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Present and Future of Personalised Medicine for Endocrine Cancers

Edited by
Cristina L. Ronchi and Barbara Altieri

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Editors

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About the Editors

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Editorial

Special Issue: Present and Future of Personalised Medicine for Endocrine Cancers

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Major technological advances in genomics have made it possible to identify critical genetic alterations in cancer, rendering oncology well along the path to personalised cancer medicine [1]. Thanks to developments in high-throughput genetics, several mutations and gene rearrangements have been identified in patients with endocrine cancers (e.g., thyroid and adrenocortical carcinoma [2–4]). This is particularly important when talking about targeted anticancer drugs that, contrary to standard chemotherapy, aim at one or more definite molecular pathway on cancer cells—so their selection is underlying patient's genetic information. In fact, new affordable individual genomic analyses, as well as the opportunity to test new compounds in primary cells, may allow a personalised management of patients with endocrine malignancies [5–7]. This approach may improve the prediction of clinical outcome and therapeutic effectiveness, as well as help to avoid the use of ineffective drugs. However, further efforts are needed to obtain an adjustment of clinical management in patients with endocrine cancers that would rely solely or in great part on molecular pattern.

The aim of this Special Issue entitled "Present and Future of Personalised Medicine for Endocrine Cancers" was to offer an overview of exciting new research in the area of endocrine tumours that may set the stage for an innovative personalised management and future precision medicine modalities for individualised care. This issue encompasses nine publications on basic, translational and clinical research in different types of endocrine malignancies, including thyroid cancer [8–11], adrenocortical neoplasms [12–14], pheochromocytoma/paraganglioma [15] and pituitary tumours [16].

Looking across diseases, some themes are recurrent, such as the efforts to identify effective biomarkers useful to improve differential diagnosis and/or prognostication of endocrine cancers [11,12,14,15] or to predict response to treatment [10,15]. More specifically, in the field of thyroid cancers, Piciu et al. evaluated the correlation among different prognostic factors in papillary thyroid cancer, including the mutation of the BRAF V600E oncogene and the pathological standardized uptake values at the F18-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) [9]. In addition, Ieni and colleagues reviewed the role of molecular variability and intra-tumoral heterogeneity for the prognostic classification and clinical management of patients with differentiated thyroid cancer (DTC) [11]. Finally, another review by Feola et al. provided an excellent overview of the predictive clinical, biochemical and molecular factors for the response to treatment with the multikinase inhibitors sorafenib and lenvatinib in radioactive iodine refractory DTC. In this setting, most promising biomarkers are those involved in the angiogenic pathways [10].

Considering adrenocortical tumours, the role of several immunohistochemical markers for the distinction of carcinomas (ACC) from adenomas (ACA) and for prognostic stratification of malignant tumours have been investigated by Angelousi and colleagues,

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demonstrating that altered reticulin pattern and p53/Ki-67 expression are useful markers for the differential diagnosis of adrenocortical tumours [14]. Another study showed that cell-to-matrix-related molecules are specifically altered in ACC when compared to ACAs and identified osteopontin and HAS-1 (Hyaluronan Synthase 1) as novel potential diagnostic and prognostic biomarkers, respectively [12].

Regarding phaeochromocytoma and paraganglioma, which rare neuroendocrine tumours with uncertain malignant potential, Winzeler et al. extensively reviewed the relevant impact of molecular profiling for the understanding of the pathogenic mechanisms and for the prognostic classification, providing current and future opportunities for precision oncology [15].

From a different point of view, Koot and team underlined the importance of considering patients' needs, preferences and values in enabling us to improve doctor–patient communication and to develop decision support tools in DTC [8]. Basile et al. performed a multicentre retrospective analysis on ACC patients treated with adjuvant mitotane, showing that extending the duration of the treatment over two years is not beneficial for patients with low to moderate risk of recurrence [13]. Finally, Duhamel and colleagues reported two case reports and a review of the literature on the efficacy of immunotherapy in aggressive pituitary tumours [16].

In general, there is agreement that further studies aimed at evaluating diagnostic, prognostic and predictive markers for therapeutic response are needed for tailoring patient management and allowing more appropriate treatment choices. We hope that future investigations, integrating modern molecular methodologies across cell and tissue models, computational approaches and prospective clinical trials, will continue to drive progress towards improved personalised management of endocrine cancers.

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Review

Precision Medicine in Phaeochromocytoma and Paraganglioma

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Abstract: Precision medicine is a term used to describe medical care, which is specifically tailored to an individual patient or disease with the aim of ensuring the best clinical outcome whilst reducing the risk of adverse effects. Phaeochromocytoma and paraganglioma (PPGL) are rare neuroendocrine tumours with uncertain malignant potential. Over recent years, the molecular profiling of PPGLs has increased our understanding of the mechanisms that drive tumorigenesis. A high proportion of PPGLs are hereditary, with non-hereditary tumours commonly harbouring somatic mutations in known susceptibility genes. Through detailed interrogation of genotype-phenotype, correlations PPGLs can be classified into three different subgroups or clusters. Thus, PPGLs serve as an ideal paradigm for developing, testing and implementing precision medicine concepts in the clinic. In this review, we provide an overview of PPGLs and highlight how detailed molecular characterisation of these tumours provides current and future opportunities for precision oncology.

Keywords: personalized medicine; neuroendocrine tumours; phaeochromocytoma; paraganglioma; molecular clusters

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1. Introduction

Precision medicine is the provision of individualised healthcare to patients based on specific characteristics that may confer susceptibility to a particular disease and/or response to specific treatment. In this context, interventions can be tailored to maximise benefit whilst reducing exposure to less efficacious or intolerable therapies and unnecessary clinical investigations. The advent of high-throughput DNA sequencing and other molecular profiling technologies has accelerated the discovery of disease causing or 'driver' genetic variants as well as other molecular biomarkers associated with disease diagnosis, prognosis and drug-response. In oncology, increased understanding of the genomic, transcriptomic and epigenetic landscapes of multiple solid tumours has paved the way for precision medicine approaches to cancer care. Today, there are select examples whereby physicians have the opportunity to exercise a precision medicine approach to clinically manage and treat patients based on their genotype or the molecular phenotype of their tumour. For example, the use of trastuzumab in HER2-positive breast cancer, vemurafenib for *BRAF*^{V600}-mutant melanoma, and olaparib for *BRCA1/2* mutated tumours. Whilst the rationales of targeted therapy in cancer remains clear, the extent to which such an approach will benefit patients on a large-scale remain uncertain and several limitations remain before widespread uptake across multiple tumour types, especially rare tumours, is realised.

Phaeochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumours with an annual incidence of 3–8 cases per 1 million per year in the general population [1]. Pheochromocytomas arise from chromaffin tissue from the adrenal medulla, whereas paragangliomas arise from neural crest derivatives of extra-adrenal sympathetic or parasympa-

thetic paraganglia [2]. The management of PPGL patients is challenging for clinicians, as these tumours exhibit marked clinical heterogeneity that includes indolent, slow growing to aggressive and metastatic tumours. Clinical signs and symptoms vary considerably depending on the localisation and size of the tumours and their hormonal activity. PPGL may be non-functional or produce catecholamines such as adrenaline, noradrenaline and dopamine. Catecholamine excess may lead to paroxysmal headache, tachycardia, sweating and hypertension [3,4], and, rarely, to lethal pheochromocytoma crisis [5].

All PPGL have metastatic potential [6] and morbidity and mortality are high in metastatic disease, which is the case in approximately 10% of pheochromocytomas and 45–40% of paragangliomas [7–9]. In addition to the clinical complexity, PPGL demonstrate considerable genetic heterogeneity and are considered the most heritable human neoplasms. Historically, patients with PPGL have been stratified according to the anatomic location, developmental origin, primary tumour size, multiplicity, metastatic behaviour or age of first diagnosis. More recently, new approaches including biochemistry, immunohistochemistry and imaging phenotypes have been proposed and used to stratify PPGL patients [10,11]. Moreover, in the area of next generation sequencing (NGS), the possibility of accurate and timely molecular characterisation through germline and somatic testing have accelerated the molecular understanding of PPGL tumourigenesis thereby providing the opportunity for a precision medicine approach to the clinical management of these tumours [12,13].

2. Genomic Landscape of PPGL

Knowledge of the genetic status of PPGL patients is key for adequate diagnosis, treatment and surveillance of affected patients and their families, and has shown to positively impact on management and patient outcome [14]. Consequently, current guidelines recommend germline genetic testing in every patient with PPGL, with NGS as the gold standard for routine diagnosis [15–17]. One third of patients are affected by a germline mutation in one of the currently known susceptibility genes [11]. Germline mutations in the *RET, VHL* and *NF1* genes predispose to phaeochromocytoma in the context of inherited tumour syndromes, including neurofibromatosis, multiple endocrine neoplasia type 2 (MEN 2) and Von Hippel Lindau syndrome (VHL) [18,19]. Non-syndromic, hereditary PPGLs are mostly due to germline mutations in the succinate dehydrogenase genes (*SDHx* group) while other familial PPGL are caused by *TMEM127* or *MAX* mutations. Newer syndromic PPGL genes include *FH* (Hereditary Leiomyomatosis and Renal Cell Cancer syndrome), *EPAS1* (mainly as a case of germline mosaicism, although *EPAS1* mutations are generally more common as somatic mutations) and *EGLN1* (associated with congenital polycythaemia). In single cases, additional PPGL susceptibility genes have been described and are implicated in mitochondrial metabolism (*MDH2, GOT2, SLC25A11, DLST*), mitogen-activated protein kinase (MAPK) signaling pathways (*MET, MERTK*) or DNA methylation (*H3F3A, DNMT3A, KIF1Bbeta*) [10,20,21].

In addition to germline genetic testing, identification of somatic mutations by sequencing tumour DNA is increasingly undertaken in both research and clinical settings. Testing can be applied to paraffin embedded tissues as well as fresh frozen samples [22] and helps to further improve knowledge of tumour biology by rapidly identifying driver mutations that may be amenable to personalised treatment strategies or inform surveillance strategies.

Up to 40% of cases without an established germline mutation may harbor a somatic mutation in one of the PPGL susceptibility genes such as *VHL, RET, EPAS1, SDHB, NF1* or in genes implicated in oncogenesis (*HRAS, TP53, CDKN2A, FGFR1*) [22–24]. The number of genetically determined tumours with a specific driver mutation rises, therefore, to approximately 70% [25]. Based on their underlying driver mutation, PPGL can be divided in three different molecular clusters [13] that reflect different mechanisms of tumourigenesis and are associated with distinct biochemical, radiological and clinical phenotypes, see Table 1 and Figure 1.

Table 1. Genes and hallmarks of tumourigenesis according to molecular clusters.

	Cluster 1	Cluster 2	Cluster 3
Genes	Cluster 1A: *SDHx*, *FH*, *MDH2*, *IDH1/2*, *SLC25A11*, *DLST*, *GOT2*, *DNMT3A*, *EGLN1*	*RET*, *NF1*, *TMEM127*, *MAX*, *HRAS*, *KRAS*, *FGFR1*, *NGRF*, *KIF1B*, *BRAF*, *MET*, *MERTK*	*MAML3*, *CSDE1*
	Cluster 1B: *VHL*, *EPAS1*, *EGLN1/2 IRP1*		
Hallmarks of tumourigenesis	Pseudohypoxia	Increased Cell proliferation	Activated Wnt/ β-catenine pathway
	Angiogenesis		
	DNA and Histone methylation	Increased cell survival	
	Metabolic reprogramming		

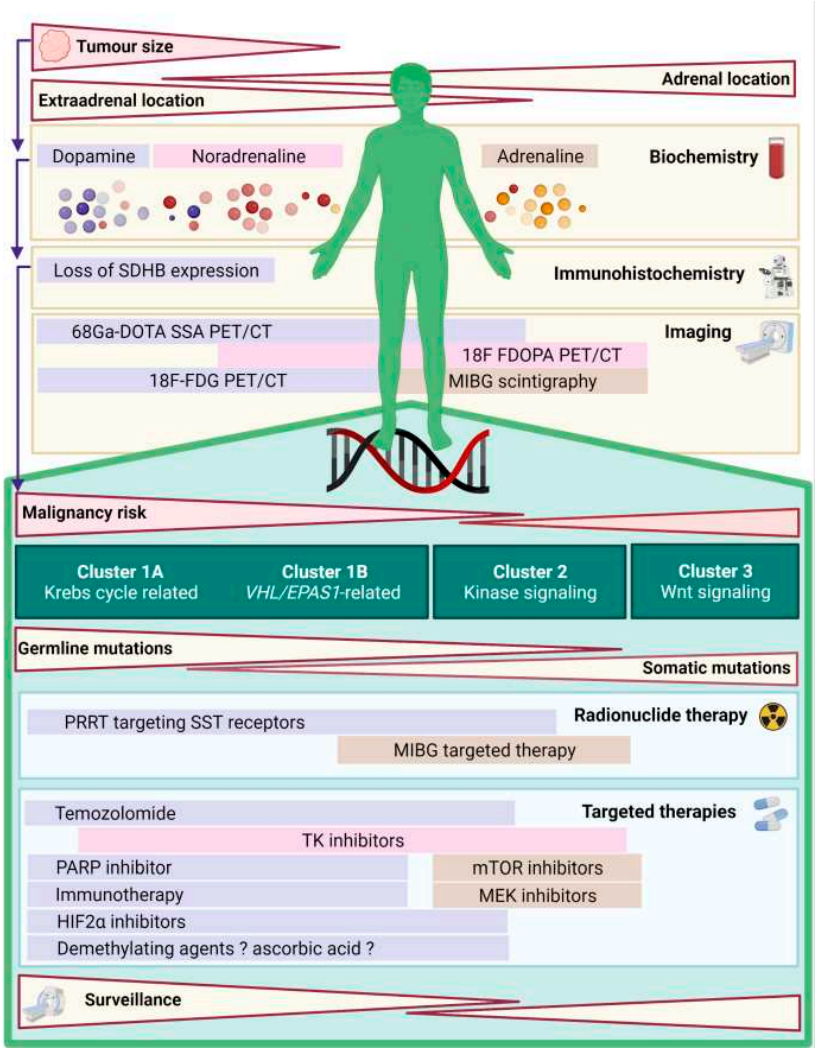

Figure 1. Provides an overview of how molecular classification can guide a personalized approach to PPGL patients. PARP: poly-(ADP)-ribose polymerase, mTOR: mechanistic Target of Rapamycin, MEK: mitogen-activated protein kinase kinase (MAP2K), TK: tyrosine kinase, MIBG: metaiodobenzylguanidine, PRRT: peptide receptor radionuclide therapy, SST: somatostatin.

3. Mechanisms of Tumourigenesis in PPGL and Molecular Clusters

The first cluster is characterised by a pseudohypoxic signature and accelerated angiogenesis promoted by abnormal stabilization of hypoxia-inducible factor (HIF) alpha transcription [26]. Pseudohypoxic PPGL can further be subdivided in Krebs cycle-related (Cluster 1A) and *VHL/EPAS1* related (Cluster 2B) tumorigenesis. Cluster 1A Krebs cycle-related tumours are secondary to mutations in *SDHx*, *FH*, *MDH2*, *GOT2*, *SLC25A11*, *DLST*

or *IDH1/2*. The *SDHx* genes (*SDHA*, *SDHB*, *SDHC*, *SDHC*, *SDHAF2*) are the most commonly affected genes and encode different units and assembly proteins of the mitochondrial enzyme succinate dehydrogenase (SDH) [18] which is implicated in the Krebs cycle and oxidizes succinate to fumarate. The disruption of Krebs cycle enzymes leads to accumulation of the oncometabolites succinate (*SDHx*), fumarate (*FH*) or 2-hydroxyglutarate (*IDH*) that drive tumorigenesis by DNA hypermethylation, inactivation of tumour suppressor genes and HIF alpha stabilisation [27].

Cluster 2B *VHL/EPAS1* related PPGL are due to mutations in the *VHL*, *EPAS1*, *EGLN1/2* and *IRP1* genes and are characterised by the key pathogenic mechanism of stabilisation and accumulation of HIF alpha proteins. The pseudo-hypoxic state of Cluster 1 tumours results in increased angiogenesis (e.g., vascular endothelial growth factor (VEGF) transcription), cellular proliferation and reduced apoptosis [28,29].

The second cluster is characterised by an upregulation of kinase signaling pathways involving the MAPK pathway and the mechanistic Target of Rapamycin (mTOR) pathway leading to enhanced cell growth and cell survival. These tumours arise as a result of gain-of-function-mutations in *RET* or mutations in genes such as *NF1*, *TMEM127*, *MAX*, *MET*, *MERTK* and *FGFR1* and *HRAS* (both somatic) [13].

Finally, the third cluster comprises genes leading to activation of the Wnt/beta-catenin pathway resulting in increased angiogenesis, cell proliferation and invasion. Perturbations of this pathway are less explored and were exclusively described in sporadic PPGL with somatic variants in *CSDE1* and *MAML3* fusion genes [13].

Generally, Cluster 1 genes are more commonly affected at the germline level, while Cluster 2 genes mutations are observed both at the germline and somatic level [13,23].

4. Clinical Phenotype According to Molecular Clusters

Besides the presence of typical syndromic features or a specific family history, a range of clinical, biochemical, histopathological or imaging characteristics may inform prediction about a genetic cause and the underlying molecular cluster, and therefore, may guide a personalized management from an early stage. Young age at presentation suggests the presence of a germline pathogenic variant. Extra-adrenal localisation and large tumour size at diagnosis may predict a germline *SDHx* gene mutation while adrenal localization may point to a Cluster 2 mutation [30]. Episodic symptoms or a higher symptom score (e.g., tremor, pallor, anxiety) are suggestive of an adrenaline producing (mostly Cluster 2 related) PPGL. Cluster 1 and possibly Cluster 3 PPGLs show a more aggressive phenotype (with the highest metastatic risk for *SDHB* mutations) compared with Cluster 2 tumours [31]. According to a recent meta-analysis, the metastatic risk for Clusters 1, 2, and 3 PPGLs are 24% (40.5% for Cluster 1A), 4.1% and 11.4%, respectively [31]. In patients with metastatic PPGL the probability of an underlying mutation in Cluster 1 genes are as high as 60% [32–34].

5. Biochemistry

The recommended test to establish the biochemical phenotype of PPGLs is the measurement of the respective O-methylated metabolites of adrenaline, noradrenaline and dopamine (metanephrine, normetanephrine and 3-methoxytyramine) ideally in plasma via liquid chromatography-tandem mass spectrometry (LH-MS) or urine [17]. Blood for plasma metanephrines should be drawn in a supine position and the patient should ideally be recumbent for at least 30 min before sampling. The sensitivity of plasma free metanephrines (as well as of 24-h urine fractionated metanephrines) is very high (96–100%) and a negative test result virtually excludes PPGL. The specificity or plasma free metanephrines is lower (around 89%) due to medication or physiological stress that may interfere with the measurement and sampling should be repeated and confirmed by another test modality (24-h urine fractionated metanephrines or clonidine test [17,35]).

From the biochemical phenotype conclusions can be drawn about molecular tumour characteristics and cell differentiation. A noradrenergic phenotype (with increased

normetanephrines levels) points to a tumour lacking the enzyme phenylethanolamine N-methyl transferase, which converts noradrenalin to adrenalin [36]. A dopaminergic phenotype (with increased 3-methoxytyramine levels) is suggestive of a deficiency in the enzyme dopamine-beta-hydroxylase, which converts dopamine to noradrenaline [37]. PPGLs of the pseudohypoxia group (Cluster 1) and especially *SDHx* mutation carriers are associated with a noradrenergic or dopaminergic phenotype reflecting the poor differentiation of paraganglia cells and reduced converting enzyme expression [38]. Some *SDHx* mutation carriers also lack the enzyme tyrosine hydroxylase (catecholamine synthesis) and are, therefore, characterised by a non-functional phenotype. Conversely, Cluster 2 tumours typically show a mixed or predominately adrenergic secretory phenotype as a consequence of more mature cell differentiation and increased expression of phenylethanolamine N-methyl transferase.

