.

Results

Cohort characteristics

The demographics of the patients based on the distribution of each institute were shown in table 1: 183 (58%) from the Centro Hospitalar Vila Nova de Gaia e Espinho- CHVNG/E, 51 (16%) were from the Centro Hospitalar Universitário de São João (Porto) (HSJ), 27 (8%) were from Trakya University, 25 (7%) were from Ipatimup, 18 (5%) were from Cukurova University and 14 (4%) were from Acibadem University. There was a prevalence of 253 (79.6%) females and 65 (20,4%) males. The cohort had an age range between 16-99 years and the mean age was 51.65 (Table-1). After histological revision, the 318 thyroid tumours were classified as follows: 212 PTCs (114 Classical variant of PTC (C-PTC), 45 invasive/infiltrative follicular variant of PTC (iFV-PTC), 26 hobnail variant PTC (HV-PTC), 10 oncocytic variant of PTC (OV-PTC), 5 papillary microcarcinoma (miPTC), 4 tall cell variant PTC (TCV-PTC), 4 Warthin-like PTC (WL-PTC), 3 solid variant of PTC (SV-PTC), 1 diffuse sclerosing variant of PTC (DS-PTC)), 39 Hürthle cell carcinoma (HCC), 30 non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), 15 follicular adenoma (FA), 14 Hürthle cell adenomas (HA), 5 follicular tumours with uncertain malignant potential (FT-UMP) and 3 well-differentiated tumours with uncertain malignant potential (WT-UMP) (Figure 2). Characteristics of the patients and tumours are summarized in Table 3. In total, the series includes 251 (78.9%) malignant, 29 (9.1%) benign, and 38 (11.9%) low-risk neoplasms. Regarding focality, 193 (64,8%) of the tumours were unifocal and 105 (35,2%) were multifocal, 233 (81,1%) were unilateral and 54 (18,8%) were bilateral. The extrathyroidal extension was evidenced in 101 (31,8%) cases. Lymphovascular invasion was present in 82 (25,8%) samples. Distant metastases were demonstrated in 4 (2,8%) cases and psammomas were present in 60 (33,1%) of the observed cases, median nodule size was 19,50 ± 8,5 mm (Table 3).

Oncocytic morphology was exhibited in 105 (33.1%) tumours using the diagnostic criteria of the 4th edition of the WHO endocrine blue book [17]. The capsule status was invasive/infiltrative in 189 (73,5%) cases (Table 3).

In a subgroup composed of 183 PTC, mutational analysis was available, and the results were as follows: *BRAF* mutation in 111 (62,3%) cases; *TERT* mutation was present in 12 (7,2%) cases; *NRAS* mutation occurred in 8 (4,5%); *HRAS* mutation in 9 (9,3%) and *KRAS* mutation in 4 (4,3%) cases.

Association between Pathological characteristics of the tumours and Intensity, extension, and H-score of 5-hmC

In our series, the nuclear 5-hmC staining intensity was distributed as follows: 37 (11,6%) cases with intensity value 0 (negative staining), 101 (31,8%) cases with intensity value 1 (weak staining), 80 (25,2%) cases with intensity value 2 (moderate staining) and 100 (31,4%) cases with intensity value 3 (strong staining) (Table 3). Regarding the nine pathological characteristics, we observed that focality, capsule status, extrathyroidal extension, lymphovascular invasion, oncocytic morphology, and biological behaviour show a statistically significant relation with 5-hmC intensity score values (Table 4).

Concerning 5-hmC staining extension, 12 (3,8%) cases showed a score between 0-25%, 23 (7,2%) cases a score between 26-50%, 29 (9,1%) cases a score between 51-75% and 254 (79,9%) cases a score between 75-100% (Table 3). Concerning the pathological characteristics evaluated and the statistical tests used there´s a relation between 5-hmC staining extension and nodule size, lymphovascular invasion, and oncocytic morphology (Table 4).

5-hmC H-Score presented 132 (41,5%) samples belonging to the interval 0-150, and 186 (58,5%) to the 151-300 range. We found a significant association between 5-hmC and binary H-Score evaluation and focality, laterality, capsule status, extrathyroidal extension, lymphovascular invasion, oncocytic morphology, and biological behaviour (Table 5).

Lower 5-hmC H-Score (evaluated as a continuous variable) was found in cases that presented the following clinicopathological characteristics: multifocality, bilaterality, the invasive and infiltrative status of the capsule, minimal and major extrathyroidal extension, the presence of lymphovascular invasion, and oncocytic morphology (Table 6). In addition, the benign biological behaviour showed a significantly higher H-Score when compared with the low-risk and malignant behaviour (Figure 3).

Regarding tumour diagnosis, follicular adenoma showed a statistically significant higher 5-hmC H Score value when compared to a classical variant of PTC, invasive/infiltrative follicular variant of PTC, an oncocytic variant of PTC, Warthin-like PTC, hobnail variant PTC, Hürthle cell carcinoma, and NIFTP. While Hürthle cell adenoma presented similar results with a classical variant of PTC, hobnail variant PTC, and Hürthle cell carcinoma (Figure 4).

Association between tumour Pathological characteristics and 5 hmC H-Score and tumour molecular status.

A subgroup of 183 cases was also analyzed due to the availability of genetic and other clinicopathological characteristics. From these 183 cases, 84 (45,9%) had H-Score values between 0-150, and 99 (54,1%) cases amongst 151-300. Cases presenting *TERT* mutation, *HRAS* mutation, and *(H, N, or K) RAS* mutation showed significantly lower levels of the 5-hmC H-Score (Table 7).

Discussion

Nowadays, the disruption of epigenetic landscapes, including aberrant DNA methylation patterns, is accepted as one of the hallmarks of cancer. DNA methylation and demethylation at the C-5 position occur in a cyclical manner involving many enzymes and proteins, influencing the regulation of gene transcription and expression. DNA methylation can be simply described as the transfer of a methyl group, conducted by DNA methyltransferases (DNMTs), from S-adenosyl methionine (SAM) to the 5′ position of a pyrimidine ring of cytosines in a CpG site. DNA methylation can be actively and passively removed, the latter occurring due to dysfunctional DNA methylation activity, by DNMTs, during cell replication, leading to a decrease in methylation levels after this division. To realize active DNA demethylation an iterative stepwise oxidization of 5-mC to 5-hmC, 5-formylcytosine (5-fC), and 5-carboxylctyosine (5-caC) by ten TET enzymes, is required [20]. Now, both 5-mC and 5-hmC in DNA and RNA have been accepted as potential epigenetic markers due to their role in various metabolic pathways in the cellular processes. However, it was only after the remarkable study of Hu et al in 2015 [21], showing that 5-hmC-DNA interactions are less prone to further oxidation than 5-mC-DNA, that 5-hmC started to be considered an ideal alternative biomarker with much more chemical stability. Following this study, growing evidence has been displayed in the literature emphasizing the specific role of the 5-hmC with the diagnostic and prognostic properties in human cancers. A decreased nuclear level of 5-hmC in comparison with normal tissue has been reported in some human cancers such as melanoma, glioma, parathyroid carcinoma, hepatocellular carcinoma, cervical carcinoma, and hematologic malignancies [6-9, 11, 22, 23]. Aligned with the previous studies in other organs, our results, in the present series of 318 thyroid tumours, show that decreased or loss of expression of 5-hmC was significantly associated, in thyroid pathology, with adverse pathological characteristics such as ETE,