Stratification according to the hormonal activity of the tumour helps to decide whether or not patients need pre-operative treatment with alpha-blockade but may also guide sequential genetic testing. Longitudinal comparison of metabolites gives information about disease control or progression [39]. In non-functional tumours surveillance is based on repeated cross-sectional imaging and the measurement of the biomarker chromogranin A [40–42]. Finally, highly elevated 3-methoxytyramine levels are associated with a more aggressive behaviour of the tumour and may suggest metastatic disease (an exception to this rule is *SDHx*-mutated, non-metastatic head and neck paraganglioma that may show elevation of 3-methoxytyramine) [43]. Such patients should undergo pre-operative staging, ideally with functional radionuclide imaging (see below), and need increased surveillance.

6. Immunohistochemistry

In general, neuroendocrine tumours are characterised by positive immunohistochemistry for chromogranin A, synaptophysin, and S100 [25]. In PPGL, a relevant metabolic biomarker is SDHB immunohistochemistry which allows for the identification of patients with mutations in the *SDHx* tumour suppressor genes [44–46]. Biallelic inactivation (e.g., germline variant and "second hit" at the somatic level) in the *SDHx* genes disrupts the SDH enzyme complex and the anchor SDHB protein [45]. Loss of SDHB protein expression is seen in PPGLs with mutations in any of the *SDH* genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*) or with somatic hypermethylation of the *SDHC* promoter region [47]. Conversely, negative immunohistochemistry for SDHB and SDHA is only seen in tumours with SDHA mutations. The sensitivity and specificity of SDHB immunohistochemistry are around 94% and 85%, respectively. In *SDHD* mutated tumours, the SDHB immunohistiochemistry may be misleading, as sometimes interpreted as immunopositive. Conversely, immunonegative results in some *VHL* and *NF1* mutated tumours compromise specificity of SDHB immunohistochemistry [47].

SDHB immunohistochemistry allows a targeted approach to genetic testing and has also a value in the pathogenicity assessment of genetic variants of unknown significance. Moreover, SDHB immunohistochemistry in PPGL is associated with metastasis and poor outcome and is, therefore, a marker of malignancy.

Immunohistochemistry may also have a role in interpreting variants in *FH* by assessing loss of expression of the fumarate hydratase protein or in screening for *MAX*- and *VHL*-related PPGLs by immunohistochemical staining for MAX and carbonic anhydrase 9 expression, respectively [48,49].

In analogy to immunohistochemistry, the measurement of metabolites such as succinate, fumarate, malate or 2-hydroxyglutarate (2HG) provides also a screening tool to identify underlying driver mutations in PPGL or to assess functionality of variants of unknown significance. PPGL with high succinate:fumarate ratios point to *SDHx* mutations, while a high fumarate:malate ratio suggests a FH mutation. High levels of 2HG (combined with a high D- to L-enantiomers ratio of 2HG) may identify patients at risk for IDH1/2 mutations [46,50].

7. The Influence of Genotype on Molecular Imaging Modality Selection in PPGL

Molecular imaging techniques have an important role in the management of PPGL in clinical practice and may be used for: (i) PPGL staging, (ii) localisation of an occult tumour(s) and (iii) theranostic applications to determine the response to radionuclide therapies.

Molecular imaging tracers specific for PPGL can be sub-classified into three main groups based on their target ligand and include: (i) catecholamine storage and synthesis; [123I-metaiodobenzylguanidine, 18F-fluorodopamine (18F-FDA) and 18F-fluorodihydroxyphenylalanine (18F-FDOPA)], (ii) glucose metabolism [18F-fluorodeoxyglucose (18F-FDG)] and (iii) somatostatin receptor [111indium–pentetreotide and gallium-68 DOTA-conjugated peptide (68Ga-DOTATATE)]. The selection of the most appropriate tracer is by patient genotype and the affect that genotype has on tumour biology, anatomical location and secretory pattern, all of which influence the expression of receptors targeted by molecular imaging tracers, thereby giving rise to a so-called molecular imaging phenotype [51].

7.1. Radiotracers Targeting Ligands Involved in Catecholamine Synthesis and Storage

Commercially available metaiodobenzylguanidine (MIBG) is radiolabelled with ^{123}I (Iobenguane) or ^{131}I and binds to the noradrenaline transporter (NET) and is stored within catecholamine secreting tumours in neurosecretory granules via vesicular monoamine transporters (VMATs). However, the sensitivity of 123/131I-MIBG scintigraphy is reduced in de-differentiated tumours where expression of the NET or VMATs are reduced or absent, therefore leading to a false-negative results. Furthermore, mutations in 'Cluster 1' genes, specifically the *SDHx* genes and *VHL*, are associated with reduced expression of the NET transporter, thereby affecting the sensitivity of 123/131I-MIBG scintigraphy for the detection of primary or metastatic PPGL in patients harbouring these gene mutations. The recognition that false negative results with 123/131I-MIBG scintigraphy may be more common in patients with metastatic PPGL or 'Cluster 1' gene mutations has informed current consensus guidelines [15]. It is now recommended that 123/131I-MIBG scintigraphy is reserved for those cases being investigated for suitability of treatment with 123/131I-MIBG radionuclide therapy, particularly in those patients with suspected *SDHx* mutations [15].

The 18F-FDA also binds to the NET transporter and therefore has the same sensitivity issues as 123/131I-MIBG for de-differentiated tumours or patients with 'Cluster 1' gene mutations. Finally, the imaging tracer 18F-fluorodopa (FDOPA) binds to the neutral amino acid transporter system L. The sensitivity of 18F-FDOPA PET-CT is also reduced in patients with 'Cluster 1' gene mutations, specifically the *SDHx* genes, and this has been attributed to the impaired catecholamine synthesis pathway and truncated citric acid cycle in SDH-deficient tumours. Therefore, patients with 'Cluster 1' gene mutations have a specific molecular imaging phenotype that includes reduced or absent avidity of tracers involved in catecholamine synthesis or storage. The opposite molecular imaging phenotype is noted for patients with 'Cluster 2 gene' mutations in whom the catecholamine synthesis pathway is often upregulated and therefore tracers binding to NET and VMATs are often more sensitive in patients with 'Cluster 2' gene mutations. Therefore, 123/131I-MIBG scintigraphy may be considered as the first-line imaging modality in patients with Cluster 2 gene mutations or sporadic tumours [52].

7.2. Tracers Targeting Glucose Metabolism

Imaging with 18F-fluorodeoxyglucose positron emission tomography (18F-FDG PET) is employed in clinical practice to probe the increased glucose use that occurs in many metabolically active tumours and cancers. The sensitivity of 18F-FDG PET-CT in the imaging of PPGL is also influenced by genotype. 'Cluster 1' tumours exhibit increased glycolysis due to pseudohypoxia leading to upregulation of glycolytic enzymes and glucose transporters and therefore have higher standard uptake values (SUVs) of 18F-FDG compared to sporadic or 'Cluster 2' tumours although FDG avidity in these tumours does not correlate with malignant potential [51].

7.3. Tracers Targeting Somatostatin Receptors

The 68Ga-labeled DOTA peptides such as DOTA(0)-Tyr(3)-octreotate (DOTATATE), DOTA(0)-Phe(1)-Tyr(3)-octreotide (DOTATOC), and DOTA(1)-Nal(3)-octreotide (DOTANOC) bind to somatostatin receptors (specifically somatostatin receptor 2) and combined with PET/CT are now the favoured molecular imaging modality for PPGL, largely replacing Technitium (Tc)-99m and Indium (In)-111-labeled Octreotide SPECT/CT [52]. The 68Ga-DOTATATE PET-CT is now recommended as the imaging modality of choice for patients with *SDHx*-mutated PPGL or sporadic or metastatic PPGL and can also predict the efficacy of peptide receptor radionuclide therapy with 177Lu-DOTATATE [52]. The 68Ga-DOTATATE can also help to differentiate between a PPGL and other tumour, such as a gastrointestinal stromal tumour (GIST) in patients with *SDHx* mutations, who are at risk of both tumour types. In such cases, 68Ga-DOTATATE may be preferred over 18F-FDG, which is frequently positive in both tumour types [53].

Finally, as 68Ga-DOTATATE is also taken up by the background normal adrenal gland, sensitivity of this tracer is reduced for patients at risk of small or bilateral phaeochromocytomas such as patients with 'Cluster 2' gene mutations. In this instance, 18F-DOPA PET-CT is preferred because of the superior tumour to background uptake of this tracer [54].

8. In Vivo Detection of Oncometabolites in PPGL

Tumorigenesis caused by Kreb's cycle gene defects are driven by accumulation of metabolites referred to as 'oncometablites', such as succinate in patients with *SDHx* mutations or fumarate in patients with *FH* mutations. The early detection of oncometabolite accumulation in tumours in vivo can facilitate early personalised management of patients with PPGL. Molecular imaging facilitates the characterisation of tumours at a molecular level and despite advances and improved sensitivity of molecular imaging tracer the ability to measure individual metabolites within a tumour is beyond the scope of these tests. However, techniques such as proton magnetic resonance spectroscopy (^{1}H-MRS) can detect metabolite accumulation in PPGL in vivo [55,56]. Our group has previously demonstrated that ^{1}H-MRS can detect succinate and fumarate in tumours in vivo and may be used for the early detection of metabolically driven tumours as well as serving as an early efficacy biomarker to monitor treatment response [55,57]. Thus, the detection of metabolite accumulation in tumours in vivo using ^{1}H-MRS, offers the opportunity to apply a non-invasive technique to stratify suitable patients with PPGL for specific targeted therapies and may serve as a pre-selection tool for recruitment into clinical trials.

9. Precision Medicine for the Treatment of PPGL

9.1. Surgery

The first-line treatment option for a primary PPGL or localised disease is surgery. The genotype, if known pre-operatively, may influence the surgical strategy as it provides information on the risk of synchronous or bilateral tumours and metastatic potential. For patients with a *SDHx* gene mutation and a large para-aortic paraganglioma, for example, an open approach may be favoured over a laparoscopic approach to facilitate clear resection margins and lymph node dissection. Whereas patients at risk of bilateral phaeochromocytoma due to mutations in the *VHL*, *RET* or *NF1* genes may be considered for a cortical sparing adrenal surgery to mitigate against the need for life-long glucocorticoid and mineralocorticoid replacement therapy [58]. Although patients with *SDHx* mutations may also develop bilateral phaeochromocytoma, a total adrenalectomy is favoured over a cortical sparing approach because of the higher malignant potential of *SDHx* mutated tumours [59].

9.2. Radionuclide Therapies

Radiopharamaceutical therapies for patients with metastatic, inoperable PPGL include ^{131}I-MIBG, ^{90}Y and ^{177}Lu-DOTATATE. The first step in evaluating the potential efficacy of radionuclide therapies is to consider imaging with 68Ga-DOTATATE PET/CT or 123/131I-MIBG scintigraphy. A radionuclide therapy should only be selected if there is evidence of

tumoral uptake of the relevant tracer in the tumors demonstrated on either cross-sectional imaging or 18F-FDG-PET/CT [60]. Therefore, genetics can influence the selection of these radiopharmaceutical therapies because of the above-mentioned affect that genotype has on the expression of radiopharmaceutical ligands such as NET in patients with 'cluster 1' gene mutations. To date, there are no phase III studies that have investigated the efficacy of ^{131}I-MIBG versus ^{90}Y- or ^{177}Lu-DOTATATE in patients with metastatic PPGL [60].

9.3. Cytotoxic Chemotherapy

Combination chemotherapy consisting of cyclophosphamide, vincristine, and dacarbazine (CVD) is the standard cytotoxic chemotherapy regime for patients with metastatic PPGL. Treatment with CVD has demonstrated partial response rates (including tumour volume and catecholamine burden) of up to 40% in unselected cohorts of patients with metastatic PPGL [61]. Anecdotal case reports have suggested that response rates to CVD chemotherapy may be superior in patients with *SDHB* gene mutation [62], and a more recent retrospective study also found that median progression free survival after CVD therapy was superior in patients with *SDHB* gene mutations and metastatic PPGL compared to those without *SDHB* gene mutations [63].

9.4. Alkylating Agents

Temozolomide is an alkylating agent originally developed as an oral alternative to intravenous dacarbazine. The expression of O(6)-methylguanine-DNA methyltransferase (MGMT) within cancer cells allows the cell to recover from the DNA damaging effects of Temozolomide, enabling the tumour to become resistant to therapeutic use of such agents. A large retrospective study demonstrated therapeutic efficacy of temozolomide in patients with metastatic PPGL and partial responses were specifically observed in patients with *SDHB* gene mutations [64]. This reported efficacy of temozolomide in *SDHB* mutated PPGL has been attributed to epigenetic silencing of the *MGMT* gene in the *SDHB* mutated tumours [64].

9.5. Tyrosine Kinase Inhibitors

PPGL are vascular tumours and studies have shown that angiogenic factors like VGEF, are preferentially expressed in PPGL [65]. Furthermore, the pseudohypoxic phenotype of 'Cluster 1' mutated PPGL promotes upregulation of vascular growth factors and, therefore, tyrosine kinase inhibitors (TKIs) have a potential role in the management of metastatic PPGL. Data from a recent phase II study investigating the role of sunitinib in the treatment of metastatic PPGL, has suggested that patients with germline mutations in *RET* or *SDHx* may benefit most [66].

10. Emerging Targeted Therapies for PPGL

10.1. Cluster 1 PPGL

10.1.1. PARP Inhibitors

Recent studies have demonstrated that accumulation of oncometabolites such as succinate, fumarate or 2-hydroxyglutarate (2-HG) may render tumours susceptible to synthetic-lethal targeting with poly-(ADP)-ribose polymerase (PARP) inhibitors [67,68]. Oncometabolite accumulation inhibits α –ketoglutarate-dependent dioxygenases and the subsequent inhibition of two key lysine demethylases; KDM4A and KDM4B, which ultimately dysregulates DNA homologous repair [68]. A currently recruiting phase II clinical trial is investigating whether the combination of the PARP inhibitor, olaparib, and temozolomide is more efficacious that temozolomide monotherapy in patients with metastatic PPGL (NCT04394858). If proven effective, there may be a role in the future for in vivo or ex vivo analysis for oncometabolite accumulation in order to stratify those patients predicted to be more responsive to PARP inhibition.

10.1.2. Immunotherapy

The pseudohypoxic tumour environment in 'Cluster 1' mutated PPGL may facilitate immune evasion through inactivation of cytotoxic T-cell lymphocytes and stimulation of immune-suppressive monocytes [69] and this finding has prompted interest in the role of immune modulating drugs for PPGL. Two phase II studies investigating the efficacy of immunotherapies such as pembrolizumab (NCT02721732) and Nivolumab plus ipilimumab (NCT02834013) in rare cancers including PPGL, are currently recruiting.

10.1.3. Demethylating Agents

Demethylating agents may have therapeutic benefit in Cluster 1 mutated PPGL because of the hypermethylation phenotype promoted by mutations in Kreb's cycle genes [70]. The role of hypomethylating agents prompted interest in new second-generation demethylating agents such as guadecitabine (SGI-110), which has superior bioavailability compared with its parent drug decitabine [71], and is licensed for use in solid tumours. However, a recent phase 2 clinical trial investigating Guadecitabine (SGI-110) monotherapy (NCT03165721) in patients with *SDHx* or *FH* mutated tumours was terminated because of low accrual. Of the nine subjects enrolled including seven with SDH-deficient GIST, one with SDH-deficient paraganglioma and one *FH*-mutated RCC, no complete or partial responses were observed [72].

10.1.4. HIF2α Antagonists

Hypoxia-inducible factor 2 α (HIF-2 α) is strikingly upregulated in 'Cluster 1' mutated PPGL and has sparked great interest in applying inhibitors of HIF as anticancer therapies. A pre-clinical study demonstrated efficacy of a small molecule HIF-2αinhibitor (PT2399) in mouse models of primary and metastatic pVHL defective clear cell renal cell carcinoma [73]. More recently, two phase II clinical trials evaluating the role of a HIF2α (PT2385) in VHL associated RCC (NCT03108066) and the effectiveness of Belzutifan/MK-6482 for the treatment of advanced PPGL and pancreatic neuroendocrine tumours, respectively, are currently ongoing (NCT04924075). These studies will hopefully inform the therapeutic utility of HIF2α inhibitors for advanced PPGL as well as tumours harbouring *EPAS1*, *VHL*, *SDHX*, *FH* and other somatic and germline 'Cluster 1' gene mutations.

10.1.5. Targeting Metabolic Reprogramming and Redox Imbalance

The metabolic vulnerability of tumours harbouring citric acid cycle gene mutations provides an opportunity for therapeutic targeting. Ex vivo metabolomics analysis on tumour samples performed by this group has demonstrated that the metabolomics fingerprint of *SDHx* mutated PPGL includes a significant reduction in metabolites such as aspartate (unpublished data). Aspartate is essential for DNA synthesis and cellular proliferation and in vitro studies have suggested that SDH deficient tumours overcome the deficiency in aspartate by up-regulating the enzyme pyruvate carboxylase [74]. A small molecule inhibitor of oxidative phosphorylation (IACS-010759) is currently being evaluated for safety in phase 1 studies (NCT02882321 and NCT03291938). This molecule arrests proliferation by inhibiting aspartate production, thereby impairing nucleotide biosynthesis and therefore may be a promising therapy for SDH deficient tumours.

Finally, impaired oxygen sensing pathways in 'Cluster 1' mutated PPGL leads to accumulation of oxygen free radicals and iron. In vitro studies have suggested that pharmacological does of ascorbic acid can induce an overload of reactive oxygen species in in *SDHB* knockdown cells, promoting apoptosis [75] and this has prompted interest in the therapeutic role of ascorbic acid alone or in combination with other therapies for 'Cluster 1' mutated PPGL.

10.2. Cluster 2 Mutated PPGL

New Kinase Inhibitors

Upregulation of signalling pathways such as the Ras/Raf/Erk or PI3K/Akt/mTOR pathways is most commonly seen in PPGL with Cluster 2 germline gene mutations e.g., *NF1*, *RET*, *TMEM127* or somatic mutations in *NF1* or *HRAS*. A phase II study investigating the benefit of the mTOR inhibitor everolimus in metastatic neuroendocrine tumours and PPGL demonstrated modest efficacy in the small number of metastatic PPGL patients enrolled [76]. Recently inhibition of MAPK signaling using mitogen-activated protein kinase kinase (MEK) inhibitors has demonstrated efficacy in the treatment of malignant peripheral nerve sheath tumours harboring *NF1* mutations in children [77] and raises the possibility that MEK inhibitors may have a therapeutic role in molecular subsets of metastatic PPGL such as those with 'Cluster 2' gene mutations (Figure 1).

11. Future Considerations for Precision Medicine in PPGL

With technological advances contributing towards an improved understanding of the molecular basis for PPGLs for a large proportion of patients, it seems that we have entered the era of precision healthcare for individuals with PPGL. Indeed, this notion is reflected in recent published guidance, which recommends that all patients with primary PPGL or metastatic PPGL have clinical germline genetic testing [13,14,16]. Such recommendation implies that a genetic diagnosis will inform personalised medicine strategies, which will guide individualised clinical management and, ultimately, improve patient outcomes. However, several challenges exist which may limit the widespread incorporation of precision medicine in routine clinical care of patients with PPGL.

In order for precision medicine to have widespread impact, genomic profiling technologies used to stratify patients need to be affordable, widely accessible and supported by the appropriate infrastructure. The requirement of tumour tissue for next generation sequencing or gene expression profiling necessitates that tumour specimens obtained through biopsy or surgical resection are sampled, collected and stored correctly to mitigate against technical failures due to poor sample quality. In many instances, due to a reluctance or inability to undertake repeated tumour biopsies because of the associated risk of catecholamine excess and need for alpha blockade, archival tissue is analysed in attempt to molecularly stratify tumours and 'match' their molecular signatures with targeted therapies. However, the genomic landscape of archival tissue may not accurately reflect tumour evolution and intra-tumoral heterogeneity in recurrent, progressive or metastatic disease. For some cancers, a 'liquid biopsy' containing circulating tumour DNA (ctDNA) has been proposed as an alternative to repeat tumour biopsies and may provide 'real-time' information regarding genomic alterations within a tumour and identify key driver mutations [78]. Recently, the diagnostic utility of the NETest, a NET-specific 51-marker gene blood assay, was investigated in 81 subjects with PPGL [79]. The investigators found that the NETest was able to diagnose PPGLs with 100% efficacy and differentiate Cluster 2 from Cluster 1 tumours thereby providing proof of concept evidence that 'liquid biopsies' may have clinical utility in the management of PPGLs. When biomarkers, such as ctDNA, or other molecular diagnostic tests, are used to stratify patients into smaller sub-groups and accompany a targeted therapy, they may be referred to as a companion diagnostic. A companion diagnostic is a regulatory approved test that has been validated, both analytically and clinically with the therapy. Companion diagnostics are often co-developed alongside a new targeted therapy in a lengthy process and usually at a considerable cost. Thus, when developed for smaller patient sub-groups with rare cancers, such as PPGLs, targeted therapies with accompanying companion diagnostics may be prohibitively expensive for some healthcare systems.

To justify the rationale and cost of precision medicine approaches to the management of PPGLs to healthcare providers, patients, regulators and payers, it is crucial that such strategies are backed by evidence that demonstrate patient benefit. That consensus guidance recommends genetic testing for the majority of patients with PPGL suggests

that confirmation and awareness of a genetic diagnosis confers greater benefit to patients compared with those unaware of their genetic status. Indeed, Buffet et al., found that PPGL patients who were informed of their positive genetic status for *SDHx* and *VHL* mutations underwent more examinations, were less likely to be lost to follow-up and had an improved 5-year survival rate in the presence of metachronous metastases when compared to a historic cohort of subjects who were not aware of their genetic diagnosis [14]. Thus, knowledge of a genetic diagnosis influences how patients engage with healthcare providers which equates to improved clinical outcomes. Additional clinical studies which prospectively follow individuals with mutations in PPGL susceptibility genes and records compliance with surveillance and corresponding clinical outcomes are warranted.

The most desired outcome of precision oncology is the ability to match the genomic profile of a tumour with a well-tolerated and efficacious targeted therapy. However, such a strategy may be difficult to implement due to a lack of available medicines that 'match' the molecular landscape of a tumour. Moreover, if a candidate therapy is available for a specific patient subgroup generating the required clinical trial evidence to support its use in the clinic can be challenging, especially for rare tumours. The traditional randomised placebo-controlled clinical trial may not be the optimal clinical trial design for studying the safety and efficacy of new investigational therapies in rare diseases. This may be due to a number of factors, which includes a small number of recruitable patients resulting in long and expensive clinical trials that may be underpowered. However, as new precision medicine-based therapies are developed, clinical trial design has also evolved, especially in oncology, to accommodate the need for innovative approaches for studying new treatments.

The 'basket' trial design is intended to study a single investigational drug across a number of tumours (tumour agnostic) that share a common feature (for example, molecular profile). Therefore, in a basket design the study population is enriched by including only those participants with biomarkers that render them most likely to respond to the intervention. In 2018, pembrolizumab was the first drug to receive FDA (Food and Drug Administration) approval for a tissue agnostic indication based on demonstrated efficacy across a number of solid tumours characterised by mismatch repair deficiency or high microsatellite instability. A similar approach could be adopted for the study of new treatments for PPGL and related tumours. For example, it is well-established that SDH deficiency not only predisposes individuals to developing PPGL but other tumours, including gastrointestinal stromal tumours (GIST) and renal cell carcinoma [80]. Thus, adoption of the 'basket' design across the spectrum of *SDHx* mutated tumours may be an effective way of studying new or repurposed drugs in this patient group. Another example of clinical trial innovation that supports the development of therapies targeting rare diseases is the use of real-world evidence (RWE). Here, advanced data analytics and RWE can be leveraged to create an observational placebo arm to support a clinical trial. Such an approach minimises the number of people required for a control arm and provides opportunity for more patients to potentially benefit from a new targeted treatment.

Finally, if precision medicine approaches to PPGL and other cancers are to become commonplace in the clinical setting, it is imperative that endocrinologists and oncologists are equipped with the knowledge required to exercise this practice. Physicians caring for these patients will need to have familiarity with genomics and genetics and the interpretation of genetic test results. They will need to possess the qualification required to accurately communicate the findings of genomic tests, and their implications, to their patients and their families. Physicians will need to understand the fundamentals of molecular technologies including their advantages and disadvantages, and maintain their knowledge of the ever evolving scientific and therapeutic landscape.

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Needs, Preferences, and Values during Different Treatment Decisions of Patients with Differentiated Thyroid Cancer

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Abstract: Background: The purpose of this study was to identify the needs, preferences, and values of patients with differentiated thyroid cancer (DTC) and the physicians treating patients with DTC regarding two different treatment decisions, namely: the extent of primary surgery (low-risk patients) and the tyrosine kinase inhibitor (TKI) treatment (high-risk patients). Methods: A qualitative study was conducted. There were two physician focus groups discussing the extent of primary surgery. One included endocrinologists (n = 4) and surgeons (n = 5), and the other included nuclear medicine physicians (n = 3) treating patients with low-risk DTC. The physicians focus group discussing waiting or starting TKIs included endocrinologists (n = 2) and oncologists (n = 5) treating patients with advanced radioactive iodide (RAI) refractory DTC. Moreover, one patient focus group per treatment decision took place. In total 13 patients and 19 physicians participated. Interviews were audio-taped, fully transcribed verbatim, and analyzed. Results: Several themes were identified. Patients, but not physicians, mentioned the importance of a strong doctor–patient relationship. Patients in both treatment decision groups wanted to receive more detailed information, whereas physicians preferred providing more general information. Patients in the TKI decision group focused on palliative care, whereas physicians focused more on the effect and benefit of TKIs. Conclusions: Considering the identified themes in DTC, based on the patients' needs, preferences, and values, enables us to improve doctor–patient communication and to develop decision support tools.

Keywords: differentiated thyroid cancer; information needs and preferences; focus group interview

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1. Introduction

Most patients with low-risk differentiated thyroid cancer (DTC) have an excellent long-term prognosis [1–4]. Up to 30% of DTC patients develop recurrent disease and/or distant metastases. Patients with distant metastases have a five-years survival rate of approximately 50%, which is worse in those with advanced radioactive iodide (RAI) refractory DTC [5,6]. Therefore, considering the relatively high survival rates for both low-risk and high-risk DTC survivors, maintaining long-term quality of life (QOL) is important.

Recently, clinical practice shifted towards more individualized approaches, and patients are involved in trade-offs between the harms and benefits of different approaches [7]. For patients with low-risk DTC and patients with RAI refractory DTC, the optimal treatment is debated, as insufficient evidence is available regarding the harms and benefits of treatments in relation to the oncological outcome. As such, some low-risk DTC patients might undergo overtreatment, thus negatively affecting QOL. The American Thyroid Association (ATA) guidelines suggest considering patient preferences, as for some low-risk

patients, thyroid lobectomy (TL) alone may be a sufficient initial treatment [8,9]. Similarly, for asymptomatic or mildly symptomatic RAI refractory DTC patients, premature starting of tyrosine kinase inhibitors (TKIs) may expose patients to side-effects and worsening QOL. The recent European Thyroid Association (ETA) guidelines stated that the decision to start TKIs should include patient-related medical factors and patient preferences with respect to treatment goals and values, as well as the acceptance of adverse effects [10]. However, a recommendation on how to shape the communication process is lacking.

Doctor–patient communication could be improved to consider the patients' perspective by using shared decision making (SDM). Accordingly, physicians provide patients with information on existing options and consider patients' needs in order to enable a personalized treatment choice [11]. In this process, important life goals are involved, and these should be explored together with the patient [12–15]. In practice, however, talking about values is difficult, and values are discussed in a minority of decision-making consultations [11]. Moreover, exploring values is complicated by different perspectives of physicians and patients [16].

To facilitate SDM in doctor–patient communication, it is important to determine the patients' needs. Decision-support tools, such as decision aids and values clarification exercises, exist to inform patients and help them explore their values [17,18]. These tools can be applied in clinical practice. These instruments are not yet available for all patients with DTC. For developing such tools, knowledge about patients' and physicians' needs is required. Previous studies have explored the patients' needs in patients with papillary microcarcinoma (PMC) [19–23] and in patients with larger low-risk DTC (>1 cm) requiring treatment [24–30]. However, particularly the differences between patients' and physicians' needs have not previously been investigated.

To reduce miscommunication between patients and their physicians, we aimed to identify the needs of DTC patients in two different treatment decision groups in preference-sensitive decision making, as stated in the ATA and ETA guidelines [9,10]: (1) the extent of primary surgery, including the need for subsequent ablation of thyroid remnants with RAI in patients with low-risk DTC (>1 cm), and (2) starting TKIs in patients with advanced RAI refractory DTC. Our aim was to, on the one hand, address the differences and similarities between the two treatment decision-making groups (regarding low-risk and high-risk patients), and on the other hand, between perspectives of physicians and patients for both decisions. In addition to previous studies, more attention was given to values communication, given the difficulty in exploring values and to help physicians to discriminate between their own and patients' values.

2. Materials and Methods

2.1. Study Design

We performed a qualitative study using semi-structured focus group interviews. The aim of the interviews was to identify in-depth needs, preferences, and values of DTC patients in two different treatment decision groups and in physicians treating DTC patients. This study is part of the COMBO study (COMmunication Booster, NCT03905369), aiming to develop, evaluate, and implement decision-support tools for DTC patients. Twelve hospitals (six academic and six non-academic) in the Netherlands participated. The Dutch patient association "Schildklier Organisatie Nederland (SON)" was also involved. The Medical Ethical Committee (CMO) of the region Arnhem–Nijmegen approved the study protocol (MEC-2018-4521). The study is in agreement with the COREQ checklist [31].

2.2. Setting

In the Netherlands, DTC patients undergo treatment in both academic (high- and low-risk patients) and non-academic hospitals (mainly low-risk patients) involving multidisciplinary teams of specialists. Long-term follow-up is generally carried out by endocrinologists. Patients with advanced RAI refractory disease requiring TKI treatment are followed-up by oncologists.

2.3. Participants

2.3.1. Patients

Two treatment decision-making groups were involved, namely: (1) patients with low-risk DTC according to the ATA criteria [9] who had surgery, and (2) patients with advanced RAI refractory DTC who started or considered TKIs. The inclusion criteria for the low-risk group were being diagnosed with DTC, having been treated with surgery within one year, and being capable of understanding their treatment trajectory as judged by their physician. Inclusion criteria for the advanced disease group were patients who started or considered TKIs within one year and were capable of understanding their treatment trajectory as judged by their physician. Six academic hospitals with expertise in DTC treatment throughout the Netherlands, as well as the Dutch patient association SON, selected patients for participation in the interviews. After physicians asked their patients to participate in the interviews, participants were approached by the researcher (A.K.) by telephone. Participation was voluntary. All participants provided written informed consent alongside answers to some demographic questions.

In total, two patient focus groups were organized. One focus group included low-risk DTC patients (n = 6) discussing thyroid lobectomy or total thyroidectomy, including the need for the subsequent ablation of thyroid remnants with RAI. The other focus group included patients with advanced disease (n = 7), discussing the watchful waiting approach or starting with TKIs decision. The patient focus group interviews took place in the main investigating center (Radboud University Medical Center). The interviewee had no treatment relationship with the participants.

2.3.2. Physicians

Physicians (endocrinologists, surgeons, nuclear medicine physicians, and oncologists) from the above-mentioned expertise centers with extensive expertise on the treatment of patients with DTC were approached by the researcher (A.K.) by email. All physicians provided verbal informed consent and answered several questions regarding their clinical experience. In total, three physician focus groups were organized. To discuss the thyroid lobectomy or total thyroidectomy decision, two focus groups were organized, as follows: one included endocrinologists (n = 4) and surgeons (n = 5), and one included nuclear medicine physicians (n = 3) treating patients with low-risk DTC. To discuss the decision between watchful waiting or starting with TKIs, another focus group was held, including endocrinologists (n = 2) and oncologists (n = 5) treating patients with advanced RAI refractory DTC.

2.4. Data Collection

An expert panel developed an interview guide (Table A1). For both patients and physicians, the interview guide contained three sections, open-ended questions, and optional questions to elaborate each topic. The three sections referred to the diagnostic, treatment, and evaluation phases of DTC care. The interviewer (A.K., first author, female, MD endocrinology, PhD student, trained in interviewing techniques by a qualitative research expert) started by explaining the process of the interview. Next, open-ended questions, not provided in advance, were asked concerning the information given by the physician during the consultation in the specific treatment phase. The open-ended questions, focused on needs, preferences, and values; communication with the health care provider; strong and weak points of the received health care; and points to improve the current health care. Finally, patients were invited to give their opinion about the content to be included in a decision-support tool. The focus group interviews were conducted between May and December 2019. All of the focus group interviews lasted between 26 and 94 min and were audio-taped. One additional observer attended each focus group and field notes were made during the focus group interviews (R.N.-M. or P.O.). A pilot for the interview was performed with the first and fourth author (female, senior researcher, experience with qualitative research). Patients did not receive questions in advance and

were not informed about the use of the framework to analyse the data using the Picker domains [32,33].

2.5. Analysis

First, all five focus group interviews were transcribed verbatim and qualitatively analyzed using ATLAS.ti, 8.4.15 [34]. Two researchers (A.K. and a second coder; female, experienced coder) independently analyzed all of the transcripts. The perspectives of patients and physicians and the two different treatment decisions were analyzed separately. The eight-dimension Picker domains were used as a basis for our analyses (Table A2) [32]. Expressed needs, preferences, and values were categorized into one of the eight Picker domains, particularly involvement in decisions and respect for preferences, coordination and integration of care, clear information, and communication and emotional support. All interviews were open coded independently by both researchers. Open coding allows for an exploration of the ideas and meaning that were contained in the raw data. Once codes were created using open coding, they were analyzed using the axial coding process. This analysis enabled researchers to identify connections between the codes [35]. For axial coding, two concept coding trees were made, one for the surgery decision and one for the TKI decision. Both researchers could add, remove, or move the codes of the coding tree. The codes were compared and discussed until a consensus was reached. Thereafter, the codes were categorized into similar themes and subthemes within one of the domains. We aimed to fit all themes into the Picker domains, and a new domain was proposed if the codes would not fit.

3. Results

All of the invited patients and physicians agreed to participate. The patient and physician characteristics are displayed in Table 1. Sixteen themes and sixty-three subthemes were categorized for low-risk DTC patients and their respective physicians. Fifteen themes and thirty-eight subthemes were categorized for patients with advanced DTC and their respective physicians. The themes and subthemes fitted within four of the eight Picker domains (Table A2) [32]; one additional domain occurred, namely values (Tables 2–5 and Figure 1). Table 2 shows the qualitative results for the surgery decision regarding "thyroid lobectomy or total thyroidectomy" and Table 3 shows several quotes fitted to the corresponding themes for the decision regarding "thyroid lobectomy or total thyroidectomy". Table 4 shows the qualitative results for the decision "to wait or start with TKIs", and Table 5 shows several quotes fitted to the corresponding themes for the decision "to wait or start with TKIs". The identified domains were as follows.

Table 1. Characteristics of participating patients and physicians.

Characteristics of Patients (n = 13)	Number n(%)
Sex:	
Male	7 (54)
Female	6 (46)
Age, mean (range), y	57 (31–84)
Caucasian	13 (100)
Married/living together	13 (100)
Educational level:	
High school or less	2 (16)
Vocational education	11 (84)
University	0 (0)
Treatment:	
Surgery	6 (46)
Thyroid lobectomy	3 (50)
Total thyroidectomy	3 (50)
Average time from surgery (mean), m	10.5
Complication rate	1 (17)

Table 1. *Cont.*

Characteristics of Patients (n = 13)	Number n(%)
TKIs	7 (54)
Not started	4 (57)
Started	3 (43)
Average time from progressive advanced disease (mean), m	6.9
Site of distant metastases	
* Lung	6 (60)
* Bone	3 (30)
* Other	1 (10)
Characteristics of physicians (n = 19)	Number n(%)
Sex:	
Male	7 (37)
Female	12 (63)
Working in university hospital	15 (100
Medical specialty:	
Endocrinologist	6 (32)
Oncologist	5 (26)
Surgeon	5 (26)
Nuclear medicine physician	3 (16)
Experience in years	
0–10	1 (5)
11–20	11 (58)
>20	7 (37)

y: years, m: months; * site of distant metastases

Table 2. Qualitative results for the surgery decision regarding "thyroid lobectomy or total thyroidectomy". Expressed needs, preferences, and values of patients and of physicians treating patients with DTC.

Information Needs for Surgery Decision Making			
Domain	**Theme**		**Description of Corresponding Items**
	Patients	**Physicians**	
1. Involvement in decisions and respect for preferences	Personalized care regarding patient values • Deliberation • Phone number of the oncology nurse for support Doctor–patient relationship Time to accept the diagnosis	Personalized care regarding patient values • Deliberation Doctor–patient relationship Time to accept the diagnosis	Patients and physicians: Physicians listen to the patient, take the patient seriously, and incorporate the patient's wishes into the treatment plan. In addition, patients should be enabled through enough time and attention given to the patient. Patients: Patients need a doctor who takes care and is available most of the time. Integrity and mutual respect are necessary for a good doctor–patient relation. Physicians: Physicians should be available to answer questions. Integrity and mutual respect are necessary for a good doctor–patient relationship. Patients and physicians: After hearing the diagnosis, most patients need time to accept the diagnosis and deal with the consequences.
2. Coordination and integration of care	Clarity about healthcare process • Follow-up • Way of informing [1] Multidisciplinary team Patient support groups • Fellow patients	Clarity about healthcare process • Follow-up • Way of informing [1] Multidisciplinary team	Patients and physicians: The healthcare path should make clear what to expect. This means all steps in follow-up should be made clear. Patients want to receive information about the healthcare process on paper or digitally. Patients and physicians: Patients need to know a person from the multidisciplinary team who can answer questions and address health-related issues. The contact-person should be regularly available. A tumor board, where physicians can discuss difficult cases with other healthcare providers, is important. Patients: When confronted with illness, patients seek professional help and advice from their doctors, and also rely on support from family members, peers, and fellow patients.

Table 2. *Cont.*

Information Needs for Surgery Decision Making			
Domain	**Theme**		**Description of Corresponding Items**
	Patients	**Physicians**	
3. Clear information and communication	Information about diagnosis • Thyroid • Thyroid cancer • (Lymph node) metastases • Tumor location • Tumor size • Tumor growth • Thyroid cancer type • Clear, neutral, and stepwise way • Diagnosis being told by physician Information about prognosis • Nothing about prognosis • Survival • Treatment opportunity • No difference in treatment outcome Information about treatment options • Thyroid lobectomy • Total thyroidectomy • Radioactive iodine • Active surveillance • SDM with physician • Format of explanation [2] Information about risks and complications • Surgery - General - Anesthesia - Second surgery • Complications - Parathyroid gland - Vocal cord - Infection • Risk of wait and see • Psychological pressure after decision • Consequence of surgery • Recurrence Information about medication • Thyroid hormone substitution • Additional supplements • Pregnancy • Need for thyroid hormone substitution treatment • Medication adjustment • Side effects • Quality of life Information about recovery after treatment Information about recovery after treatment • Possible (negative) effects associated with each treatment - Changes in daily life - Weight loss/gain - Recurrence - Psychological aspects - Low calcium level - Fluctuations in thyroid function • Duration of recovery	Information about diagnosis • Thyroid cancer • Thyroid cancer type • Clear, neutral and stepwise way • Diagnosis being told by physician Information about prognosis • Survival • Treatment opportunity • No difference in treatment outcome Information about treatment options • Radioactive iodine • Active surveillance • SDM with physician Information about risks and complications • Surgery - Second surgery • Complications - Parathyroid gland - Vocal cord - Tracheostoma • Psychological pressure after decision • Radioactive iodine Information about medication • Medication adjustment • Quality of life Information about recovery after treatment • Duration of recovery	Patients: Patients need clear, honest, and complete information about the diagnosis. Physicians need to tell every aspect of the diagnosis. Information on the internet should be of good quality. Physicians: Patients need short and general information about the diagnosis. If not, there are concerns about the amount of information that will be forgotten. Patients and physicians: Information about the prognosis needs to be honest. To talk about treatment opportunities and outcomes is important. Especially when there is no difference in treatment outcomes. Patients: Clear and detailed information about different treatment options is important in SDM. With clear information, patients can deliberate which option fits them best. Physicians: To talk about all treatment options is important in SDM. With clear but short information, patients are able to deliberate which option fits them best. Patients and physicians: Information about the risks and complications during and after treatment is important in order to make a considered decision. The amount and consequences of complications in daily life are essential in SDM. Patients: Before patients can make a decision, it is important to have clear and extensive information about the medication. What are risks and benefits of this medication. In addition, the impact on quality of life is an important part of information about medication. Physicians: Before patients can make a decision, it is important to have complete information about the medication. Especially about the difficulties of medication adjustment. In addition, the impact of quality of life is an important part of information about medication. Patients: The possible effects after treatment are important. Patients specifically want to know what changes will take place in daily life. Physicians: Physicians mention that information about recovery is most important to patients.
4. Emotional support	Personalized psychological support for emotional problems • Reassurance • Multidisciplinary consultation		Patients: To offer psychological care to every patient is important. There should be the option to involve family and friends, for example the option to bring relatives to hospital appointments. Reassure patients through clear communication and the possibility of discussing with other healthcare providers.