invasive/infiltrative capsule status, LVI, bilaterality, multifocality, and tumour malignancy. In addition, in a subgroup of 183 PTC cases, we have access to mutational status for *RAS* gene family members (*H-RAS, N-RAS, AND K-RAS*), *BRAF* and *TERT* promoter, and additional pathological characteristics and we observed a significantly lower expression of 5-hmC in cases with *TERTp* mutations, as well as in cases with distant metastasis. Finally, our results revealed for the first time a link between 5-hmC expression and Oncocytic morphology.

In our study, information about tumour size was available for all cases and grouped based on the cut-off value of 4 cm as suggested by AJCC/TNM Staging System 8th edition. We found a statistical association between the extension score of 5-hmC and the tumour size (*p*-value 0.003). LVI also presented a statistically significant dependence in the evaluation of 5-hmC intensity, extension, and H-score where its presence was associated with lower 5-hmC values. In accordance with these results, Tong et al [13], compared the expression of 5-mC and 5-hmC in 88 PTC, 20 MNG, and in the adjacent normal tissue, based on a 3-tiered H-score evaluation. They found that PTC presented significantly less 5-hmC than MNG. Curiously, for 5-mC, there was no difference noted. This result supports the sensitivity of 5-hmC as a distinct epigenetic marker establishing more stable DNA interactions over 5-mC, as was shown in the literature [20]. Tong et al [13] also compared the H-score of PTC with and without lymph node metastasis and, in line with our results, found that the group with lymph node metastasis presented a lower score. Curiously, they didn't find statistically significant differences concerning size (micro-PTC vs macro-PTC) [13].

Our results also showed a decreased level of 5-hmC expression in the group of tumours with invasive/infiltrative growth pattern being the statistical difference significant for intensity (*p*-value <0.001) and H-score (*p*-value <0.001). Curiously when we compare the invasive (n=87) versus infiltrative (n=102) growth pattern, 5-hmC expression was similarly distributed across the score evaluations of intensity, extension, and H-score. An interesting study by Seok et al [15] collected 40 cases of follicular-patterned thyroid neoplasm and grouped them as a group I (NIFTP, 5 cases), group II (encapsulated FVPTC with capsular invasion, 14 cases), group III (infiltrative FVPTC, 10 cases) and group IV (PTC with a predominantly follicular pattern and well-formed papillae (<1%), 11 cases). All cases were analyzed for 5-hmC immunohistochemistry using the H-score and 34 cases were evaluated for BRAF mutation analysis. 5-hmC was highly preserved in group I, II, and III whereas Group IV cases were noted with moderately reduced nuclear 5-hmC [15].

In our cohort, *TERT promoter* mutation was found in 12 cases out of 183 PTCs and 9 of them presented low H-score (*p*-value 0.003). Similarly to our results, in 2020, Oishi et al [16] showed that *TERT* promoter mutated PTCs and ATCs have significantly decreased nuclear 5-hmC levels than the normal thyroid tissues and PTCs with wild type *TERT* promoter and this result was confirmed by ELISA in the same study. Later, the same authors added 63 PTCs with wild-type *TERT* promoter, ten PTCs with *TERT* promoter mutations (among which seven had -124 and three had -146), and four ATC to perform 5-hmC expression by IHSC, and no difference was found in the two groups. The authors also compared the clinicopathologic variables and verify that larger tumour size and advanced AJCC/TNM stage were associated with lower 5-hmC positivity scores [16]. In the same subgroup of our cohort, *RAS* and *H-RAS* mutations were associated with the highest H-score, reaffirming the association of these mutations and diagnosis with better prognostic that has been demonstrated in the literature [19].

Our analysis also verified an association between both multifocality (p-value 0.007) and bilaterality (p-value 0.043) with decreased 5-hmC H-Score values. This association in thyroid tumours has not been described in the literature, something that can be explained by the recent start of research and few information available about 5-hmC in thyroid. However, these results follow the ones described before as the loss of this marker is one more time related with clinicopathological features that have been associated with poorer prognosis and the need for more aggressive treatment [24,25]

In our results, 5-hmC expression was significantly lower when we compare benign tumours with low-risk (*p-value* <0.01) and malignant thyroid tumours (*p-value* <0.01), while no differences were observed when comparing low-risk tumours versus malignant ones. Seok et al [14] analyzed the place of 5-hmC together with *IDH1* mutation focusing only on an ATC cohort composed of 9 cases that occurred de novo and 15 ATCs that were derived from either PTC (12 cases) or FTC (3 cases). H-score was found markedly reduced in ATC, and moderately reduced in PTC and FTC components. In contrast, in the nonneoplastic thyroid, the expression was highly preserved. The study emphasizes the role of 5-hmC in multistep carcinogenesis of thyroid tumour as it has shown in other human cancers. Also, the positioning of low-risk tumours closer to the malignant ones in our study highlights the current concern with overdiagnosis and overtreatment in thyroid tumours, with low-risk tumours being increasingly portrayed with a good prognosis, although collateral malignant lesions and metastatic disease have also been described [26-28]. The malignant potential of this class needs further study and 5-hmC could be a biomarker for more aggressive cases.

None of the previously mentioned studies addressed specifically the thyroid tumors with oncocytic morphology, as well as the accepted separate class of Hürthle cell tumours (HA, HCC).

In our cohort, 33% of the cases presented oncocytic morphology, including, well-differentiated tumours with oncocytic morphology (n=52) and real oncocytic tumours as known as "Hürthle cell tumours" (14 HA, 39 HCC), according to the 4th edition of the WHO Classification of Endocrine and Neuroendocrine Tumors. There was a strong statistical association between oncocytic morphology and 5-hmC intensity, extension, and H-score evaluation (*p-value*s <0.001, <0.001, and 0.037, respectively). This association remained as a "trend" in the Man-Whitney test evaluation with the *p*-value 0.06 presenting an association between this morphology and lower H-Score values, which leads us to hypothesize that perhaps the genetic variations/mutations in mtDNA and the metabolic alterations characteristic of these tumours may play a relevant role in this association [29-34].