Table 2. *Cont.*

Information Needs for Surgery Decision Making			
Domain	**Theme**		**Description of Corresponding Items**
	Patients	**Physicians**	
5. Values	Regarding functioning in daily life • Work • Sport • Holiday • Future • Family • Quality of life • Pregnancy Regarding behavior • Self-determination • Medical decision by physician • Fear - Side-effects • Attitude • Had cancer before Medical values • Information • Decision • enetics • Medication • Quick treatment • Recovery • Conservative surgery • Extensive surgery	Regarding functioning in daily life • Quality of life Regarding behavior • Fear - Recurrence - Voice • Had cancer before Medical values • Information • Medication • Quick treatment • Conservative surgery	Patients and physicians: Values are about "what matters to me". Values are an important part of health care decisions. Strengthening and clarifying patients' values and preferences in the consultation is important. Values deliberation is a core step in the consultation, where the values of physicians and patients come together to reach a treatment decision

SDM—shared decision making; [1] To inform patients by paper or digitally/e-mail; [2] Treatment options have to be told in a detailed way.

Table 3. Quotes of patients and physicians corresponding to some of the themes for the surgery decision regarding "thyroid lobectomy or total thyroidectomy".

Domain	Theme	Quotes Patients	Quotes Physicians
1. Involvement in decisions and respect for preferences	Time for processing the diagnosis	"It is important that you have time for processing the diagnosis and that you can think about the treatment options."	"The diagnosis of thyroid cancer can cause anxiety and uncertainty about the future. Therefore, it is important to give patients time to process the diagnosis and make the right decision."
2. Coordination and integration of care	Multidisciplinary team	"An important part of the decision making was the involvement of a tumor board.""An oncology nurse is important for practical issues."	"When we talk about a patient in our tumor board, it is possible to discuss the different treatment options and to decide if a patient is suitable for shared decision making.""We have an oncology nurse who takes excellent care of our patients. Patients feel reassured by having a contact person."
3. Clear information and communication	Information about prognosisInformation about medication	"I only remembered that it was treatable.""It took a year to adjust on thyroid medication, it is necessary to receive information about this process in advance."	"I think it is important to tell patients about their type of cancer and that there are excellent treatment options with a very good prognosis.""It is important to say something about the adjustment of thyroid medication and the possibility of reducing quality of life."
4. Emotional support	Personalized psychological support for emotional problems	"I wavered for a very long time, I was afraid to make the wrong treatment decision. When I discussed this with my physician, she reassured me and helped me with my decision."	-

Table 4. Qualitative results for the advanced disease decision regarding "wait or start with TKIs". Expressed information on the needs, preferences, and values of patients with and of physicians treating patients with DTC.

Information Needs for TKI Decision Making			
Domain	**Theme**		**Description of Corresponding Items**
	Patients	**Physicians**	
1. Involvement in decisions and respect for preferences	Personalized care regarding patient values • Deliberation • Phone number of the oncology nurse for support Doctor–patient relationship Time to accept the diagnosis	Personalized care regarding patient values • Deliberation • Phone number of the oncology nurse for support Doctor–patient relationship	Patients and physicians: Healthcare providers listen to the patient, take the patient seriously, and incorporate the patient's wishes into the treatment plan. In addition, patients should be enabled so that there is enough time and attention for the patient. Patients: Patients need a doctor who takes care and is available most of the time. Integrity and mutual respect are necessary for a good doctor–patient relationship. Physicians: Physicians should be available to answer questions from patients. Integrity and mutual respect are necessary for a good doctor–patient relationship. Patients: After hearing the diagnosis, most patients need time to accept the diagnosis and deal with the consequences.
2. Coordination and integration of care	Clarity about healthcare process • Follow-up • Way of informing[1] Multidisciplinary team Patient support groups • Fellow patients	Clarity about healthcare process • Follow-up • Way of informing [1] Multidisciplinary team	Patients and physicians: The healthcare path should make clear what to expect. This means all steps in follow-up should be made clear. Patients want to receive information about the healthcare process on paper or digitally. Patients and physicians: Patients need to know a person from the multidisciplinary team to answer questions and address health-related issues. The contact-person should be regularly available. In addition, involving oncology nurses might result in saving time during the consult with the doctor, and their involvement is described as a more personal contact. A tumor board, where physicians can discuss difficult cases with other healthcare providers. Patients: When confronted with illness, patients seek professional help and advice from their doctors, and also rely on support from family members, peers and fellow patients.

Table 4. *Cont.*

Information Needs for TKI Decision Making			
Domain	**Theme**		**Description of Corresponding Items**
	Patients	**Physicians**	
3. Clear information and communication	Information about diagnosis • Thyroid cancer • (Lymph node) metastases • Tumor growth Information about prognosis • Palliation Information about treatment options • Active surveillance • SDM with physician • TKIs • Information by physician Information about medication • Side effects • Quality of life • Dosage • Information by physician • When to provide information • When to start with medication • TKIs • Sources of information [3] Information about recovery after treatment • Possible (negative) effects associated with each treatment - Changes in daily life	Information about diagnosis • Tumor growth Information about prognosis • Beneft of treatment • Palliation • Communication of risk information [2] Information about treatment options • Active surveillance • SDM with physician • Possibility to stop with TKIs • There is no wrong decision Information about medication • Side effects • Quality of life • Dosage • Information by physician • When to provide information • When to start with medication • TKIs • Sources of information [3]	Patients: Patients need clear, honest, and complete information about the diagnosis. Physicians need to discuss every aspect of the diagnosis. Information on the internet should be of a good quality. Physicians: Patients need short and general information about the diagnosis. If such information is not given, physicians raise concerns about the amount of information that will be forgotten. Patients: Information about the prognosis needs to be honest. After all, it is a palliative treatment. Talking about treatment opportunities and outcomes is important. Physicians: Information about the prognosis needs to be honest. Physicians want to focus on the benefits of treatment, but after all, it is a palliatve treatment. To talk about treatment opprtunities and outcomes is important. Patients: Clear and detailed information about different treatment options is important in SDM. With clear information patients can deliberate which option fits best. Physicians: To talk about all treatment options is important in SDM. With clear, but short information, patients are able to deliberate which option fits best. Patients: Before patients are able to decide, it is important to have clear and extensive information about the medication. What are the risks and benefits of this medication. In addition, the impact on quality of life is an important part of the information about medication. Physicians: Before patients are able to decide, it is important to have complete information about the medication. Especially about the side effects. In addition, the impact on quality of life is an important part of information about medication. Patients: The possible effects after treatment are important. Patients especially want to know what changes will occur in daily life.
4. Emotional support, empathy and respect	Personalized psychological support for emotional problems • Reassurance • Multidisciplinary consultation • Family	Personalized psychological support for emotional problems • Reassurance	Patients: To offer psychological care to every patient is important. There should be the option to involve family and friends, for example the option to bring relatives to hospital appointments. Reassure patients by clear communication and the possibility of discussing with other healthcare providers. Physicians: Reassure patients by clear communication and the possibility of discussing with other healthcare providers.

Table 4. *Cont.*

Information Needs for TKI Decision Making			
Domain	**Theme**		**Description of Corresponding Items**
	Patients	**Physicians**	
5. Values	Regarding functioning in daily life • Work • Sport • Holiday • Future • Family • Quality of life • Autonomy Regarding behavior • Self-determination • Deferral of medical decision to physician • Fear - Side-effects - Symptoms - Dying • Done everything Medical values • Information • Decision	Regarding functioning in daily life • Work • Holiday • Family Regarding behavior • Fear - Dying • Done everything	Patients and physicians: Values are about "what matters to me". Values are an important part of health care decisions. Strengthening and clarifying patients' values and preferences in the consultation is important. Values deliberation is a core step in the consultation, where values of physicians and patients come together to reach a treatment decision.

SDM—shared decision making; [1] To inform patients by paper or digitally/e-mail; [2] Visually inform patients about survival and recurrence; [3] Different sources of information were the pharmacy, package leaflet of the medicine, and the internet.

Table 5. Quotes of patients and physicians corresponding to some of the themes for the advanced disease decision regarding "wait or start with TKIs".

Domain	Theme	Quotes Patients	Quotes Physicians
1. Involvement in decisions and respect for preferences	Time for processing the diagnosis	"The fact that you switch from an endocrinologist to an oncologist with a waiting room full of patients with cancer was a real change for me."	
2. Coordination and integration of care	Multidisciplinary team	"A case manager can help, advise, and reassure when there are problems."	"We discuss all patients in our tumor board and decide if whether they are a candidate for treatment with TKIs."
3. Clear information and communication	Information about prognosis Information about medication	"You know that it is not a curative treatment and that there will be a moment when the medication is not longer working or that you have to stop because of the side effects." "Information about the side effects was clear and detailed." "What are the side effects and what can you expect?"	"It is important to inform patients about the possible benefit, but also that it is not a curative treatment." "I especially talk about the side effects and quality of life."
4. Emotional support, empathy and respect	Personalized psychological support for emotional problems	"Family means everything, my granddaughter is always aware that I cannot do everything. They give so much love."	"Patients fear death, so your role as a physician who supports and reassures is important."
5. Values	Regarding functioning in daily life	"Quality of life is the most important value in many different aspects of life. Maintaining quality of life is important for being able to participate in sport and work, but especially for experiencing family events."	"What I think is important, some patients are still working and that is also quality of life. Patients just want to keep doing social things, work, to go on a holiday."

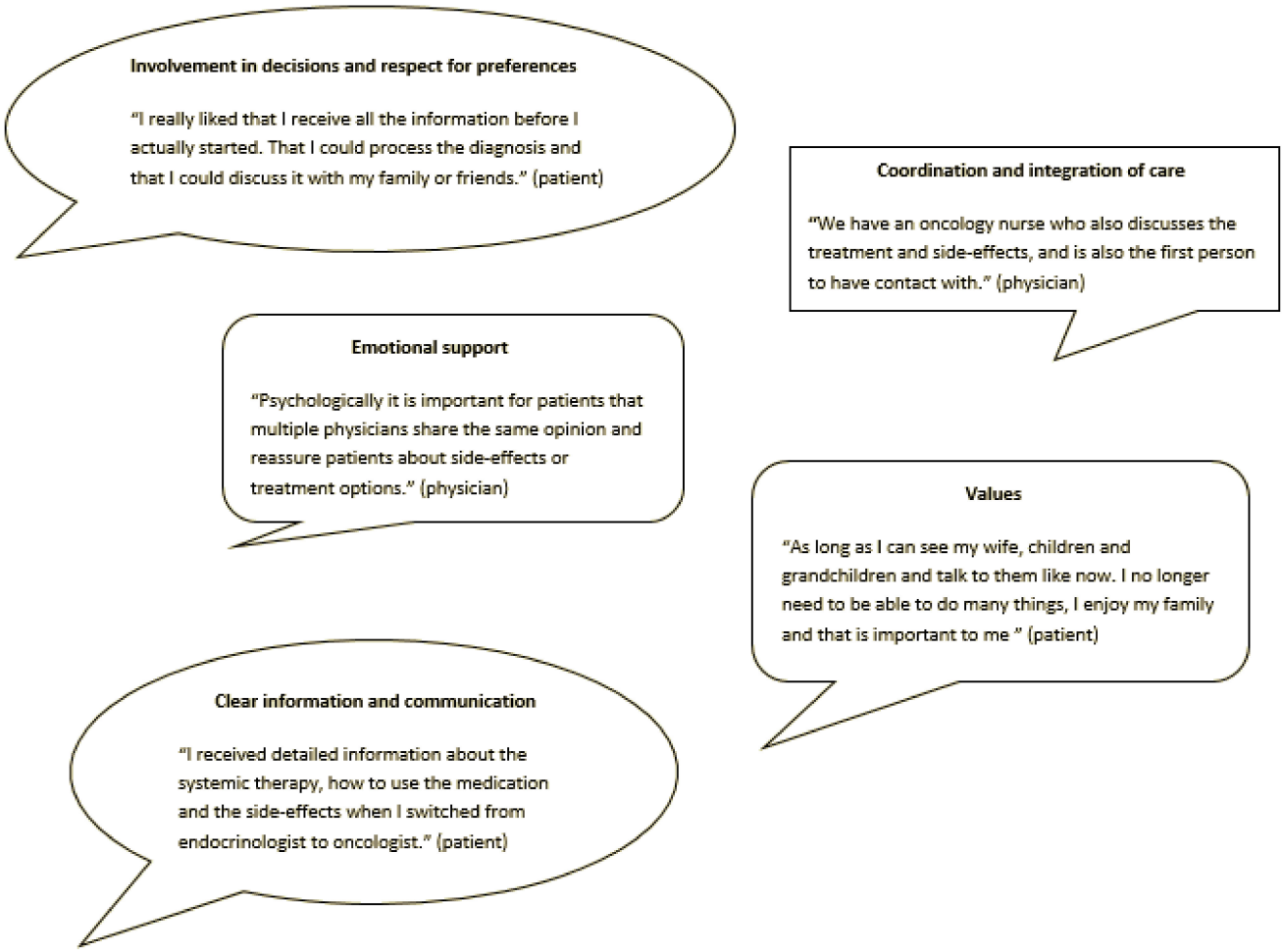

Figure 1. Quotes expressed by patients with DTC and by physicians treating patients with DTC for each Picker domain.

3.1. Involvement in Decisions and Respect for Preferences

The importance of a good doctor–patient relationship was most often mentioned by patients with advanced RAI refractory DTC. Patients indicated the importance of discussing the treatment steps with family and friends, and listening to patients' needs and preferences. They indicated needing a doctor who takes care and is available most of the time. Integrity and mutual respect were necessary for a good doctor–patient relationship. For patients treated with surgery, it was important to be involved in their treatment process and to discuss the options with their physician. The patients in both treatment decision groups needed time to cope with the diagnosis and its consequences. Physicians in both treatment decision groups mentioned that patient involvement in the treatment process was important. The difference between patients and physicians in this domain was the importance of a good doctor–patient relationship. This theme was rarely mentioned by physicians.

3.2. Coordination and Integration of Care

Themes mentioned by patients and physicians for both treatment decisions were the involvement of a multidisciplinary team and information process. It was important to have a contact person for questions and problems. Patients and physicians for both decisions mentioned that they considered an oncology nurse as the most suitable multidisciplinary team member. A tumor board should be available to discuss difficult cases with other healthcare providers. For both patients and physicians, paper and digital ways of informing

were considered equally important, and adequate ways to inform patients. In this domain there were no differences between patients and physicians.

3.3. Clear Information and Communication

Patients in both decision groups needed clear, honest, and complete information. They also wanted to receive detailed information. The two important themes for patients in the surgery decision group were general information about DTC and treatment options. For patients in the TKI decision group, general information about advanced RAI refractory DTC and medication was important. In both groups, patients were not at all or only slightly satisfied with the amount of information received. They wanted to receive more information. Patients in both groups felt they were not involved in decision making, "there was nothing to choose". Important themes for physicians discussing the surgery decision also involved general information about DTC and treatment options. Physicians mentioned most often "giving information about the prognosis", especially if patients were considered to benefit from treatment with TKIs. The main difference in this domain was the detailed information patients wanted to receive, whereas physicians wanted to give more general and short information. In the TKI treatment decision group, physicians wanted to give information about the benefit of therapy, whereas patients were more focused on palliative care.

3.4. Emotional Support, Empathy and Respect

Patients mentioned the importance of offering psychological care. In both decision groups, emotional support and the involvement of family was important, as well as reassurance by health care providers. In both groups, patients felt reassured by their physician, they also felt that there was sufficient psychological support. Only physicians in the TKI decision group mentioned reassurance by health care providers. The vast majority of involved physicians did not mention emotional support or psychological care, which was the main difference between patients and physicians in this domain. Although few physicians did not mention it often, they apparently succeeded in reassuring the patients.

3.5. Values

Values were an important part of health care decisions, "what matters to me". For patients who started or considered TKIs, the values mentioned most often involved functioning in daily life, for example, maintaining QOL and family milestones. In the surgery decision group, values regarding medical outcomes involved recovery and physical changes after treatment. In addition, the value "I need surgery, there is something in my body that does not belong there" was often mentioned. Physicians in the TKI decision group believed that values about daily life were important. Physicians in the surgery decision group mentioned medical values most often. The main difference was that patients wanted to discuss values more.

4. Discussion

This qualitative study investigated the perspectives of patients with low-risk (>1 cm) DTC and advanced tumors, regarding the extent of surgery or starting TKIs. We studied the differences between physician and patient perspectives, as well as the differences and similarities between the aspects involved in these two decisions. The main themes emerging from both patient and physician interviews were the involvement of a multidisciplinary team and a way of being informed, general information on DTC and treatment options, reassurance by health care providers, medical values, and values regarding functioning in daily life. The main differences in perspective between patients and physicians were that only patients mentioned the importance of a good doctor–patient relationship, patients desired detailed information about their diagnosis, patients focused on palliative treatment whereas physicians focused on the benefit of therapy, and only patients mentioned the need for psychological care and emotional support. These differences provide valuable

information on the perspective of patients with DTC and physicians that can be used when designing decision aid instruments. These differences are elaborated below.

Regarding the doctor–patient relationship, patients in both treatment decision groups appreciated a good doctor–patient relationship, whereas physicians did not mention this often. Pitt et al. also showed that patients with low-risk DTC (>1 cm) wished for a strong patient–surgeon relationship, in line with the findings in patients with other malignancies [24]. This was particularly important for patients with DTC, who remained in follow-up and required medical guidance and support for many years, even after remission.

Regarding the desire to receive detailed information, patients from both decision groups wanted to receive more detailed information about DTC, treatment options, and aftercare, whereas physicians from both decisions groups preferred providing more general and short information. We hypothesize that this difference arises from the fact that physicians are limited with respect to the available consultation time and have concerns about how much of the information is remembered. However, ensuring that the information is individualized and patients are involved in the treatment process was a strong preference of our participants. This preference is corroborated by studies focusing on needs in DTC survivors after primary treatment. Such studies show that survivors usually receive information related to their diagnosis, prognosis, and primary treatment; however, information on long-term effects, recurrence, and aftercare is scarce [25–28,30]. Likewise, a systematic review of Hyun et al. showed that cancer survivors in general perceive many unmet needs, and these needs extend to aftercare [29]. Previous studies among other cancer types also showed the importance of providing patients with disease specific information about cure, spread of disease, and side effects. There are some possible explanations for this need for information: (1) it can increase trust in the caring physician and reduce possible feelings of uncertainty and doubt, and (2) it might be used as a coping strategy to gain control and to understand what is happening to body and mind [36–39].

Regarding the difference in the TKI decision group, physicians focused more on the effect and benefit of TKIs, whereas patients were focused more on palliative care. Physicians may focus more on the effects and benefits of TKIs because they prescribe TKIs when they believe there is a positive effect and benefit of therapy. We hypothesize that the physicians' knowledge of the progression free survival (PFS) benefit shown in patients treated with TKIs in the randomized controlled trials plays an important role in this result. This could particularly be the case when patients may not be sufficiently aware of these results [40,41]. Another explanation for the differences in focus is that patients' awareness of having an incurable disease might generate more thoughts regarding their quality of life and the availability of palliative care. No other studies focused specifically on the needs and preferences in patients with advanced metastatic DTC. Because DTC is generally a slowly progressive cancer type, patients with metastatic disease often have long-time survival while maintaining a good QOL. Therefore, a comparison with other patients with disseminated malignant tumors could not be used to corroborate our findings, which highlights the relevance of the present DTC-specific findings.