Actually, cellular metabolism must play a very important role in regulating cellular epigenetics in general and DNA 5-hmC levels in particular. As it was explained above, the 1st step of DNA demethylation is orchestrated by TET enzymes and their functions go with Fe+2 and α-ketoglutarate (*α-KG*)- dependent dioxygenase activities [29]. In fact, it was been shown that mutations in 'metabolic enzymes', such as isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH), and fumarate hydratase (FH) condition the levels of some cellular metabolites, notably alpha-ketoglutarate (alpha-KG), succinate and fumarate levels, modifying TET enzymes activity and thus leading to changes in cellular epigenetics and likely contribute to tumourigenesis. [30-32]. While IDH mutations act through the reduction of alpha-KG availability, the accumulation of fumarate and succinate due to FH and SDH mutations leads to TET inhibitory competition [30-32]. The isocitrate dehydrogenase (IDH) mutations described by Shi et al [30] in gliomas and Murugan et al [31] in thyroid, as well as fumarate hydratase (FH) and succinate dehydrogenase (SDH) mutations described by Xiao et al [32] might negatively influence this TET enzyme and consequently the loss of 5-hmC.

Interestingly, in ongoing work in our group, we found that in cytoplasmic hybrid (cybrid) cell lines harboring mutations in mtDNA genes, there are significant changes in alpha-KG and succinate levels (data not shown) associated with the presence of mtDNA mutations. Since mtDNA mutations are the most characteristic genetic alteration of Hürthle cell tumours, we suggest that this could explain the reduction in 5-hmC levels in Hürthle cell tumours, through

the reduction in alpha-KG levels, but it remains to be understood what role 5-hmC might play in the etiopathogenesis of Hürthle cell/oncocytic thyroid tumours.

Conclusion

Our study reveals that DNA demethylation via 5-hmC has a prominent role in the oncogenesis and progression of thyroid tumours as it has been shown in different tumour types. The presented cohort strengthens the association of loss of 5-hmC and adverse pathological and clinical characteristics of the tumour as well as the diagnostic power segregating benign cases from low-risk and malignant ones. This might lead to 5-hmC as a potential preoperative marker for fine needle aspiration materials in the future. The specific epigenetic mechanisms behind 5-hmC need to be further investigated based on the phenotypic differences of thyroid tumours, suggesting the place of 5-hmc specifically for Hürthle cell tumours. Only through this, unlatching a novel therapeutic avenue for thyroid carcinoma by targeting 5-hmC would be possible.

Statements and Declarations

Conflict of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Funding: This work was supported by Portuguese funds through FCT—Fundação para a Ciência e a Tecnologia—in the framework of a Ph.D. grant to SC (SFRH/BD/147650/2019). This article is partly supported by the project “Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets”—NORTE-01-0145-FEDER-000051, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Figure Captions

Figure 1. Microscope images of stained tumor cells. A - FA (follicular adenoma) and H score is 300; B - HA (Hürthle cell adenoma) and H-score 300; C - WT-UMP (well-differentiated tumour with uncertain malignant potential) with oncocytic morphology and H score is 300; D - FT-UMP (follicular tumour with uncertain malignant potential) with oncocytic morphology and H score is 300; E - NIFTP (Non invasive follicular thyroid neoplasm with papillary like nuclear features) and H score is 170; F - HV-PTC (Hobnail variant PTC) and H score is 120; G - invasive FV-PTC (Follicular variant of PTC) and H score is 240 and H - HCC (Hürthle cell carcinoma) and H score is 2.

Figure 2. Distribution of the histological diagnosis of the case cohort; FA – Follicular adenoma; HA - Hürthle cell adenoma; NIFTP – Non invasive follicular thyroid neoplasm with papillary like nuclear features; FT-UMP - Follicular tumours with uncertain malignant potential; WT-UMP - Well-differentiated tumours with uncertain malignant potential; mi-PTC – Microcarcinoma; OV-PTC – Oncocytic variant of PTC; HV-PTC - Hobnail variant PTC; FV-PTC - Follicular variant of PTC; HCC - Hürthle cell carcinoma; C-PTC - Classical variant of PTC; TCV-PTC - Tall cell variant PTC; DS-PTC - Diffuse sclerosing variant of PTC; WL-PTC - Warthin-like PTC; SV-PTC - Solid variant of PTC.

Figure 3. Box plot of H-score evaluation of 5-hmC for biological behaviour.

Figure 4. Box plot of H-score evaluation of 5-hmC for tumour diagnosis. FA – Follicular adenoma; HA - cell adenoma; NIFTP – Non invasive follicular thyroid neoplasm with papillary like nuclear features; FT-UMP - Follicular tumours with uncertain malignant potential; WT-UMP - Well-differentiated tumours with uncertain malignant potential; mi-PTC – Microcarcinoma; C-PTC - Classical variant of PTC; FV-PTC - Follicular variant of PTC; DS-PTC - Diffuse sclerosing variant of PTC; TCV-PTC - Tall cell variant PTC; OV-PTC – Oncocytic variant of PTC; WL-PTC - Warthin-like PTC; SV-PTC; Solid variant of PTC;.HV-PTC - Hobnail variant PTC; HCC - Hürthle cell carcinoma;

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Tables and Figures

Table 1. Institutional Demographics

Institution	Number of Cases	Female	Male	Age, Mean (Range), yrs
A	183	153	30	51.9 (68)
B	51	43	8	55.6 (75)
C	27	20	7	47.0 (57)
D	25	19	6	60.4 (63)
E	18	12	6	39.2 (45)
F	14	6	8	44.3 (60)
Total	318	253	65	51.65 (83)

Table 2. Scoring System

Intensity (I)			**Extension (E)**			**H-Score**
Staining strength	**Score**		**% of stained tumour cells**	**Score**		**Binary evaluation**
Absent	0		**≤25**	0		≤150
Weak	1		**26-50**	1		
Moderate	2		**51-75**	2		151-300
Strong	3		**≥76**	3		

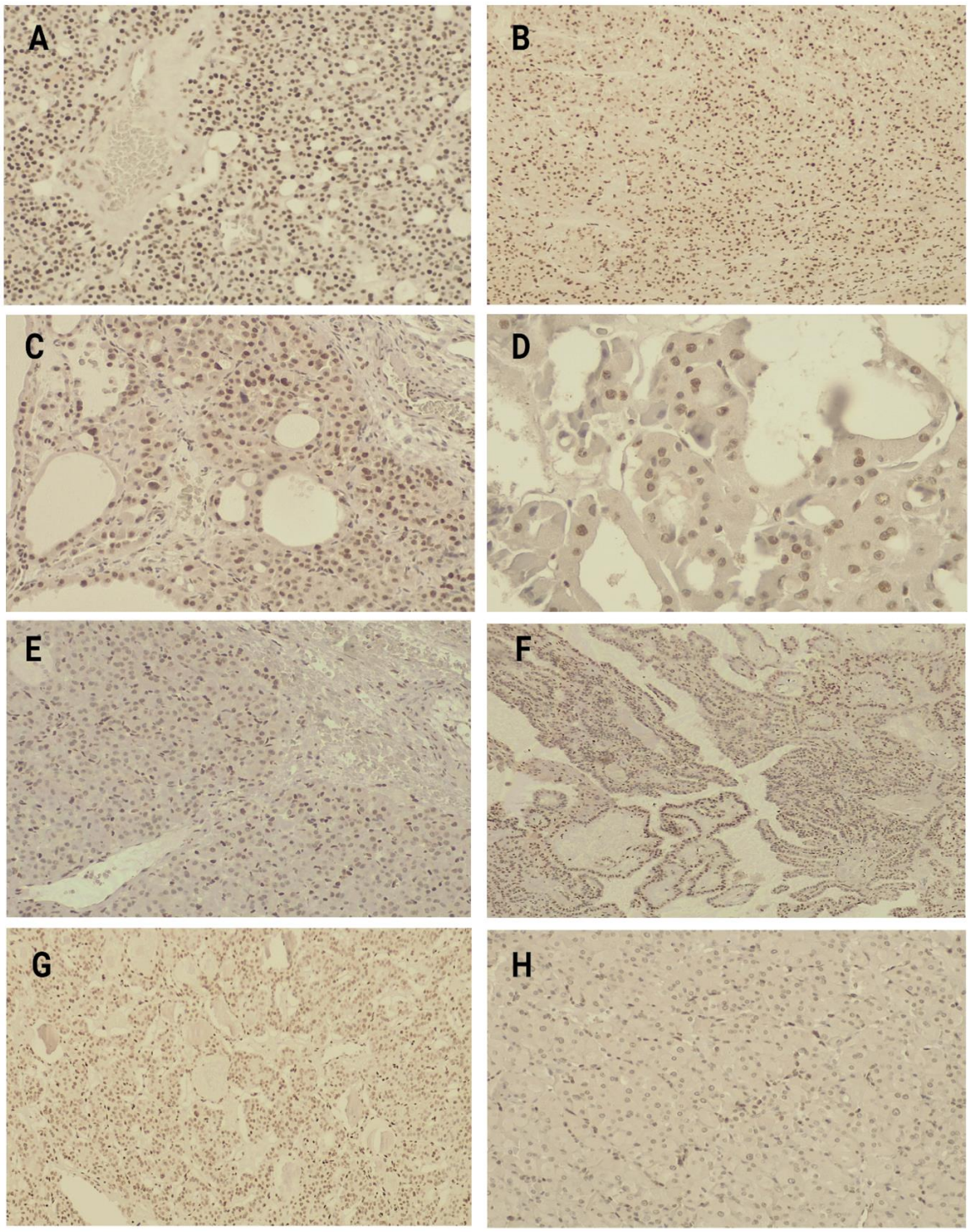

Figure 1.

Table 3. Cohort description: patient characteristics, clinicopathological features, genetical evaluation, and scoring systems

Characteristics	n	(%)	n (Total)	Characteristics	n	(%)	n (Total)
Gender				**Distant Metastasis**			145
Female	253	(79.6%)		*Yes*	4	(2.8%)	
Male	65	(20.4%)		*No*	141	(97.2%)	
Age				**TERT mutation**			167
Mean ± std (yrs)	51.65 ± 16.50			*Yes*	12	(7.2%)	
Size				*No*	155	(92.8%)	
Median ± IR ÷2 (mm)	19.50 ± 8.5			**BRAF mutation**			178
Focality			298	*Yes*	111	(62.3%)	
Unifocal	193	(64.8%)		*No*	67	(37.6%)	
Multifocal	105	(35.2%)		**NRAS mutation**			178
Laterality			287	*Yes*	8	(4.5%)	
Unilateral	233	(81.1%)		*No*	170	(95.5%)	
Bilateral	54	(18.8%)		**HRAS mutation**			97
Capsule Status			257	*Yes*	9	(9.3%)	
Invasive/Infiltrative	189	(73.5%)		*No*	88	(90.7%)	
No	68	(26.5%)		**KRAS mutation**			93
Extrathyroidal Extension			318	*Yes*	4	(4.3%)	
Minimal/Major	101	(31.8%)		*No*	89	(95.7%)	
No	217	(68.2%)		**5-hmC Intensity**			318
Lymphovascular Invasion			318	*0*	37	(11.6%)	
Yes	82	(25.8%)		*1*	101	(31.8%)	
No	236	(74.2%)		*2*	80	(25.2%)	
Oncocytic Morphology			315	*3*	100	(31.4%)	
Yes	105	(33.1%)		**5-hmC Extension**			318
No	212	(66.9%)		*≤25*	12	(3.8%)	
Biological Behaviour			318	*26-50*	23	(7.2%)	
Benign lesions	29	(9.1%)		*51-75*	29	(9.1%)	
Low-risk neoplasms	38	(11.9%)		*76-100*	254	(79.9%)	
Malignant neoplasms	251	(78.9%)		**5-hmC H-Score**			318
Psammoma Bodies			181	*≤150*	132	41.5%	
Yes	60	(33.1%)		*151-300*	186	58.5%	
No	121	(66.8%)					

n: number of cases

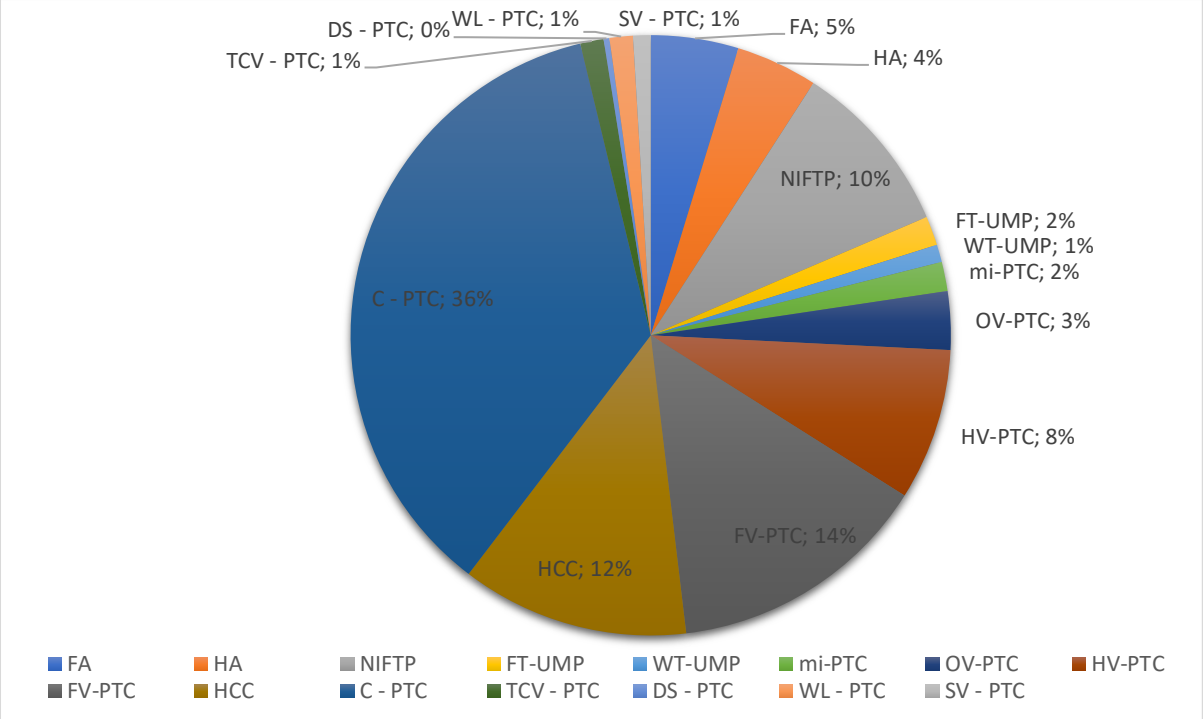
DS - PTC; 0%
WL - PTC; 1%
SV - PTC; 1%
FA; 5%
TCV - PTC; 1%
HA; 4%
NIFTP; 10%
FT-UMP; 2%
WT-UMP; 1%
mi-PTC; 2%
C - PTC; 36%
OV-PTC; 3%
HV-PTC; 8%
FV-PTC; 14%
HCC; 12%
FA
HA
NIFTP
FT-UMP
WT-UMP
mi-PTC
OV-PTC
HV-PTC
FV-PTC
HCC
C - PTC
TCV - PTC
DS - PTC
WL - PTC
SV - PTC