Our results indicate the importance of talking with patients about their values: values regarding treatment and decision, and values about daily life were mentioned by both patients and physicians. Identifying such values is relevant because, in practice, values are voiced or discussed in a minority of consultations [11], and to talk about values is the most difficult part of doctor–patient communication. Other studies have showed the importance of talking about values, e.g., the systematic review of Hyun et al. found that psychosocial information and supportive care needs may be insufficiently met in DTC survivors [29]. The emotional reaction "I need surgery, there is something in my body that does not belong there" was also found by Pitt et al. [39]. In other cancers, the importance of the involvement of family and friends was also found [36–38].

5. Strengths and Limitations

A strength of our study is that, on the one hand, it contrasts differences and similarities between two treatment decision-making groups (regarding low-risk and high-risk patients), and on the other hand, it contrasts differences between the perspectives of physicians and patients for both decisions. Our study has some limitations. Because of logistic difficulties, only one focus group interview per treatment decision could be organized. Therefore, our results may not give an understanding of all of the issues involved [42]. Furthermore, physicians and patients with DTC in other cultural or geographical settings may have needs that we did not identify, which could impact generalizability. In general, the results of a qualitative study cannot be generalized, although the results can be of major importance for the specialists in the field. This underscores the need for similar cross-cultural validation studies in other countries.

6. Conclusions

In conclusion, this study illustrates the needs, preferences, and values in patients with DTC in two different treatment decision groups in both low-risk and high-risk patients. While many of these are recognized and are overlapping with those of the physicians treating patients with DTC, some are clearly different and potentially not sufficiently addressed in daily practice. Communication may be improved by (1) meeting patients' needs with respect to stage-specific information provision about the disease and its consequences, (2) raising awareness among the physicians to inquire about and address patients' needs with respect to emotional and psychological support, and (3) addressing patients' concerns about palliative care. This may help physicians to improve their communication and better meet patient needs. The results of this study can also be used to develop decision support tools to make current doctor–patient communication more SDM-based.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the Medical Ethical Committee (CMO) of the region Arnhem–Nijmegen (protocol code MEC-2018-4521, 14-01-2020).

Informed Consent Statement: Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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Appendix A

Table A1. Interview guide.

Initial Questions			
Topic		**Patients**	**Physicians**
Introduction		Explaining the interview	Explaining the interview
Information	Diagnosis Surgery TKIs	What is your diagnosis and what kind of treatment did you receive? What did the physician tell you about the diagnosis? What did the physician tell you about the surgery? OR What did the physician tell you about the TKIs?	- What do you tell patients about the diagnosis? What do you tell patients about the surgery? OR What do you tell patients about TKIs?
Evaluation	Importance Sufficient	What information provided by the physician was most the important to make a treatment decision? What aspects of the information provision did you like or dislike?	What information do you think is most important for patients? To what extent are patients informed after visiting the physician?
Experience	Decision-making capability	How should this information be presented? Would you like to be involved in decision making?	How should this information be presented? How do you feel about involving patients in decision-making during the consultation?
Values		What are important values?	What are important values for patients?
Conclusion		Do you have any additional remarks?	Are there any additional remarks?

Appendix B

Table A2. Picker dimensions.

Picker Dimensions
1. Involvement in decisions and respect for preferences
2. Effective treatment delivered by trusted professionals
3. Continuity of care and smooth transitions
4. Involvement and support for family and carers
5. Clear information, communication and support for self-care
6. Fast access to reliable healthcare advice
7. Emotional support, empathy, and respect
8. Attention to physical and environmental needs

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Review

Predictors of Response and Survival to Multikinase Inhibitors in Radioiodine Resistant Differentiated Thyroid Cancer

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Abstract: Sorafenib and lenvatinib are the only multikinase inhibitors (MKIs) approved for the treatment of radioactive iodine refractory differentiated thyroid cancer (RR-DTC). Although they have been demonstrated to improve progression free survival and overall response rate, the risk of toxicities is very high, worsening patients' quality of life. Therefore, predicting MKI treatment outcomes in the setting of RR-DTC is very challenging for optimizing patients' management. The current review provides an overview of the predictive factors for the response and survival of sorafenib and lenvatinib in RR-DTC. In this setting, a systemic therapy should be considered after conducting a multidisciplinary discussion aimed at evaluating the risk-benefit ratio of the treatment and taking into account several clinical, biochemical, and molecular factors. Age, performance status, and cancer-related symptoms are the most important clinical markers to be considered prior to starting MKI treatment, together with tumor burden. Some tissue and circulating biomarkers have been investigated, those involved in the angiogenic pathways being the most promising. Finally, prospective clinical trials aimed at evaluating predictive markers for therapeutic response are needed for tailoring patient management and allowing more appropriate treatment choices.

Keywords: multikinase inhibitors; sorafenib; lenvatinib; differentiated thyroid cancer; radioiodine resistance; predictive marker; predictors; response to treatment; survival

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1. Introduction

The differentiated thyroid cancer (DTC) represents the most common type (>90%) of tumor originating from the follicular epithelium, including the papillary and the follicular histotypes [1]. The majority of DTC can be successfully treated by thyroidectomy, radioactive iodine (RAI) therapy, and thyroid stimulating hormone (TSH)-suppressive therapy, with L-thyroxine showing favorable prognosis [2]. Only less than 5% of all cases, but 60–70% of metastatic DTC, lose the ability to uptake and concentrate RAI and to produce tireoglobulin (Tg), becoming RAI refractory (RR)-DTC [3]. RR-DTC has a poor prognosis, with a 10-year survival rate less than 20% and a mean life expectancy of 3–5 years [4]. The definition of RR-DTC is still debated, but current guidelines include four categories: (1) the absence of RAI uptake in all lesions on scintigraphy; (2) the absence of RAI uptake in some but not all lesions; (3) disease progression despite RAI uptake; and (4) reaching the maximum recommended activity of RAI [1].

Recently, the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) approved two multikinase inhibitors (MKIs) for the treatment of RR-DTC, namely sorafenib and lenvantinib.

Sorafenib is an oral kinase inhibitor of vascular endothelial growth factor receptor (VEGFR)-1, -2, and -3, rearranged during transfection receptor protein (RET) -including

RET/PTC-, rapidly accelerated fibrosarcoma kinase (RAF) -including BRAF V600E-, and platelet-derived growth factor receptor (PDGFR) beta (Figure 1).

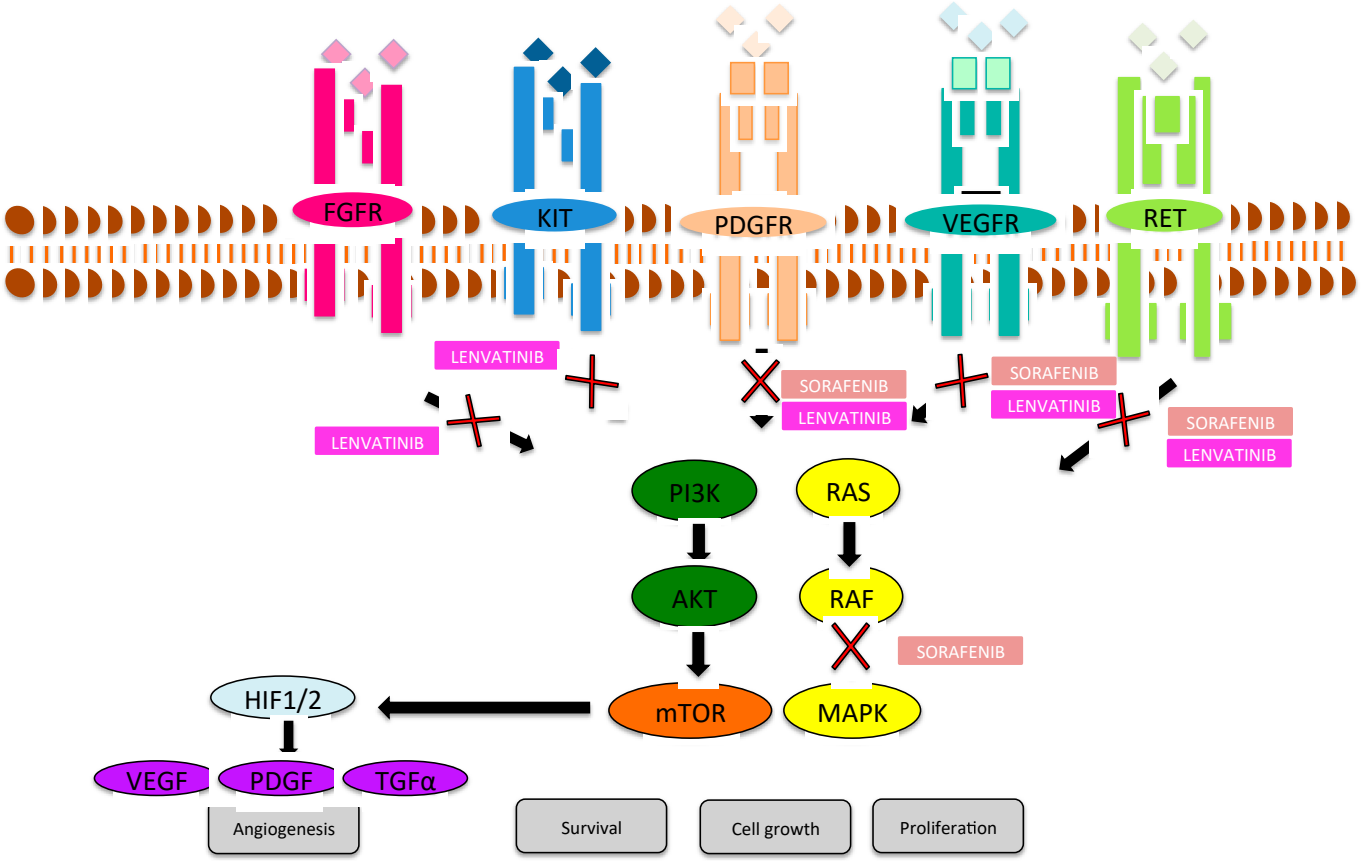

Figure 1. Molecular pathways of multikinase inhibitors (MKIs) in radioactive iodine refractory differentiated thyroid cancer. The MKIs (sorafenib, lenvatinib) block signaling from the tyrosine kinase receptors, preventing cell survival, growth, proliferation and angiogenesis. Abbreviations: AKT, protein kinase B; FGFR, fibroblast growth factor receptor; HIF, hypoxia-inducible factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase; RAF, rapidly accelerated fibrosarcoma kinase; RAS, rat sarcoma protein; RET, rearranged during transfection receptor protein; TGF, tumor growth factor; VEGFR, vascular endothelial growth factor receptor. The signaling pathways inhibited by the indicated drugs are represented by crossed-out arrows.

For this reason, given the effectiveness of sorafenib on the RAF-1 serine/threonine kinase, in preclinical and clinical models sorafenib was experimented in inhibiting the growth of anaplastic thyroid cancer (ATC) [5,6]. Lenvatinib is an oral inhibitor of VEGFR-1, -2, and -3, fibroblast growth factor receptor (FGFR) 1 through 4, PDGFR alfa, RET, and KIT (Figure 1). Lenvatinib was also administered for ATC [7], given the multitarget effect oriented on molecular basis [8]. Sorafenib and lenvatinib showed significant improvements in progression free survival (PFS) and overall response rate (ORR) in patients with progressive RR-DTC (compared to placebo) in the DECISION and the SELECT trials, respectively [9,10]. Although they provide new therapeutic strategies against RR-DTC, unfortunately the high risk of toxicities could impair patients' quality of life [9,10].

Therefore, the clinical management of RR-DTC is challenging and the choice of whether and when to start a target therapy should be performed in a multidisciplinary setting by an expert panel. In this context there is a growing need to understand how to predict MKI treatment response to better define which patient could benefit more from this kind of therapy.

To address this issue, we performed a review assessing the predictors of MKI clinical benefit in the setting of progressive RR-DTC, aiming at personalizing patients' management.

2. Materials and Methods

We performed a keyword based PUBMED search, using relevant keywords [(predictive OR marker OR biomarker) AND (sorafenib OR lenvantinib) AND (differentiated thyroid cancer)]. The search was last updated on April 2021, and only English language studies were considered. Titles and abstracts have been screened for articles selection, identifying only those that dealt with potentially relevant factors predicting treatment outcome with sorafenib or lenvatinib in progressive RR-DTC. The selected abstracts were further assessed for a full-text evaluation. Finally, 21 papers (7 sorafenib; 14 lenvatinib) were included in the review. Predictive factors have been divided in those predicting radiological response (RECIST criteria) and those predicting survival response (prognosis).

3. Sorafenib

Sorafenib is a MKI previously approved for the treatment of renal cell carcinoma (2005) and hepatocellular carcinoma (2007). It has been approved by FDA in 2013 and by EMA in 2014 for the treatment of locally recurrent or metastatic, progressive DTC.

Sorafenib inhibits multiple intracellular (c-CRAF, BRAF and mutant BRAF) and cell surface kinases (KIT, FLT- 3, RET, RET/PTC, VEGFR-1, VEGFR- 2, VEGFR- 3, and PDGFR-ß) that are involved in tumor cell signaling, angiogenesis, and apoptosis (https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/021923s020lbl.pdf, accessed on 1 April 2021).

Its effectiveness and tolerability have been evaluated in a phase 3 double-blind randomized trial (DECISION NCT00984282), enrolling 417 patients with progressive RR-DTC [9]. The trial demonstrated an improvement in PFS (10.8 months vs. 5.8 months for sorafenib and placebo arms, respectively) and ORR (12% vs. 1% for the sorafenib and placebo arms, respectively), whereas there were no significant differences in the overall survival (OS) between sorafenib and placebo arms. More than 60% of patients receiving sorafenib presented adverse events (AEs) responsible of drug withdrawal or dose reduction. The most frequent reported AEs (on. The most frequent reported AE dodiarrhea, alopecia, weight loss, hypertension, rash, decreased appetite, stomatitis, nausea, pruritus, and abdominal pain. Other significant AEs included squamous cell carcinoma of the skin and hypocalcemia [9].

According to data sheet, the recommended dose and schedule is 400 mg (two 200 mg tablets) taken twice daily without food. (https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/021923s020lbl.pdf, accessed on 1 April 2021).

3.1. Predictive Markers of Radiological Response

Four studies [11–14] investigated the role of potential predictive factors of radiological response to sorafenib (Table 1).

Table 1. Predictive markers of radiological response to sorafenib and lenvatinib in progressive RAI-refractory DTC. * (cycle 2), § (cycle 3), # (cycle 4-5-6), • mean ± standard deviation. DCR, disease control rate; DOR, duration of overall response; MTS, maximum tumor shrinkage; OR, objective response rate; R, responders; NR, non-responders.

First Author, Year (Ref)	Study Design (Trial Name)	N° of Patients	Median Follow-Up (Months)	Biomarker	Type of Marker	Statistical Analysis	Significance	Endpoint
				Sorafenib				
Marotta V, 2013 [12]	Retrospective, longitudinal study	17	15.5	(1) Baseline Tg (2) Tg response (3) Baseline average SUV max (PET-FDG)	(1) Circulating (2) Circulating (3) Functional imaging	ANOVA to compare R and NR	(1) $p < 0.001$ (2) $p < 0.01$ (3) $p = 0.001$	Radiological response
Yarchoan M, 2016 [14]	Phase 2 study (NCT00654238)	40	NA	nuclear pAKT	Tissue	ANOVA to compare R and NR	$p < 0.01$	Radiological response
Kim M, 2017 [11]	Retrospective multicenter cohort study	98	12.3	Tg decrease $\geq$ 60%	Circulating	Subgroup analyses and Cox proportional hazard model	$p = 0.044$	Disease control duration
Marotta V, 2017 [13]	Single center study	17	17	(1) VEGFA SNPs: AA/CC genotype (2) VEGFR-2 SNPs: AA + AT genotype	Genetic	Chi-square test and Odds Ratio to compare the rate of PR between groups	(1) $p = 0.022$ (2) $p = 0.036$	Radiological response
				Lenvatinib				
Cabanillas M.E, 2015 [15]	Open-label, single-arm, phase 2 trial (NCT00784303)	58	14	(1) Tg decrease (2) Baseline Ang2 (3) Baseline IL-10 (4) Baseline fms-related tyrosine kinase 3 ligand	Circulating	Wilcoxon signed-rank test for paired samples	(1) $p = 0.028$ *, $p = 0.002$ §, $p = <0.001$ # (2) $p = 0.034$ (3) $p = 0.032$ (4) $p = 0.041$	(1) MTS (2) ORR (3) ORR (4) ORR

Table 1. *Cont.*

First Author, Year (Ref)	Study Design (Trial Name)	N° of Patients	Median Follow-Up (Months)	Biomarker	Type of Marker	Statistical Analysis	Significance	Endpoint
Robinson B, 2016 [16]	Exploratory analysis from SELECT trial (NCT0132155)	261	17.1	(1) Basal body weight (2) Baseline ECOG status (3) Baseline tumor size	(1) Clinical (2) Clinical (3) Radiological	Multivariate Cox regression model	(1) $p = 0.035$ (2) $p = 0.007$ (3) $p < 0.001$	Radiological response
Tahara M, 2017 [17]	Exploratory analysis from SELECT trial (NCT0132155)	261	17.1	(1) Baseline Ang2 (2) Baseline VEGF	Circulating	Cox proportional hazards model and Multivariate analysis	(1) $p < 0.0001$ (correlation for each), p interaction = 0.018 (2) $p = 0.0082$, $p = 0.0009$ (correlation)	MTS, ORR
Gianoukakis A.G, 2018 [18]	Analysis from SELECT trial (NCT0132155)	261	17.1	Disease burden	Radiological	Cox proportional hazards	NA	DOR
Lee E.K, 2019 [19]	Multicenter retrospective study (NCC2017-0162)	57	8.6 ± 7.2 •	Tumor doubling time	Radiological	Pearson Chi square test between R and NR lesions	$p = 0.02$	Radiological response

Among the circulating biomarkers, baseline Tg levels and Tg response to treatment have been widely explored. A retrospective study from Marotta and coworkers found that baseline Tg levels were significantly higher in patients who showed disease progression compared with responders. Moreover, the decrease in serum Tg levels was significantly greater in patients who achieved clinical benefit compared with non-responders [12]. The role of Tg response was confirmed in a Korean study in which patients with a longer disease control duration (in a Korean study in which patients on-responders n widely explored. A retrospective study from Marotta and c [11].

Considering the role of MAPK and AKT/PI3K pathways in the progression of DTC, Yarchoan et al. investigated the molecular tumor markers from these two pathways in a phase 2 study evaluating the effectiveness of sorafenib in the treatment of RR-DTC. The authors found that low tumor expression of nuclear phospho-AKT (pAKT) was associated with partial response (PR) to sorafenib [14].

Moreover, since sorafenib showed both anti-proliferative and anti-angiogenic effects, the possible role of germline VEGF-A and VEGFR-2 single nucleotide polymorphisms (SNPs) in predicting objective response in RR-DTC patients has been explored [13]. In the study from Marotta et al. the AA/CC genotype of the VEGF-A SNPs and the AA + AT genotype of the VEGFR-2 SNP proved statistically significant association with the achievement of PR [13].

Finally, 18F fluoro-D-glucose (18-F FDG) positron emission-tomography (PET)-computed-tomography (CT) has been suggested as a useful tool in predicting radiological response, being baseline average SUVmax significantly higher in patients who showed disease progression compared with responding subjects [12].

3.2. Predictive Markers of Survival

Several studies evaluated the role of prognostic factors for better PFS and OS after sorafenib treatment (Table 2).

Table 2. Predictive markers of survival in progressive RAI-refractory DTC treated with sorafenib or Lenvatinib. DSS, disease specific survival; OS, overall survival; PFS, progression free survival.