Figure 2.

Table 4. Analysis of immunohistochemical scoring of 5-hmC in clinicopathological characteristics

Pathological Characteristics	**Extension Score 0 (0 – 25%)**	**Extension (Score 1) (25 – 50 %)**	**Extension (Score 2) (50 – 75 %)**	**Extension (Score 3) (75 – 100 %)**	**p-value**
Size					
< 40	11	18	18	219	0.003*
≥ 40	1	5	10	26	
Lymphovascular Invasion					
Yes	6	10	12	54	0.003
No	6	13	17	200	
Oncocytic Morphology					
Yes	5	15	15	70	<0.001
No	7	7	14	184	
Pathological Characteristics	**Intensity Score 0**	**Intensity Score 1**	**Intensity Score 2**	**Intensity Score 3**	**p-value**
Focality					
Unifocal	19	54	54	66	0.053
Multifocal	13	44	21	27	
Capsule Status					
Invasive/Infiltrative	26	65	52	46	<0.001
No	4	11	7	46	
ETE					
Minimal/Major	18	43	32	8	<0.001
No	19	58	48	92	
Lymphovascular Invasion					
Yes	17	30	15	20	0.006
No	20	71	65	80	
Oncocytic Morphology					
Yes	22	29	17	37	<0.001
No	14	72	63	63	
Biological Behaviour					
Benign lesions	0	3	0	26	<0.001
Low-risk neoplasms	4	8	10	16	
Malignant neoplasms	33	90	70	58	

P-values were determined using a Chi-square test.Fisher´s Exact test was used when indicated (*).

Table 5. Analysis of H-score evaluation of 5-hmC in clinicopathological characteristics			
Pathological Characteristics	**H-Score 0-150**	**H-Score 151-300**	***p-value***
Focality			
Unifocal	68	125	**0.007**
Multifocal	54	51	
Laterality			
Unilateral	86	147	**0.043**
Bilateral	28	26	
Capsule Status			
Invasive/Infiltrative	86	103	**<0.001**
No	14	54	
ETE			
Minimal/Major	56	45	**0.001**
No	76	141	
Lymphovascular Invasion			
Yes	45	37	**0.004**
No	87	149	
Oncocytic Morphology			
Yes	52	53	**0.037**
No	79	133	
Biological Behaviour			
Benign lesions	4	25	**<0.001**
Low-risk neoplasms	10	28	
Malignant neoplasms	118	133	

P-values were determined using a Chi-square test.

Table 6. Comparison of means of H-score evaluation of 5-hmC for clinicopathological characteristics		
Pathological Characteristics	**Mean Rank**	**Mann-Whitney Test p-value**
Focality		
Unifocal	158,06	**0.02**
Multifocal	133,77	
Laterality		
Unilateral	148,45	**0.059**
Bilateral	124,81	
Capsule Status		
Invasive/Infiltrative	114,11	**<0.001**
No	170,38	
ETE		
Minimal/Major	119,25	**<0.001**
No	178,24	
Lymphovascular Invasion		
Yes	132,64	**0.002**
No	168,83	
Oncocytic Morphology		
Yes	145,38	**0.062**
No	165,75	
Pathological Characteristics	**Mean Difference** ($1^{st} - 2^{nd}$)	**Tukey HSD Test p-value**
Biological Behaviour		
Benign – Low-risk	84,256	**<0.001**
Benign – Malignant	105,617	**<0.001**
Low-risk – Malignant	21,361	**0.262**

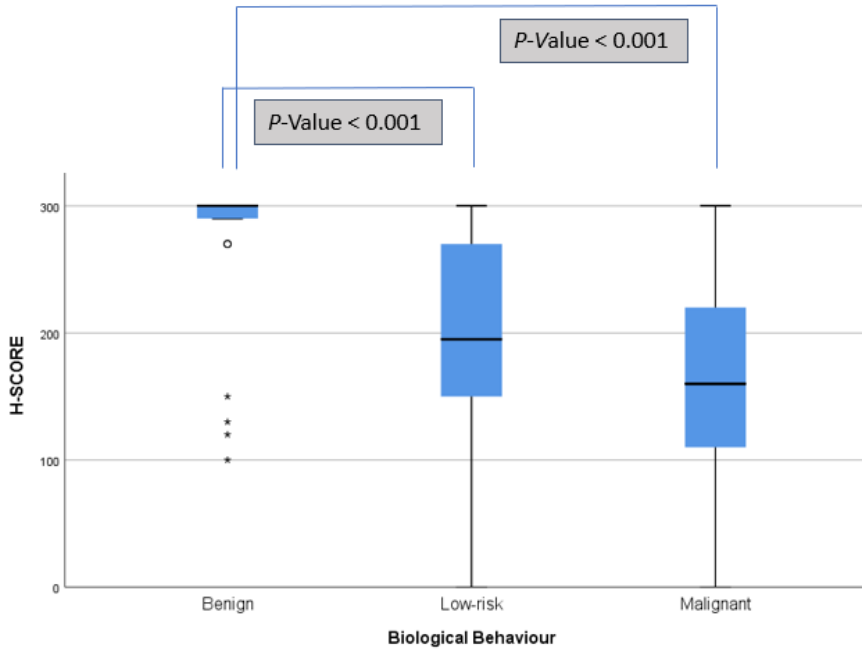
P-Value < 0.001
P-Value < 0.001
H-SCORE
300
200
100
0
Benign
Low-risk
Malignant
Biological Behaviour

Figure 3.

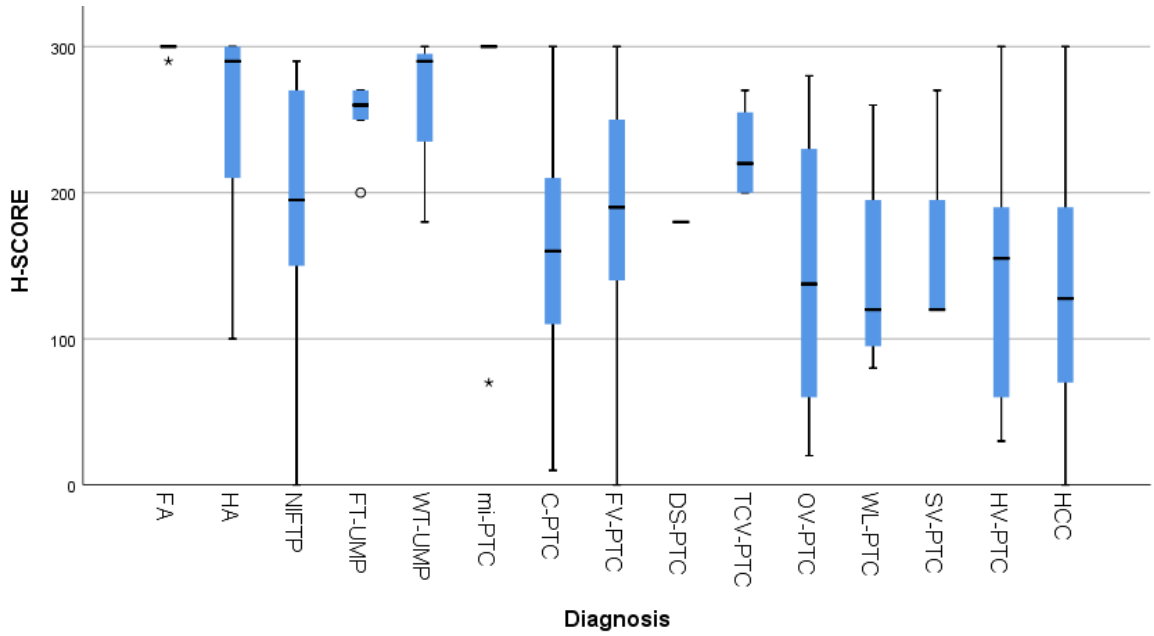
H-SCORE
300
200
100
0
FA
HA
NIFTP
FT-UMP
WT-UMP
mi-PTC
C-PTC
FV-PTC
DS-PTC
TCV-PTC
OV-PTC
WL-PTC
SV-PTC
HV-PTC
HCC
Diagnosis

Figure 4.

Table 7. **Comparison of H-score evaluation of 5-hmC with clinicopathological characteristics for a subgroup of 183 patients**			
Pathological Characteristics	H-Score 0-150	H-Score 151-300	*p-value*
Focality			
Unifocal	42	72	**0.002**
Multifocal	42	27	
Laterality			
Unilateral	57	19	**0.003**
Bilateral	88	8	
Psammoma bodies			
Present	34	26	**0.051**
Absent	50	71	
Distant metastasis			
Yes	4	0	**0.046***
No	64	77	
TERT mutation			
Yes	9	3	**0.030**
No	66	89	
HRAS mutation			
Yes	0	12	**0.001***
No	39	46	
RAS mutation			
Yes	7	18	**0.058**
No	75	80	

P-values were determined using a Chi-square test. Fisher´s Exact test was used when indicated (*).

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	8
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	8
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	9
Objectives	3	State specific objectives, including any prespecified hypotheses	10
Methods			
Study design	4	Present key elements of study design early in the paper	10
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	10
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	10
		(*b*) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	10/11
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10!11
Bias	9	Describe any efforts to address potential sources of bias	10/11
Study size	10	Explain how the study size was arrived at	10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	12
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12
		(*b*) Describe any methods used to examine subgroups and interactions	12
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how matching of cases and controls was addressed	
		(*e*) Describe any sensitivity analyses	

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	13
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	13
		(b) Indicate number of participants with missing data for each variable of interest	13
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	13
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	14
		(*b*) Report category boundaries when continuous variables were categorized	14
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	15
Discussion			
Key results	18	Summarise key results with reference to study objectives	15/16
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	18
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15/16
Generalisability	21	Discuss the generalisability (external validity) of the study results	19
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Reporting Guidelines – Examples

1. "In this work, we evaluated the levels of 5-hmC, by immunohistochemistry, in a retrospective series of 318 thyroid tumours, including benign, low-risk, and malignant tumours, classified according to the 4th edition of WHO, and correlated its expression with demographic and clinicopathological features of the patients and tumours, aiming to verify whether 5-hmC levels can be used as a diagnostic, prognostic or therapeutic marker.
Our data show a significant association between loss of expression of 5-hmC and extrathyroidal extension, invasive/infiltrative capsule status, lymphovascular invasion, bilaterality, multifocality, tumour malignancy, and an unprecedented link with oncocytic morphology. Additionally, in a subgroup of 183 papillary thyroid carcinoma (PTC) cases, we also observed a statistically significant loss of 5-hmC in cases with TERT promoter mutations and distant metastasis."

2. "Alterations of global 5-hmC levels begin to be described as an epigenetic marker of cancer and the loss of 5-hmC has already been reported in some cancer models such as melanoma, brain tumours, hematologic malignancies, bile duct, lung, and ovarian cancer, and hepatocellular carcinoma cancer acting as a promoter of neoplastic transformation and progression of neoplastic cells [6-12].
Regarding thyroid tumours, the role of 5-hmC remains to be clarified. There are few studies, and some data begin to emerge, based on relatively small and unrepresentative series, with regard to the tumour pattern diversity [13-16]."

3. "To better understand the diagnostic and prognostic role of the 5-hmC in thyroid tumourigenesis, our group collected a multi-institutional comprehensive data set."

4. "The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases."

5+6. "A total of 318 patients from six tertiary centers were included in the study (Centro Hospitalar de Vila Nova de Gaia e Espinho (n=183), Hospital São João (n=51), Trakya University (n=27), IPATIMUP (n=25), Çukurova University (n=18), Acibadem University (n=14)) (Table 1). The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases. The histology of all tumour samples was reviewed independently by an endocrine pathologist (S.C.), and the thyroid tumour classification was performed according to the 4th edition of WHO Classification of Endocrine and Neuroendocrine Tumors [17]."

7. "From the 318 patients, 318 tumours were evaluated, and the following clinicopathological characteristics were collected: age, sex, size, focality, laterality, oncocytic morphology, biological behaviour, extrathyroidal extension, capsule status, and lymphovascular invasion."