First Author, Year (Ref)	Study Design (Trial Name)	N° of Patients	Median Follow-Up (Months)	Biomarker	Type of Marker	Statistical Analysis	Significance	Endpoint
				Sorafenib				
Marotta V, 2013 [12]	Retrospective, longitudinal study	17	15.5	(1) Baseline Tg (2) Tg response	Circulating	Log-rank test	(1) $p = 0.04$ (2) $p = 0.01$	PFS
Kim M, 2017 [11]	Retrospective multicenter cohort study	98	12.3	(1) Absence of disease-related symptoms (2) Lung metastasis (3) Daily maintenance dose $\geq$ 600 mg (4) Tg decrease $\geq$ 60%	(1) Clinical (2) Clinical (3) Clinical (4) Circulating	Subgroup analyses and Cox proportional hazard model	(1) $p = 0.041$ (2) $p = 0.048$ (3) $p = 0.005$ (4) $p = 0.012$	PFS
Marotta V, 2017 [13]	Single center study	17	17	(1) VEGF-A SNPs: AA/CC genotype (2) VEGFR-2 SNPs: AA + AT genotype	Genetic	Log-rank test	(1) $p = 0.006$ (2) $p < 0.001$	PFS
Capdevila J, 2019 [20]	*Post-hoc analysis* From DECISION trial (NCT00984282)	125	16.2	BRAF like gene expression profile	Tissue	Multivariate Cox proportional hazard models	BRAF like *vs.* RAS like $p = 0.015$ BRAF like *vs.* noBRAL like $p = 0.015$	PFS
Kim MJ et al., 2019 [21]	Retrospective	85	19.1	Tumor doubling time	Imaging	Cox proportional hazard model	$p < 0.01$	PFS
Oh HS, 2019 [22]	Multicenter, retrospective cohort study	98	12.3	(1) No cancer-related symptoms (2) Maximal diameter of target lesion	(1) Clinical (2) Imaging	Cox proportional hazard model	(1) $p = 0.048$ (2) $p = 0.029$	OS

Table 2. *Cont.*

First Author, Year (Ref)	Study Design (Trial Name)	N° of Patients	Median Follow-Up (Months)	Biomarker	Type of Marker	Statistical Analysis	Significance	Endpoint
				Lenvatinib				
Cabanillas M.E, 2015 [15]	Open-label, single-arm, phase 2 trial (NCT00784303)	58	14	(1) Baseline Ang2 (2) Baseline EGF	Circulating	Univariate Cox proportional hazard models	(1) $p = 0.011$ (2) $p = 0.033$	PFS
Robinson B, 2016 [16]	Exploratory analysis from SELECT trial (NCT0132155)	261	17.1	(1) Basal body weight (2) Baseline ECOG status (3) Baseline tumor size	(1) Clinical (2) Clinical (3) Radiological	Multivariate Cox regression model	(1) $p = 0.04$ (2) $p = 0.03$ (3) $p = 0.03$	PFS
Tahara M, 2017 [17]	Exploratory analysis from SELECT trial (NCT0132155)	261	17.1	(1) Baseline Ang2 (2) Tie2	Circulating	Cox proportional hazards model and log-rank tests Multivariate analysis	(1) $p < 0.0001$ (correlation) p interaction = 0.018 (2) $p = 0.038$ (correlation)	PFS
Sugino K, 2018 [23]	Cohort study	29	14.7	Cancer-related symptoms	Clinical	Univariate analysis	(1) $p < 0.02$ (2) $p < 0.01$	(1) PFS (2) OS
Wirth L.J, 2018 [24]	Multicenter, double-blind SELECT trial (NCT0132155)	261	17.1	Treatment emergent hypertension	Clinical	Univariate and multivariate Cox proportional hazards models	(1) $p < 0.01$ (only univariate) (2) $p = 0.04$ (multivariate)	(1) PFS (2) OS
Tahara M, 2019 [17]	Exploratory analysis from SELECT trial (NCT0132155)	261	17.1	Dose interruption (<10% versus 10% of total treatment duration)	Clinical	Multivariate Cox regression model	$p = 0.0004$	PFS
Suzuki C, 2019 [25]	Retrospective cohort study	26	26.7	(1) Tumor related symptoms (2) Bone metastasis (3) Sum of diameters of target lesions (4) Maximum tumor diameter (5) Tumor growth slope	(1) Clinical (2) Radiological (3) Radiological (4) Radiological (5) Radiological	A stepwise Cox proportional hazards	(1) $p < 0.01$, $p = 0.05$ (2) $p < 0.01$ (3) $p = 0.02$, $p = 0.03$ 4) $p = 0.04$ 5) $p = 0.03$	PFS, OS

Table 2. *Cont.*

First Author, Year (Ref)	Study Design (Trial Name)	N° of Patients	Median Follow-Up (Months)	Biomarker	Type of Marker	Statistical Analysis	Significance	Endpoint
Song E, 2020 [26]	A Korean multicenter study	43	16	(1) Tumor growth slope before lenvatinib initiation (2) The sum of the largest diameters of target lesions (3) Tg doubling time	(1) Radiological (2) Radiological (3) Circulating	Univariate regression analysis	(1) $p = 0.003$ (2) $p = 0.043$ (3) $p = 0.024$	PFS
Fukuda N, 2020 [27]	Retrospective cohort study	33	15.4	Neutrophil-to-lymphocyte ratio (NLR)	Circulating	Fisher's exact test to compare outcomes according to the NLR values at the start of treatment	$p < 0.05$	OS
Takahashi S, 2020 [28]	All-case post-Marketing Observational Study	442 DTC	12	(1) Body weight, (2) ECOG PS score (3) Tumor diameter prior to lenvatinib administration (4) Tumor invasion to the carotid artery, jugular artery, trachea, skin, or other region	(1) Clinical (2) Clinical (3) Radiological (4) Radiological	Multivariate Cox regression analyses	NA	OS
Ahmaddy F, 2021 [29]	Retrospective cohort study	22	17	Response according to mPERCIST (1) at 3 months (2) at 6 months	Functional imaging	Log rank test	(1) $p = 0.008$, $p = 0.003$ (2) $p = 0.015$, $p = 0.001$	PFS, DSS
Taylor M., 2021 [30]	Retrospective analysis of SELECT trial	248	17.1	NLR	Circulating	Cox proportional hazard model	(1) $p < 0.001$ (2) $p = 0.029$	(1) PFS (2) OS

Clinical features have been advocated as potential predictors of survival and the absence of disease-related symptoms prior to sorafenib administration was associated with a better PFS [11] and OS [22] than symptomatic disease, suggesting the need to start the treatment before the onset of a clinically relevant disease.

Moreover, clinicians should taking into account the daily sorafenib maintenance dose, being a dose ib mainteassociated with better PFS [11].

The tumor burden was demonstrated to be an important prognostic factor, and while patients with lung metastasis alone had a better prognosis [11], the maximal diameter of target lesion was significantly associated with a minimally increased risk of death [22]. Furthermore, tumor doubling time, reflecting the tumor growth rates, was associated with a worse survival outcome in terms of PFS [21].

Considering the role of the mutational status on DTC progression, there is a great interest in finding tissue biomarkers that could correlate with outcome or predict benefit from sorafenib therapy. In a post hoc exploratory RNA-seq analysis using tumor samples from patients enrolled in the DECISION trial, the RNA-expression profiles and PFS were found to significantly correlate. In the sorafenib arm, patients harboring the BRAF-like profile had a significantly better survival than those with RAS-like and NoBRaL profiles [20].

Finally, the VEGF-A and VEGFR-2 SNPs (AA/CC genotype of the VEGF-A and the AA + AT genotype of the VEGFR-2), which have been demonstrated to correlate with tumor response, were also shown to be associated with a better PFS [13].

4. Lenvatinib

Lenvatinib is a MKI which received FDA and EMA approvals in 2015 for the treatment of locally recurrent or metastatic progressive DTC. Lenvatinib selectively inhibits the kinase activities of VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4), in addition to other proangiogenic and oncogenic pathway-related receptor tyrosine kinases, including FGFR1, 2, 3, and 4, the PDGFR alfa, KIT, and RET (https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/206947s007lbl.pdf, accessed on 1 April 2021).

Its effectiveness and tolerability have been evaluated in a phase 3 randomized, double-blind, multicenter study (SELECT trial NCT01321554), including 392 patients with progressive RR-DTC, that demonstrated significant improvement in PFS and a high ORR among patients receiving treatment with lenvatinib compared with those receiving placebo [10]. The median PFS was 18.3 months in lenvatinib group and 3.6 months in placebo group. The ORR was 64.8% for patients receiving lenvatinib and 1.5% for patients receiving placebo. The incidence of treatment-emergent AEs of all grades was higher in the lenvatinib group (97.3%) compared with the placebo group (59.5%) [10]. Hypertension was the most common treatment-emergent AEs associated with lenvatinib treatment [24]. The other common any-grade AEs in lenvatinib-treated patients included proteinuria, diarrhea, fatigue, rash, and palmar-plantar erythrodysesthesia syndrome [31].

As per the data sheet recommendations, the daily dose is 24 mg once daily and it might be modified according to the dose/toxicity ratio (https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/206947s007lbl.pdf, accessed on 1 April 2021).

4.1. Predictive Markers of Radiological Response

In the last years, several potential predictive factors of response to lenvatinib have been evaluated including clinical, radiological, and circulating markers (Table 1).

The major clinical factors have been investigated in an exploratory analysis from SELECT trial, in which baseline Eastern Cooperative Oncology Group (ECOG) performance status and body weight were found to be significantly associated with percent tumor size reduction in the multivariate model [16]. Baseline tumor size, measured by summing target lesions diameters, was also found to be a predictive factor of radiological response [16]. The role of disease burden was confirmed in another analysis from the SELECT trial, in which the median duration of response was shorter in patients with greater disease burden [18]. Moreover, in a Korean multicenter retrospective study, patients with rapidly progressive

disease and a shorter initial tumor doubling time (<6 months in patient-based assessment) were more likely to respond to lenvatinib [19].

Similarly to sorafenib, Tg has been advocated as a potential circulating biomarker. An open-label, single-arm, phase 2 trial found that Tg decrease showed a statistically significant correlation with the maximum tumor shrinkage, beginning on cycle 2 and lasting at several additional assessment points [15]. In this study, circulating cytokines (low baseline levels of IL-10 and high baseline levels of fms-related tyrosine kinase 3 ligand) as well as angiogenic factors (low baseline levels of angiopoietin-2 -Ang2-) correlated with improved ORR after lenvatinib therapy [15]. The role of angiogenic factors was further investigated in an exploratory analysis from a SELECT trial in which low baseline Ang2 level was found as a predictive biomarker of maximum tumor shrinkage for patients in lenvatinib group. Although baseline Ang2 and VEGF levels correlated with ORR, neither were predictive of ORR to lenvatinib [17].

4.2. Predictive Markers of Survival

Several studies evaluated prognostic markers of survival during lenvatinib treatment (Table 2).

In addition to radiological response, baseline body weight and ECOG status were associated with PFS in an exploratory analysis from SELECT trial [16]. Recently, these data have been confirmed in all-case post-marketing observational study by Takahashi et al., in which in multivariate analysis both these parameters were demonstrated as baseline prognostic factors affecting OS in patients with RR-DTC [28].

Similarly to sorafenib, the presence of disease-related symptoms [23,25] and a high tumor burden [25,26,28] were associated with poorer PFS and OS. Some studies highlighted the importance of tumor rate growth as a key factor to predict the outcome of lenvatinib treatment [16,25,26]. Indeed, Song and coworkers showed that patients with faster tumor growth at baseline and after the initiation of treatment had poorer survival [26]. Conversely, Suzuki at al found no association of tumor growth at baseline with PFS, but found that tumor growth after the initiation of lenvatinib and the ratio between these two parameters were associated with PFS [25]. Accordingly, in the paper of Robinson et al., in a multivariate model the percent change in tumor size at the first radiological tumor assessment was a marginally significant positive predictor for PFS ($p = 0.06$) [16].

Other clinical features have been extrapolated from the SELECT trial and advocated as predictors of survival to lenvatinib. Specifically, treatment emergent hypertension [24] and a dose interruption <10% [17] were associated with better survival outcomes.

Recently, the role of functioning imaging with 18F-FDG PET-CT was evaluated to improve treatment personalization [29]. In the paper from Ahmaddy et al., all responders to lenvatinib (according to RECIST criteria) showed a decline in nearly all PET-parameters from baseline to the three month follow-up and from baseline to the six month follow-up. At both the three and six months follow-ups, non-responders according to mPERCIST showed significantly worse survival outcomes [29].

Among circulating factors, the role of Tg, angiogenic and immune markers have been evaluated.

Song et al. showed that Tg doubling time was associated with PFS, but the analysis was confined to 34 out of 43 patients (79%) with negative Tg antibodies [26]. In an open-label, single-arm, phase 2 trial, Cabanillas et al. found that low baseline Ang2 and high baseline epidermal growth factor (EGF) levels were correlated with survival in a univariate analysis [15]. Subsequently, an exploratory analysis from the SELECT trial confirmed the role of baseline Ang2 and Tie2 as prognostic factors [17].

Finally, in a recent study Fukuda et al. explored the role of neutrophil-to-lymphocyte ratio (NLR) as a predictor of survival to lenvatinib therapy and showed that the median OS was significantly longer in the lower NLR group (<3) than in the higher NLR group when starting lenvatinib treatment [27]. These data have been confirmed by Taylor and coworkers in a post hoc analysis from a SELECT trial in which they found that patients with

a baseline NLR ≤S3 had better PFS and OS than patients with a baseline NLR > 3 [30,31]. These findings suggest that NLR could be used as an indicator for starting lenvatinib treatment.

5. Final Remarks

The clinical management of RR-DTC still represents a challenge to the decision concerning whether and when a target therapy should be performed in a multidisciplinary setting by an expert panel.

In the last years, there has been a growing interest in finding new biomarkers for RR-DTC therapy, which are strongly needed to customize treatment strategies.

The current review summarizes the literature evidence on potential predictors of radiological response and survival outcomes in patients with progressive RR-DTC, candidate to MKI treatment. In the setting of progressive RR-DTC, a systemic therapy should be considered after the evaluation of risk-benefit ratio of treatment and taking into account patients characteristics, tumor features, tissue, and circulating biomarkers.

5.1. Patients Characteristics

Clinicians should consider the age and the general status of the patients prior to starting MKI treatment. The incidence of AEs is more frequent in older than in younger patients, with a higher need of dose adjustments, although no significant differences were observed in PFS between the two groups [9,28].

The baseline ECOG score and body weight have been demonstrated to be predictive factors of radiological response as well as prognostic factors for lenvatinib treatment in an exploratory analysis from the SELECT trial. This was also confirmed in a Japanese real-world clinical setting [16,28], suggesting that patients with a good performance status could benefit more from the treatment. In accordance with these findings, an Italian real-world experience with lenvatinib showed less favorable efficacy outcomes than the registration trial, probably due to a negative selection of the study population that included patients with worse clinical features [32].

According to these findings, the absence of cancer-related symptoms is another crucial prognostic clinical factor in the decision-making process leading to the initiation of MKI treatment. Indeed, asymptomatic patients seem to benefit more from sorafenib and lenvatinib therapy [11,22,23,25], suggesting that MKIs should be started prior to the occurrence of cancer-related symptoms.

Finally, the genetic background could influence the response to MKIs. A set of SNPs of the VEGF-A and VEGFR-2 genes, which represent the most important angiogenic regulators, could be useful to predict radiological response and survival outcome of sorafenib treatment [13]. Patients harboring a genetic background associated with less efficient angiogenic mechanisms seem to be more responsive to sorafenib [13].

5.2. Tumor Features

The role of tumor burden in predicting MKI treatment outcome has been evaluated in several RR-DTC settings. A low tumor burden has been correlated with better tumor response to lenvatinib [16,18] and has been associated with better survival outcome [11,22]. More debated is the role of tumor rate growth, since highly proliferative tumors seem to be more responsive to lenvatinib [19]. However they have been associated with a worse prognosis [21,25,26].

Recently, the role of 18F-FDG PET-CT as a useful tool for predicting tumor response has been advocated and baseline SUVmax has been found to correlate with radiological response to sorafenib treatment [12]. Moreover, the assessment of lenvatinib response by (mPERCIST) appeared to be stronger correlated with survival outcomes than RECIST, improving treatment individualization through the selection of patients with an increased likelihood of benefit from lenvatinib [29]. These findings suggest that functional imaging should be included in the diagnostic work-up of patients with RR-DTC candidate to MKIs.

5.3. Tissue Biomarkers

Different tissue biomarkers have been explored as potential predictors of response to MKIs. Lower nuclear pAKT tumor expression has been associated with higher response rate to sorafenib [14]. Several genetic alterations can involve the PI3K/AKT signaling pathway, leading to DTC pathogenesis and progression. Therefore, it is possible that increased pAKT expression in tumor cells represents a mechanism of escape to sorafenib [14]. These findings suggest that a combination therapy with sorafenib and an inhibitor of the PI3K/AKT signaling pathway could be considered in RR-DTC patients.

Subgroup analyses of the DECISION and SELECT trials, evaluating the effectiveness of sorafenib and lenvatinib treatment, respectively, did not show any difference in efficacy in relation to BRAF or RAS genetic alterations. Conversely, RNA-seq analysis from DECISION trial were associated with a BRAF-like profile and a better outcome of sorafenib treatment compared with RAS-like and NoBRaL profile, suggesting that the expression profile may be useful for a better disease characterization before recommending systemic therapy with MKIs [20].

5.4. Circulating Biomarkers

Serum Tg is a well-recognized marker of disease after thyroidectomy, since detectable levels are correlated with persistent loco-regional or metastatic disease [33]. However, the role of baseline Tg and Tg decrease in predicting radiological response and survival to MKI treatment is a very debated and controversial issue. Low baseline Tg level [12] and a greater Tg decrease seem to be associated with a higher benefit [11,12,15,26], but some studies failed to find this association [9,11,34,35]. Moreover, during treatment, transient Tg oscillations are a frequent phenomenon that may not necessarily reflect morphologic tumor progression [36]. Long-term follow-up studies will be useful to clarify the prognostic value of Tg.

Considering the mechanism of action of MKIs, some studies have focused on the angiogenic pathways to find new biomarkers. Increased VEGF expression is significantly associated with angiogenesis and advanced-stage RR-DTC. Another molecular driver of tumor growth in DTC is FGF/FGFR. Finally, Ang2 is a regulator of angiogenesis that has been demonstrated to be a predictive marker in different cancer settings. Low Ang2 could be useful as circulating marker to select patients who will benefit more from lenvatinib treatment [17].

Recently, NLR has received a great interest as predictive marker in oncology, reflecting the anti-tumor immunity status. A lower NLR has been associated with better survival outcome to lenvatinib, providing a useful and feasible tool to select patients' candidacy for MKI treatment [27,30].

Several factors including clinical, molecular, circulating, and tumor markers could help in selecting patients who will benefit more from MKI treatment. However, the majority of data emerged from secondary analyses of SELECT and DECISION trials or from small retrospective studies. Therefore, the lack of trials aiming at investigating predictive biomarkers of MKI effectiveness limits the clinical applicability of these findings, which should be considered as a basis for further prospective multicenter studies.

6. Conclusions

Nowadays, sorafenib and lenvatinib are the only MKIs which have received approval for the treatment of patients with progressive RR-DTC. Unfortunately, MKI treatment is burdened with important AEs, potentially affecting patients' quality of life. Thus, evaluating the risk-benefit ratio is mandatory before starting treatment.

Evidence from the available literature shows that several factors have been advocated as potential predictors of response to MKIs, although none of them has been validated as an ideal biomarker.

Nevertheless, these factors could help the clinician in selecting patients with RR-DTC candidate to MKIs. Published data agree in suggesting that patient clinical status and

the presence of tumor-related symptoms should be taken into account, preferring to treat those asymptomatic patients in better clinical condition. Moreover, tumor burden should also guide the choice of MKIs, with a lower burden associated with a better radiological response and survival outcome.

Prospective studies aiming at validating biomarkers predicting MKI tumor response and prognosis are strongly needed to personalize therapy of patients with progressive RR-DTC.

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Differential Expression Profiles of Cell-to-Matrix-Related Molecules in Adrenal Cortical Tumors: Diagnostic and Prognostic Implications

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Abstract: The molecular mechanisms of adrenocortical carcinoma development are incompletely defined. De-regulation of cellular-to-extracellular matrix interactions and angiogenesis appear among mechanisms associated to the malignant phenotype. Our aim was to investigate, employing PCR-based array profiling, 157 molecules involved in cell-to-matrix interactions and angiogenesis in a frozen series of 6 benign and 6 malignant adrenocortical neoplasms, to identify novel pathogenetic markers. In 14 genes, a significant dysregulation was detected in adrenocortical carcinomas as compared to adenomas, most of them being downregulated. Three exceptions—hyaluronan synthase 1 (HAS-1), laminin α3 and osteopontin genes—demonstrated an increased expression in adrenocortical carcinomas of 4.46, 4.23 and 20.32-fold, respectively, and were validated by immunohistochemistry on a series of paraffin-embedded tissues, including 20 adenomas and 73 carcinomas. Osteopontin protein, absent in all adenomas, was expressed in a carcinoma subset (25/73) ($p = 0.0022$). Laminin α3 and HAS-1 were mostly expressed in smooth muscle and endothelial cells of the vascular network of both benign and malignant adrenocortical tumors. HAS-1 was also detected in tumor cells, with a more intense pattern in carcinomas. In this group, strong expression was significantly associated with more favorable clinicopathological features. These data demonstrate that cell-to-matrix interactions are specifically altered in adrenocortical carcinoma and identify osteopontin and HAS-1 as novel potential diagnostic and prognostic biomarkers, respectively, in adrenal cortical tumors.

Keywords: adrenal cortex; carcinoma; angiogenesis; gene expression; osteopontin; hyaluronan synthase 1

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1. Introduction

Adrenocortical carcinoma (ACC) is a rare tumor of the adrenal cortex which accounts for no more than 0.2% of all malignancies. Its differential diagnosis from adrenal cortical adenomas (ACA) has been classically based on several pathological and clinical parameters [1]. None of these is per se indicative of malignancy and is most commonly used in combination in a variety of scoring methods, the Weiss system being the most widely employed. However, such systems are occasionally challenging to apply and/or time consuming despite several implementations introduced in recent years to provide clinically

relevant information [2–4]. Complementary to histomorphological evaluation, several markers of malignancy have been reported, including p16, p53, topoisomerase II alpha [5–7] and Ki-67. In the latter, cut-off values able to discriminate between benign and malignant lesions were set from 2.5% to 4% according to the different authors, whereas in the ACC group, cut-offs ranging from 15 to 20% have been associated with prognosis [7–12].

Molecular techniques offer the possibility of a distinction between ACA and ACC and, more importantly, an intrinsic profiling of ACC, as consistently different and specific molecular signatures were identified in these tumors, bearing both diagnostic and prognostic implications [13–17]. At the gene expression level, IGF-2 gene up-regulation represents the most specific molecular signature of ACC, a feature related to genetic alterations at the IGF-2 locus reported in a high proportion of hereditary and sporadic ACC [18–20]. IGF-2 immunohistochemical determination has been also proposed as a diagnostic marker of ACC [21]. Among other genes differentially expressed between ACA and ACC, osteopontin and serine threonine kinase 15 were significantly up-regulated (up to 20-fold) in the latter group [17]. In addition, other differential molecular alterations between ACA and ACC were identified within the microRNA or long-non-coding RNA profiles, gene methylation status [22–24] or in the expression levels of single biomarkers, including livin/BIRC7 [25], NOTCH pathway [26] and CYP2W1 [27].

However, a detailed comparative investigation of molecules involved in cell-to-matrix interactions and angiogenesis in ACA and ACC is missing. Individual molecules, only have been investigated in adrenocortical tumors, including vascular endothelial growth factor (VEGF), gelatinases, matrix metalloproteinases and others [28,29]. Serum and tissue VEGF protein levels were found significantly increased in malignant tumors [29–31]. In addition, matrix metalloproteinases and vascular growth factors, aside from being of interest from a pathogenetic and diagnostic point of view, represent promising targets for targeted therapies [32,33].

By means of a selective PCR-based gene array profiling, our study aimed at analyzing the differential expression of genes involved in cell-to-cell and cell-to-matrix interactions, as well as angiogenic processes, in a series of adrenocortical tumors, with the specific purpose of identifying target genes of potential pathogenetic and diagnostic interest in ACC. The decision to investigate such a family of target genes stemmed from a previous publication from our group on the adverse prognostic role of matrix metalloproteinase type 2 (MMP2) expression in ACC [34], as well as from the reported high expression of osteopontin in adrenocortical tumors [17,35].

2. Materials and Methods

2.1. PCR-Based Gene Expression Profile

Frozen tissues from 12 adrenocortical tumors, including six ACC and six ACA, were analyzed using a PCR-array based method. Total RNA was extracted using QIAzol lysis Reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Clone DNA was transcribed using 500 µg/mL oligodT (Roche, Mannheim, Germany) and 500M-MLV RT (200 U/µL) (Invitrogen, Carlsbad, CA, USA) according to standard protocols.

Expression profiling was detected in duplicate by two gene Array sets for real-time PCR, which includes SYBR Green-optimized primer sets for 12 housekeeping gene probes and a thoroughly researched panel of 157 relevant genes involved in cell-to-cell and cell-to-matrix interactions and angiogenesis (SuperArray Bioscience Corporation, Frederick, MD, USA; codes PAHS-013 and PAHS-024), following the manufacturer's instructions (Supplementary Table S1). PCR reactions were carried out in a fluorescence-based real-time apparatus (ABI PRISM 7900 Sequence Detection System, Taqman; Applied Biosystems, Foster City, CA, USA), and each 96-well plate was analyzed simultaneously under uniform cycling conditions.

2.2. Osteopontin, Laminin α3 and HAS-1 Protein Expression in Adrenocortical Tumors: Case Selection and Immunohistochemistry

Based on the data obtained by gene expression profiling (see below), we aimed at validating the three proteins that resulted in being over expressed in ACC samples. Twenty ACA and 73 ACC tumor tissue samples were analyzed. The former included 42 ACC on tissue micro array (prepared as described elsewhere [36]) and 31 on whole slides. All cases were retrieved from the files at the Pathology Units of AOU San Luigi, Orbassano, and the "Città della Salute e della Scienza", Turin, hospitals. All adrenocortical neoplasms were independently classified by three of us (MV, ED and MP), according to the Weiss system. For the series of 31 ACC cases analyzed on the whole section, clinical information included sex, age, functional status, tumor weight, tumor size, individual Weiss parameters and overall Weiss score, ENSAT stage, type of tumor recurrence (local and or distant metastases), adjuvant mitotane administration and follow up. All cases were surgical specimens of primary tumor resection. Eighteen patients showed disease progression. Among the seventeen cases with available information on the type of progression, 5 had distant metastases, whereas 12 had local (intra-abdominal) dissemination. Adjuvant mitotane was administered in 23 out of 27 patients with this information available. Before starting the analyses, all samples were anonymized by a staff member of the pathology division not involved in the project, and the study was approved by the Institutional Review Board of the San Luigi Hospital (Protocol AMPRECCO, No. 128/2010).

Five micron-thick paraffin sections were collected onto charged slides and processed for osteopontin (polyclonal antibody, diluted 1:650; Sigma Aldrich, Milano, Italy) and Laminin α3 (1:100, rabbit polyclonal antibody, #PA5-38937, Thermofisher, Waltham, MA, USA) using Ventana BenchMark AutoStainer (Tucson, AZ, USA), whereas hyaluronan synthase 1/HAS-1 (1:250, rabbit polyclonal antibody, #272680, Abcam, Cambridge, UK) was assessed using the Dako Omnis platform (Agilent Technologies, Santa Clara, CA, USA). Appropriate positive and negative controls were included for each immunohistochemical run. Based on the results obtained, immune reactions were scored as follows: osteopontin was scored as negative or positive in the cytoplasm of tumor cells, laminin α3 was scored in vascular structures, only, into a semiquantitative scale based on density of the vascular network (from 0 to 3+), whereas HAS-1 was both scored in vascular structures (same as above) and in tumor cells based on staining intensity (from 0 to 3+).

2.3. Statistical Analysis

All data were analyzed with GraphPad Prism 9.0 (GraphPad Softwares, San Diego, CA, USA). A level of $p < 0.05$ was considered statistically significant. Differences in mean gene expression levels between ACC and ACA groups in PCR-array experiments were evaluated by Student's *t*-test. The differential protein expression of immunohistochemical markers in adrenocortical tumors, as well as the correlation between their expression and clinico-pathological parameters, were estimated by Chi-square test. Univariate disease free and overall survival analysis of positive and negative tumors for each marker and individual clinical and pathological parameters was based on the Kaplan–Meier product limit estimate of disease free and overall survival distribution. Unadjusted differences between survival curves were tested using the LogRank test. Multivariate Cox regression model was used to assess the association of HAS-1 expression and other clinical and pathological variables with disease free and overall survival by means of the SPSS statistical software version 22 (IBM corporation, Armonk, NY, USA).

3. Results

3.1. Gene Expression Profile of Cell-Matrix Interactive and Angiogenic Proteins in Adrenocortical Carcinomas as Compared to Adenomas

A list of all genes that were significantly differentially regulated between adrenocortical carcinoma and adenoma groups is reported in Table 1. For most of the genes analyzed, a general trend (even for those genes with a fold change not reaching the statistical sig-

nificance) to down-regulation in ACC as compared to ACA was observed. Exceptions were represented by three genes which were significantly up-regulated in the ACC group. Namely, hyaluronan synthase 1, laminin α3 and osteopontin genes, with a fold change of 4.46, 4.23 and 20.32 times, respectively. Genes just below the statistical significance in their expression fold change between the two groups (with a p value >0.05 but <0.1, all down regulated) included members of the chemokine family (CXCL-3, -6 and 9 genes), integrin subunits α8, αν and β3, matrix metalloproteinases and related inhibitors (MMP-2, TIMP-1, -2 and 3), epithelial growth factors (TGFα and EREG-epiregulin genes) and the Tie-2/Tek endothelium-specific receptor tyrosine-kinase.

Table 1. Adrenocortical carcinoma versus adenoma significant fold differences in gene expression among 157 molecules investigated.

Gene Name	Description	*t*-Test (*p*-Value)	Fold Up/Down Regulation
BFGF/FGFB	Fibroblast growth factor 2	0.0484	−5.66
DHAND2/Hed	Heart and neural crest derivatives expressed 2	0.0234	−13.61
HAS1	Hyaluronan synthase 1	0.0287	4.46
F-TCF/HGFB	Hepatocyte growth factor	0.0489	−9.02
IL-1/IL1-BETA	Interleukin 1, β	0.0448	−8.00
LAMA	Laminin, α_2	0.0391	−16.00
E170/LAMNA	Laminin, α_3	0.0409	4.23
KIAA1907	Laminin, α_5	0.0280	−3.86
COX1/COX3	Prostaglandin-endoperoxide synthase 1 (cyclooxygenase)	0.0442	−5.20
BNSP/BSPI	osteopontin, bone sialoprotein I	0.0450	20.32
CLEVER1/FEEL1	Stabilin 1	0.0215	−7.45
CED/DPD1	Transforming growth factor, β 1	0.0365	−5.53
THBS/TSP	Thrombospondin 1	0.0456	−10.82
DIF/TNF-alpha	Tumor necrosis factor (TNF superfamily, member 2)	0.0270	−11.66

3.2. Osteopontin, Laminin α3 and HAS-1 Protein Expression in Adrenocortical Tumors

By immunohistochemistry, osteopontin was found to be expressed in 25/73 (34%) ACC samples, whereas it was absent in all 20 ACA tested (p = 0.0022) (Table 2). The pattern of staining in ACC tumor cells was cytoplasmic, either diffuse or with a peculiar dot-like paranuclear appearance, or a combination of the two (Figure 1) at comparable frequencies. No specific correlation was observed between the presence or absence of staining nor the two staining patterns or the intensity of staining with morphological or clinical features in the 31 ACC patients with clinical information available.

Table 2. Immunoexpression of Laminin α3, HAS-1 and osteopontin in a series of 73 adrenocortical carcinomas and 20 adrenocortical adenomas.

	IHC Semiquantitative Score	* ACC (#73)	§ ACA (#20)	*p* Value
Laminin α3 (vascular network)	0	13 (17.8%)	3 (15%)	0.04
	1+	20 (27.4%)	11 (55%)	
	2+	23 (31.5%)	6 (30%)	
	3+	17 (23.3%)	0	
HAS-1 (tumor cells)	0	42 (57.5%)	13 (65%)	0.55
	1+	17 (23.3%)	5 (25%)	
	2+	7 (9.6%)	2 (10%)	
	3+	7 (9.6%)	0	
HAS-1 (vascular network)	0	12 (16.4%)	2 (10%)	0.51
	1+	21 (28.8%)	9 (45%)	
	2+	18 (24.7%)	5 (25%)	
	3+	22 (30.1%)	4 (20%)	
Osteopontin (tumor cells)	negative	48 (65.8%)	20 (100%)	0.0022
	positive	25 (34.2%)	0	

Abbreviations: IHC: immunohistochemistry; * ACC: adrenocortical carcinoma, 31 cases on whole section and 42 on TMA; § ACA: adrenocortical adenoma (all on whole section).

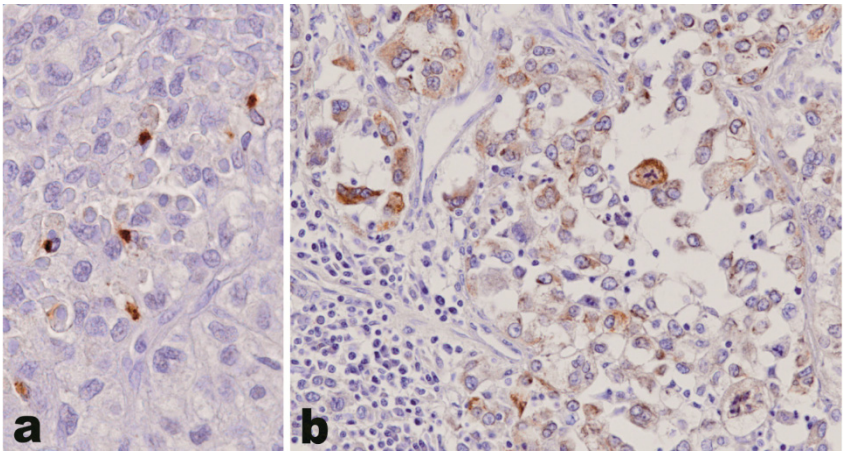

Figure 1. Osteopontin expression in adrenocortical carcinoma. The pattern of staining in adrenocortical carcinoma tumor cells is cytoplasmic, either with a peculiar dot-like paranuclear appearance (**a**) or diffuse within the cytoplasm (**b**). The intratumoral lymphocytes (**b**, bottom left) are un-reactive (original magnification 400×).

Laminin α3 was expressed both in ACA and ACC, but with a statistically significant increase of density of staining in ACC. The positive staining was observed in both ACA and ACC exclusively in vascular structures, whereas tumor cells were consistently negative in both tumor types (Figure 2a,b). As for osteopontin, no correlation between laminin α3 and clinical or pathological parameters in the series of ACC was observed. HAS-1 did not show a statistically different prevalence of staining in ACA and ACC and was expressed both in vascular structures and in tumor cells, in this latter case with a diffuse cytoplasmic pattern with occasional membrane reinforcement (Figure 2c,d).

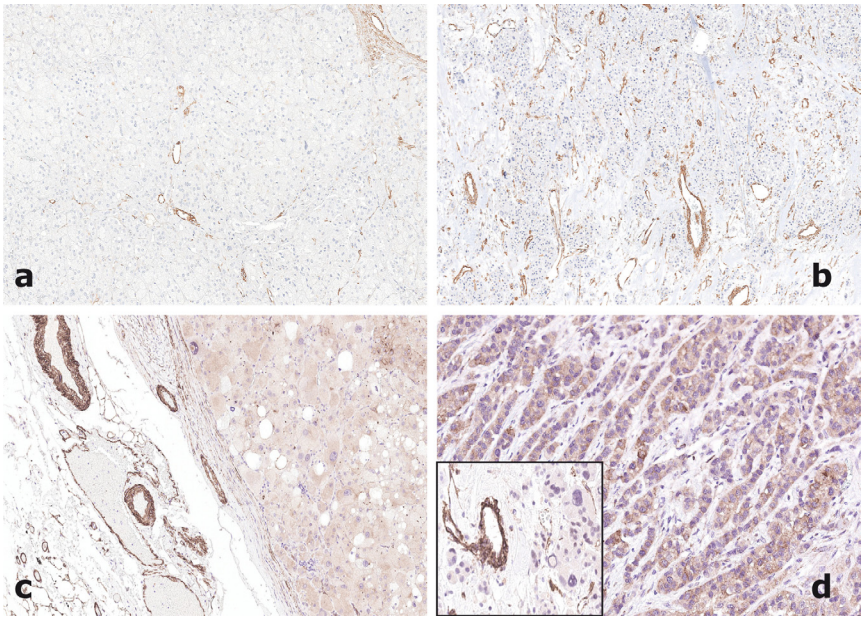

Figure 2. Laminin α3 immunoexpression is restricted to the vascular network of both an adrenocortical adenoma with rare and thin vessels (**a**, 100×) and an adrenocortical carcinoma containing numerous small and medium size vessels (**b**, 100×). Hyaluronan synthase-1 is also strongly expressed by smooth muscle cells of vessel walls in both adrenocortical adenoma (**c**, 100×) and carcinoma (**d** inset, 200×). In addition, tumor cells of adenomas present more frequently a negative or weakly positive pattern of staining (**c**, 100×), while carcinoma cells may show a strong cytoplasmic reactivity (**d**, 100×).

HAS-1 was also detectable in the cytoplasm of peritumoral normal adrenal cells, with a more intense staining in cells of the fasciculata zone. HAS-1 positivity in vascular structures was not associated with any of the clinical or pathological variables in the series of 31 ACCs with clinical and pathological annotates.

By contrast, HAS-1 negative expression in tumor cells was significantly associated with features of aggressiveness, such as higher mitotic index, presence of atypical mitoses, and poor disease outcome (Table 3). Negative HAS-1 staining in tumor cells was also more frequently observed in female patients and at lower age of onset.

Table 3. Major clinico-pathological features according to HAS-1 positivity in tumor cells (#31 cases on whole section).

Parameter		HAS-1 Positive	HAS-1 Negative	*p* Value
Sex	M	8	6	0.02
	F	7	10	
Age	<46 *	3	10	0.02
	≥46	12	6	
Functional status	Nonfunctioning	8	10	0.62
	Functioning †	7	6	
Tumor weight (g)	<275 *	10	9	0.55
	≥275	5	7	
Tumor size (cm)	<11 *	10	6	0.10
	≥11	5	10	

Table 3. *Cont.*

Parameter		HAS-1 Positive	HAS-1 Negative	*p* Value
Mitoses	<8 *	10	4	0.02
	≥8	5	12	
Atypical mitoses	absent	11	6	0.045
	present	4	10	
Necrosis	absent	4	1	0.12
	present	11	15	
Venous invasion	absent	2	6	0.12
	present	13	10	
Sinusoid invasion	absent	8	9	0.87
	present	7	7	
Capsular invasion	absent	8	8	0.85
	present	7	8	
Nuclear atypia	absent	3	4	0.74
	present	12	12	
Weiss score	3–6	8	7	0.59
	7–9	7	9	
ENSAT stage	I	4	0	0.068
	II	10	13	
	III	1	3	
Mitotane treatment (4 cases missing)	no	3	1	0.60
	yes	12	11	
Status	NED	10	3	0.0069
	AWD/DOD	5	13	
Median DFS	months	not reached	49	0.040
Median OS	months	not reached	17	0.197

Legend. DFS: disease free survival; OS: overall survival; *: median value; †: functioning tumors were defined according to the presence of an evident clinical syndrome and included among the hormones produced cortisol, aldosterone, and androgens; NED: no evidence of disease; AWD: alive with disease; DOD: dead of disease.

Disease free (DFS) and overall (OS) survival analyses did not show any significant difference in whether patients were positive or negative for osteopontin or laminin α3. Laminin α3 scores 0–1 had a median DFS and OS of 62 months and "undefined", respectively, as compared to median DFS and OS of 30 and 61 months, respectively, in cases with 2–3 scores (all *p* values not significant). Osteopontin expression was also not associated with survival, with median DFS of 37 and 22 months and median OS "undefined" and of 61 months, in negative and positive cases, respectively (all *p* values not significant). HAS-1 density of expression in the vascular network was not associated to a specific survival, with median DFS of 22 and 49 months and median OS both "undefined", in cases with score 0–2 and with score 3, respectively (all *p* values not significant). Interestingly, negative HAS-1 expression was associated with shorter survival (Figure 3). In fact, a trend towards significance was observed for OS, with a 49-month median survival in negative cases as compared to "undefined" in positive cases (Log rank test *p* value 0.197, HR 2.15). Moreover, DFS was significantly different in HAS-1 negative and positive cases, with a median survival of 17 and "undefined", respectively (Log rank test *p* value 0.040, HR 2.77).