8. "The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases (…)
Evaluation of immunohistochemical staining
The immunoreactivity was present at the nucleus of cancer cells and was semi-quantitatively evaluated for each tumour sample. Immunostaining evaluation (by S.C. and V.M.) was based on the intensity and distribution of the staining, without the knowledge of any clinical information of the cases."

9. "The histology of all tumour samples was reviewed independently (…) This work was approved by the Ethics Committee for Health (CES) of the Hospital Center of São João/Faculty of Medicine of the University of Porto (CES 66–19). All the procedures described in this study were done in accordance with national ethical standards (Law n° 12/2005) and Helsinki declaration. (…) Immunostaining evaluation (by S.C. and V.M.) (…) without the knowledge of any clinical information of the cases."

10. "A total of 318 patients from six tertiary centers were included in the study (Centro Hospitalar de Vila Nova de Gaia e Espinho (n=183), Hospital São João (n=51), Trakya University (n=27), IPATIMUP (n=25), Çukurova University (n=18), Acibadem University (n=14)) (Table 1)."

11+12. "Statistical analysis was performed with IBM SPSS Statistics version 26 (IBM, New York, NY, USA). Results were expressed in absolute frequency, percentage, mean ± standard deviation (Std), and median ± interquartile range (IR) ÷ 2. Univariate analysis and correlation were performed using a chi-squared test, Fisher´s exact test, and Pearson correlation test. Independent-sample t-test; Mann–Whitney test; One way ANOVA and Tukey HSD tests were applied whenever possible. Statistical significance was accepted with a two-tailed p-value < 0.05."

13. "The demographics of the patients based on the distribution of each institute were shown in table 1 (…)"

14 + 15. "There was a prevalence of 253 (79.6%) females and 65 (20,4%) males. The cohort had an age range between 16-99 years and the mean age was 51.65 (Table-1). After histological revision, the 318 thyroid tumours were classified as follows: 212 PTCs (114 Classical variant of PTC (C-PTC), 45 invasive/infiltrative follicular variant of PTC (iFV-PTC), 26 hobnail variant PTC (HV-PTC), 10 oncocytic variant of PTC (OV-PTC), 5 papillary microcarcinoma (miPTC), 4 tall cell variant PTC (TCV-PTC), 4 Warthin-like PTC (WL-PTC), 3 solid variant of PTC (SV-PTC), 1 diffuse sclerosing variant of PTC (DS-PTC)), 39 Hürthle cell carcinoma (HCC), 30 non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), 15 follicular adenoma (FA), 14 Hürthle cell adenomas (HA), 5 follicular tumours with uncertain malignant potential (FT-UMP) and 3 well-differentiated tumours with uncertain malignant potential (WT-UMP) (Figure 2). Characteristics of the patients and tumours are summarized in Table 3. In total, the series includes 251 (78.9%) malignant, 29 (9.1%) benign, and 38 (11.9%) low-risk neoplasms. Regarding focality, 193 (64,8%) of the tumours were unifocal and 105 (35,2%) were multifocal, 233 (81,1%) were unilateral and 54 (18,8%) were bilateral. The extrathyroidal extension was evidenced in 101 (31,8%) cases. Lymphovascular invasion was present in 82 (25,8%) samples. Distant metastases were demonstrated in 4 (2,8%) cases and psammomas were present in 60 (33,1%) of the observed cases, median nodule size was 19,50 ± 8,5 mm (Table 3). Oncocytic morphology was exhibited in 105 (33.1%) tumours using the diagnostic criteria of the 4th edition of the WHO endocrine blue book [17]. The capsule status was invasive/infiltrative in 189 (73,5%) cases (Table 3). In a subgroup composed of 183 PTC, mutational analysis was available, and the results were as follows: BRAF mutation in 111 (62,3%) cases; TERT mutation was present in 12 (7,2%) cases; NRAS mutation occurred in 8 (4,5%); HRAS mutation in 9 (9,3%) and KRAS mutation in 4 (4,3%) cases."

16. "Regarding the nine pathological characteristics, we observed that focality, laterality, capsule status, extrathyroidal extension, lymphovascular invasion, oncocytic morphology, and biological behaviour show a statistically significant relation with 5-hmC intensity score values (Table 4). Concerning 5-hmC staining extension, 12 (3,8%) cases showed a score between 0-25%, 23 (7,2%) cases a score between 26-50%, 29 (9,1%) cases a score between 51-75% and 254 (79,9%) cases a score between 75-100% (Table 3). Concerning the pathological characteristics evaluated and the statistical tests used there´s a relation between 5-hmC staining

extension and nodule size, lymphovascular invasion, and oncocytic morphology (Table 4). 5-hmC H-Score presented 132 (41,5%) samples belonging to the interval 0-150, and 186 (58,5%) to the 151-300 range. We found a significant association between 5-hmC and binary H-Score evaluation and focality, laterality, capsule status, extrathyroidal extension, lymphovascular invasion, oncocytic morphology, and biological behaviour (Table 5). Lower 5-hmC H-Score (evaluated as a continuous variable) was found in cases that presented the following clinicopathological characteristics: multifocality, bilaterality, the invasive and infiltrative status of the capsule, minimal and major extrathyroidal extension, the presence of lymphovascular invasion, and oncocytic morphology (Table 6). In addition, the benign biological behaviour showed a significantly higher H-Score when compared with the low-risk and malignant behaviour (Figure 3). Regarding tumour diagnosis, follicular adenoma showed a statistically significant higher 5-hmC H Score value when compared to a classical variant of PTC, invasive/infiltrative follicular variant of PTC, an oncocytic variant of PTC, Warthin-like PTC, hobnail variant PTC, Hürthle cell carcinoma, and NIFTP. While Hürthle cell adenoma presented similar results with a classical variant of PTC, hobnail variant PTC, and Hürthle cell carcinoma (Figure 4)."

17. "A subgroup of 183 cases was also analyzed due to the availability of genetic and other clinicopathological characteristics. From these 183 cases, 84 (45,9%) had H-Score values between 0-150, and 99 (54,1%) cases amongst 151-300. Cases presenting TERT mutation, HRAS mutation, and (H, N, or K) RAS mutation showed significantly lower levels of the 5-hmC H-Score (Table 7)."

18. "our results, in the present series of 318 thyroid tumours, show that decreased or loss of expression of 5-hmC was significantly associated, in thyroid pathology, with adverse pathological characteristics such as ETE, invasive/infiltrative capsule status, LVI, bilaterality, multifocality, and tumour malignancy. In addition, in a subgroup of 183 PTC cases, we have access to mutational status for RAS gene family members (H-RAS, N-RAS, AND K-RAS), BRAF and TERT promoter, and additional pathological characteristics and we observed a significantly lower expression of 5-hmC in cases with TERTp mutations, as well as in cases with distant metastasis. Finally, our results revealed for the first time a link between 5-hmC expression and Oncocytic morphology."

19. "None of the previously mentioned studies addressed specifically the thyroid tumors with oncocytic morphology, as well as the accepted separate class of Hurthle cell tumours (HA, HCC). (…) in ongoing work in our group, we found (…) (data not shown)"

20. "Following this study, growing evidence has been displayed in the literature emphasizing the specific role of the 5-hmC with the diagnostic and prognostic properties in human cancers. A decreased nuclear level of 5-hmC in comparison with normal tissue has been reported in some human cancers such as melanoma, glioma, parathyroid carcinoma, hepatocellular carcinoma, cervical carcinoma, and hematologic malignancies [6-9, 11, 22, 23]. Aligned with the previous studies in other organs, our results, in the present series of 318 thyroid tumours, show that decreased or loss of expression of 5-hmC was significantly associated, in thyroid pathology, with adverse pathological characteristics such as ETE, invasive/infiltrative capsule status, LVI, bilaterality, multifocality, and tumour malignancy. In addition, in a subgroup of 183 PTC cases, we have access to mutational status for RAS gene family members (H-RAS, N-RAS, AND K-RAS), BRAF and TERT promoter, and additional pathological characteristics and we observed a significantly lower expression of 5-hmC in cases with TERTp mutations, as well as in cases with distant metastasis. Finally, our results revealed for the first time a link between 5-hmC expression and Oncocytic morphology "

21. “The presented cohort strengthens the association of loss of 5-hmC and adverse pathological and clinical characteristics of the tumour as well as the diagnostic power segregating benign cases from low-risk and malignant ones. This might lead to 5-hmC as a potential preoperative marker for fine needle aspiration materials in the future. The specific epigenetic mechanisms behind 5-hmC need to be further investigated based on the phenotypic differences of thyroid tumours, suggesting the place of 5-hmc specifically for Hurthle cell tumours. Only through this, unlatching a novel therapeutic avenue for thyroid carcinoma by targeting 5-hmC would be possible.”

22. “Funding: This work was supported by Portuguese funds through FCT—Fundação para a Ciência e a Tecnologia—in the framework of a Ph.D. grant to SC (SFRH/BD/147650/2019). This article is partly supported by the project “Cancer Research on Therapy Resistance: From Basic Mechanisms to Novel Targets”—NORTE-01-0145-FEDER-000051, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).”

Instructions for Authors

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Contents

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Manuscript Submission

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References

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- This result was later contradicted by Becker and Seligman (1996).
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  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

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- Article by DOI

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- Book chapter

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- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation

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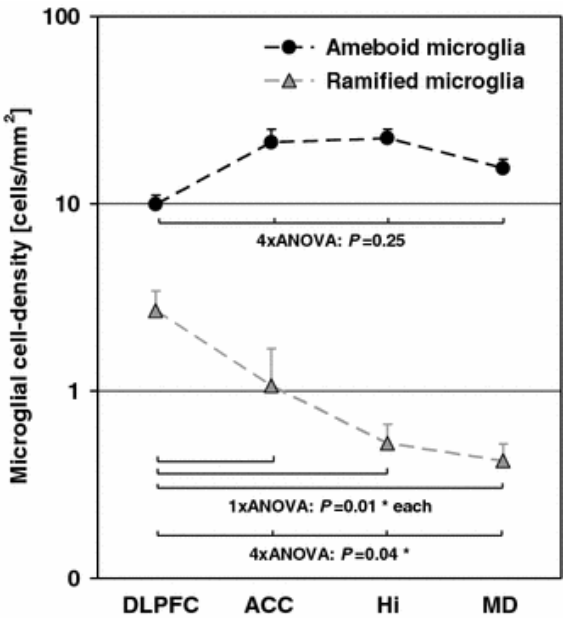

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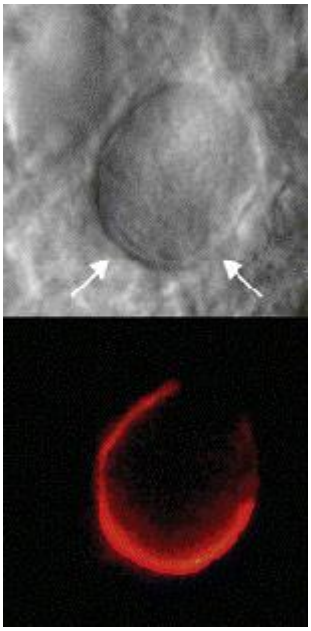

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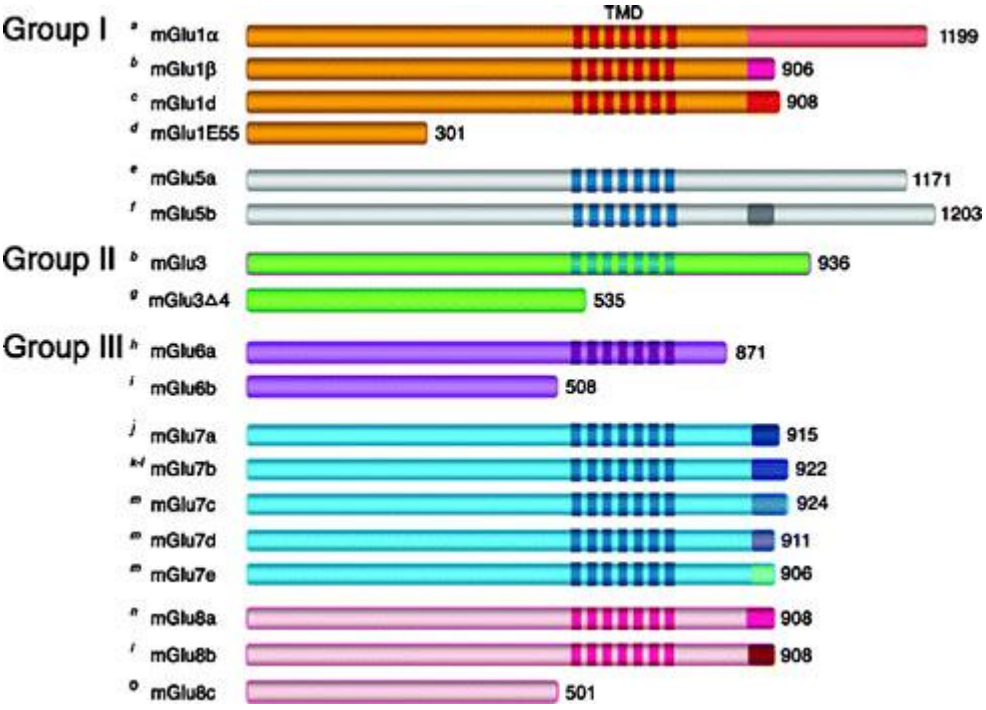

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Editors-in-Chief: Richter, K.; Van de Kuilen, J.-W.
ISSN: 0018-3768 (print version)
ISSN: 1436-736X (electronic version)
Journal no. 107