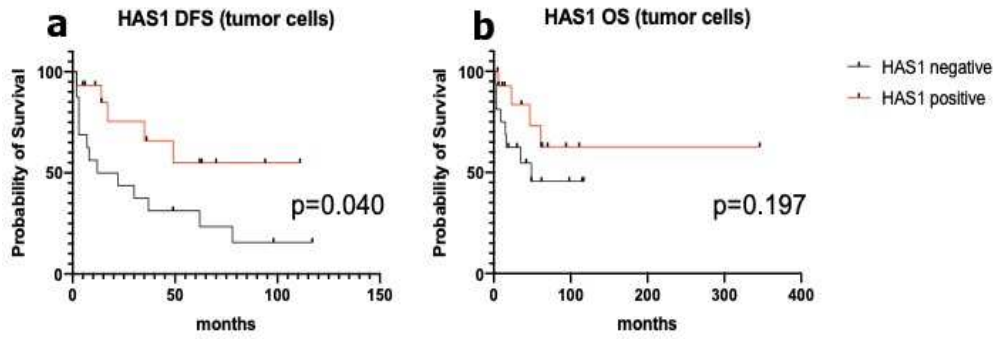

Figure 3. Univariate disease free (**a**) and overall (**b**) survival analysis in HAS-1 tumor cell positive and negative adrenocortical carcinomas.

To test the interference of other clinical and pathological factors with HAS-1 in univariate survival analyses, all variables statistically associated with HAS-1 tumor cell positive expression having a *p*-value of <0.2 (see Table 3) were tested in univariate disease free and overall survival analyses. Among those, age, ENSAT stage presence of necrosis and presence of vascular invasion failed to show statistical significance in both disease free and overall survival analyses. By contrast, male sex, size above median (11 cm), mitotic index above median (11 mitotic figures in 50 high power fields) and presence of atypical mitoses were all associated with shorter survival with a *p*-value < 0.2 (*p* values of 0.085, 0.011, 0.088 and 0.078, respectively) and were included in multivariable cox regression analysis together with HAS-1. Independent adverse prognostic impact in terms of disease free survival was retained for male sex and large size (0.036 and 0.028, respectively) only. At overall survival analysis, size above median and mitotic index above median were associated with a *p*-value < 0.2 (*p*-values of 0.082 and 0.084, respectively) and were included in multivariable cox regression analysis together with HAS-1. None of the parameters reached statistical significance, tumor size showing the most significant *p*-value (0.065).

4. Discussion

By means of a selective PCR-based gene array approach, we here demonstrated a differential expression of several genes involved in angiogenesis and cell-to-matrix interactions in ACC as compared to ACA. Previous molecular studies in adrenocortical tumors showed peculiar gene expression profiles in ACC as compared to ACA, up-regulation of IGF-2 gene and down-regulation of genes involved in steroidogenesis being the most specific molecular signatures of ACC [17,19,20]. The major advantage of wide gene expression profiling is to generate a large amount of information, thus providing a molecular classification of tumors. On the other hand, data from different studies are not easily comparable and it is sometimes difficult to transpose such complexity of information onto a diagnostic ground. Our approach was to screen a limited number of genes involved in cell-to-matrix interactions and angiogenesis that the previous literature supported to be altered in adrenal cancer [28–31,34,35], to search for potential pathogenetic or clinically relevant biomarkers. In general, most of the genes that showed a differential expression in ACC as compared to ACA are active regulators of angiogenesis (such as Fibroblast growth factor 2, Hepatocyte growth factor, Prostaglandin-endoperoxide synthase 1, Transforming growth factor, β 1 and Thrombospondin 1). Moreover, several other angiogenesis-regulating genes showed an altered regulation in ACC as compared to ACA, while not reaching statistical significance. Notably, the vast majority of these genes showed a peculiar down regulation. This finding is partially surprising, but it might fit with the consistent down-regulation of several growth factor receptors described by Giordano and co-workers in ACC [17] and it might represent either the consequence of the different vascular network in ACA vs. ACC or the consequence of decreased tissue expression. Due to the heterogeneous biological functions of these molecules, it is difficult, however, to hypothesize the biological meaning of our findings, in the lack of functional experiments that represents a major limitation

of our study. Moreover, among the genes that showed no differential or slightly lower expression in ACC, the various matrix metalloproteinases, their specific inhibitors, and VEGF isoforms should be mentioned since their related proteins have been found to be over-expressed in ACC tumor cells [29–31]. In this respect, the absence of a differential expression of the corresponding genes between ACA and ACC in our series may more probably reflect a balance between the integrity of the vascular network and neo-angiogenic capabilities in the two tumor types, the equilibrium being more shifted to the former in ACA and to the latter in ACC [31].

An altered expression of laminin isoforms was observed in the current series of ACC, with a higher expression of the α3 and lower expression of α2 and α5 laminin isoforms, as opposed to ACA. This observation is interesting since α2 and α5 laminin isoforms are consistently expressed in the basal membrane of the normal adult adrenal cortex, whereas α3 isoform is virtually absent [37], and might be related to structural alterations of the basal membrane in ACC tissue [38]. In fact, the reduced expression of α2 and α5 laminin isoforms may be correlated with the morphological finding of reticulin framework disruption in carcinomas [38], while overexpression of laminin α3 might be related to the higher expression levels of this isoform observed in the dense intratumoral vascular network of ACC.

With regard to other molecules than laminin α3 having an increased expression in ACC, osteopontin had the highest fold difference, a finding in agreement with previous gene array data [17] and with the report of Weisman and co-workers [35]. It was therefore further analyzed in a large series of adrenocortical tumors by means of immunohistochemistry to test its tissue localization pattern. Osteopontin expression was restricted to a fraction of malignant cases, being absent in all ACAs, at variance with the findings of Weismann and co-workers who observed a heterogeneous expression of OPN in both ACA and ACC, with a staining often restricted to single cells or small clusters. In our series, however, osteopontin provided a specificity of 100% but a sensitivity of 34% only, thus limiting its value as a diagnostic tool in the differential diagnosis between benign and malignant adrenocortical tumors.

Osteopontin is a secreted phosphoprotein that binds multiple integrins including αvβ1, αvβ3, and αvβ5 [35] and is involved in several cellular processes, such as cell attachment, spreading and migration, homing of lymphocytes and other hematopoietic cells and vascular remodeling [39,40].

Moreover, osteopontin has been demonstrated to be over-expressed in many human tumors, including carcinomas of the breast [41], lung [42], ovary [43], stomach [44], liver [45], and prostate [46] as well as mesothelioma [47], and it was proposed as a potential prognostic marker in many of these neoplasms. In particular, osteopontin expression is induced by hypoxia and its prognostic role in solid tumors is partially explained by its interplay with other hypoxia-related molecules and angiogenic factors, at least in head and neck and prostate cancer [48,49].

Interestingly, in different cellular models osteopontin up-regulates the expression of hyaluronan synthases. In breast cancer cells, osteopontin has been shown to upregulate HAS-2, another enzyme involved in the synthesis of hyaluronan, a major component of the extracellular matrix, with a role in several cellular functions, including cell proliferation and migration [50]. Moreover, in osteoarthritic-derived chondrocytes, osteopontin has been shown to promote the expression of the HAS-1 gene [51]. In the present series, HAS-1 was identified by immunohistochemistry in the smooth muscle and endothelial cells of blood vessels of both benign and malignant adrenocortical tumors, with no differences except for the denser vascular network in ACC. In addition, HAS-1 was also detected in the tumor cells of a fraction of ACC (42%), but only occasionally and weakly in adenoma tumor cells (although this difference was not statistically significant). This finding is not in agreement with gene expression data on the frozen series and claims that HAS-1 has no role as a diagnostic marker in the differential diagnosis between ACA and ACC. However, the lack of a direct correlation between HAS-1 gene and protein data in ACC vs. ACA might be

explained by the wide heterogeneity of protein distribution and intensity in blood vessels, as well as in tumor cells. In fact, although with no statistical significance comparing the different subgroups, both in tissue blood vessels and in tumor cells, ACC showed a higher rate of HAS-1 intense (3+ score) staining, which might explain the overexpression observed at the gene level. More intriguingly, HAS-1 expression in ACC tumor cells was associated with a lower mitotic rate, a lower prevalence of atypical mitotic figures and a better outcome. The prognostic significance of HAS-1 in cancers is equivocal and has been studied either in cancer cells or in cancer stroma, including cancer-associated fibroblasts [52–54]. However, in agreement with our findings, a negative prognostic role of HAS-1 downregulation in cancer cells has been described in lung cancer [55] and melanoma [56]. Survival analysis in our study has several limitations, including the small sample size and incomplete data on progression that could not allow a disease-specific survival, but overall survival only. The association of negative HAS-1 tumor cell expression with shorter disease free survival, that showed statistical significance in univariate analysis, could not be validated as an independent factor in multivariate analysis due to the possible interference with other significant negative prognostic factors such as tumor size. Nevertheless, even though our data need to be confirmed in a larger series, the prognostic impact of HAS-1 expression claim a role for the interplay between cancer cells and the stromal environment as a relevant field of investigation in ACC, which is at variance with several other cancer models, has not yet been explored in detail.

5. Conclusions

In conclusion, the present paper showed that ACC is associated to an altered expression of several genes involved in angiogenesis and cell-to-cell/cell-to-matrix interactions, possibly as the result of unbalanced vasculature and neo-angiogenic properties. Among these, osteopontin is selectively expressed in ACC tumor cells and represents a potential diagnostic marker, although with a remarkably low sensitivity, whereas negative HAS-1 expression in tumor cells characterizes a subset of ACC with a more aggressive clinical outcome.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jpm11050378/s1, Table S1: Panel of 157 genes involved in cell-to-cell and cell-to-matrix interactions and angiogenesis tested in the present study.

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Review

Intratumoral Heterogeneity in Differentiated Thyroid Tumors: An Intriguing Reappraisal in the Era of Personalized Medicine

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Abstract: Differentiated thyroid tumors (DTTs) are characterized by significant molecular variability in both spatial and temporal intra-tumoral heterogeneity (ITH), that could influence the therapeutic management. ITH phenomenon appears to have a relevant role in tumor growth, aggressive behavior and drug resistance. Accordingly, characteristics and consequences of ITH in DTTs should be better analyzed and understood in order to guide clinical practice, improving survival. Consequently, in the present review, we investigated morphological and molecular ITH of DTTs in benign, borderline neoplasms and in malignant entities, summarizing the most significant data. Molecular testing in DTTs documents a high risk for recurrence of cancer associated with $BRAF^{V600E}$, RET/PTC 1/3, ALK and NTRK fusions, while the intermediate risk may be related to $BRAF^{K601E}$, H/K/N RAS and PAX8/PPARγ. In addition, it may be suggested that tumor genotype is associated with peculiar phenotype.

Keywords: intratumoral heterogeneity; thyroid tumor; BRAF; RET/PTC rearrangements; RAS mutation

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1. Introduction

Intratumoral heterogeneity (ITH) represents a crucial determinant to explain the appearance of therapeutic resistance and treatment failure, resulting in poor prognosis and outcome. This intralesion mechanism is defined as diversity observed within a tumor since mosaics of different neoplastic clones are present in the same tumor at varying time [1]. ITH can exist either between geographical areas of the same tumor (spatial heterogeneity) or between different lesions that appear over time locally or distantly (temporal heterogeneity) (Figure 1) [1,2].

Temporal ITH leads to discordance between the primary tumor and the metastatic lesion, and it can stem from either two mutations in different clones in the primary tumor, one clone disseminating to the metastatic site or from a new mutation occurring in the metastatic lesion [3]. ITH may determine the development of different cell subpopulations which in turns may influence the response of a tumor to changes within the microenvironment [3,4]. In addition, this phenomenon may create a neoplastic diffusion throughout the body, realizing metastatic deposits or acquiring resistance to therapeutic agents. Therefore, ITH analysis can provide relevant information to define innovative and patient-tailored therapeutic strategies, based on detection of specific molecular alterations [2,4–6]. So far, ITH has been addressed at both morphological and molecular levels with different

methods [7–10]. Specifically, microdissection is essential to define morphological heterogeneity, which includes histotype, tissue composition, inflammatory reaction, center and borders of tumors [6,8]. Indeed, at a microscopic level, pathologists can recognize different histological patterns in each tumor, with specific morphological characteristics, such as necrosis, apoptosis, fibrosis, hemorrhagic areas, stromal reaction and neo-angiogenesis. An operative workflow to analyze ITH in tumors should be based on morphology, requiring an examination of extensive neoplastic areas to identify the different histological portions of the tumor, analyzing at least two or more representative different portions obtained by microdissection procedure (Figure 2). However, the intratumoral differentiation (well-, moderately-, poorly-) is frequently focal, leading to morphofunctional differences. Furtherly, the choice of tissue specimens may depend on the location of the tumor infiltration front, with elements able to invade capsule and stroma, in contrast to central neoplastic section (Figure 2). Then, the dissected portions have to be passed in a tube for the RNA/DNA sequencing as well as proteomic profiling (Figure 2).

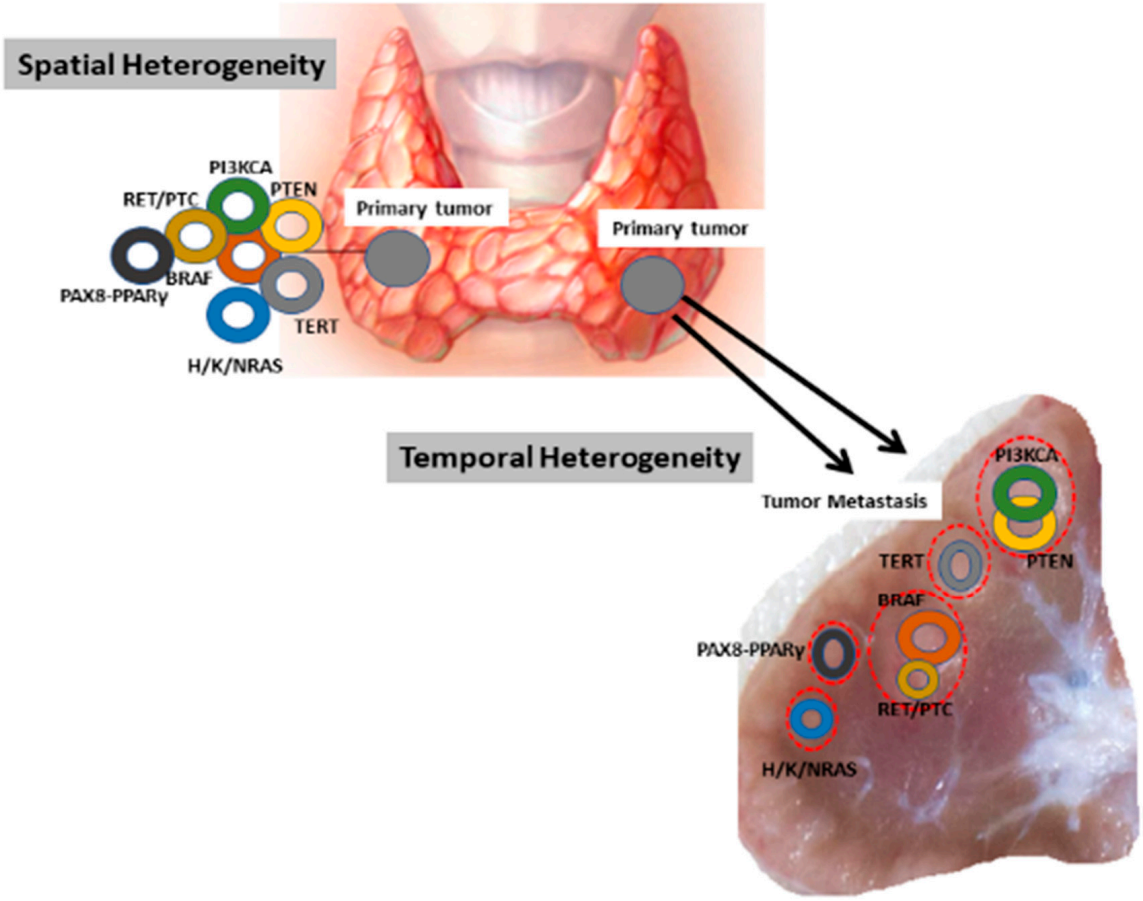

Figure 1. Spatio-temporal heterogeneity in primary and corresponding lymph node metastasis in thyroid tumors.

In addition to the inter-tumor heterogeneity, namely diversity between individuals having the same tumor type, ITH may result in different histological and cytological patterns in the same tumor, negatively impacting on the patient's prognosis [11–13]. At the molecular level, genetic and epigenetic heterogeneity can be present [14]. Particularly, immunohistochemistry, in situ hybridization methods and next generation sequencing by detecting mutations of the driver genes secondary to genetic instability may help reveal clonal and/or non-clonal heterogeneity, which are associated to phenotypic alterations driving neoplastic progression and resistance to targeted therapy [6,15–19].

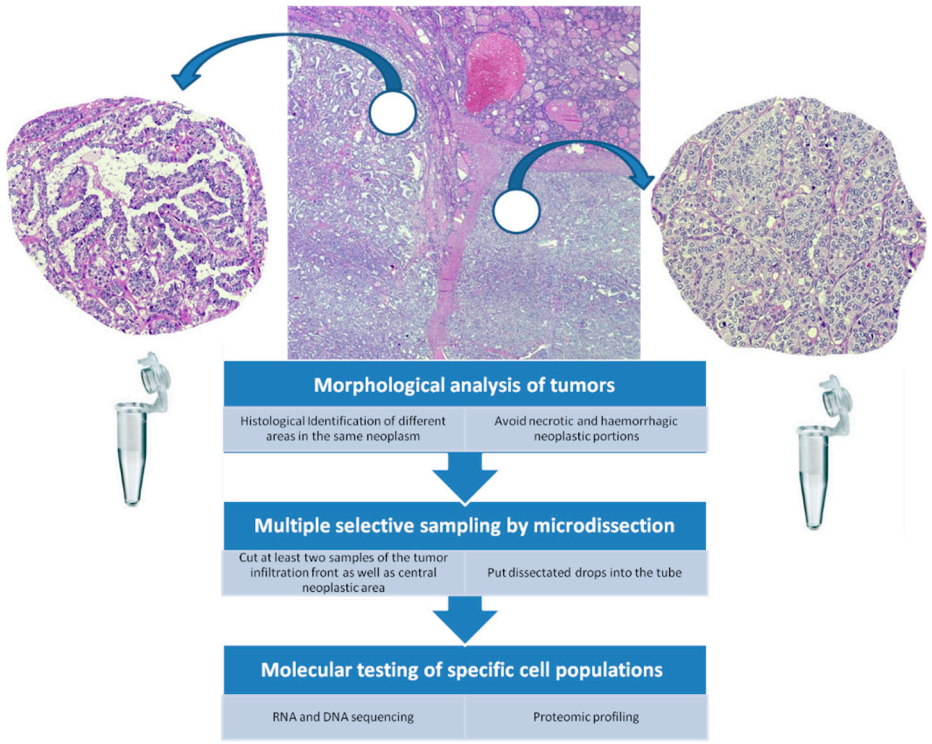

Figure 2. A schematic proposed workflow to identify ITC and to perform molecular tests in DTC.

One of the most evident examples of ITH is encountered in thyroid carcinomas and adenomas. Although the majority of differentiated thyroid carcinomas (DTCs) show an indolent behavior with an excellent prognosis, as documented by a 10-years survival rate of 90%, approximately 10% of them are aggressive, tend to recur and lead the patient to death [20–24]. In this regard, a broad ITH is evident, with histotypes spanning from thyroid papillary microcarcinoma through anaplastic carcinoma, the latter representing the late and fatal stage of carcinogenesis [25–29].

The present paper should be considered as a review in order to furnish the "state of art" regarding ITH in differentiated thyroid tumors (DTCs). The major endpoint is to comparatively analyze morphological and molecular ITH of differentiated thyroid tumors (DTTs), either follicular adenoma (FA) either DTCs in order to evaluate their behavior, identifying markers for therapeutic approaches and making individualized their management. Regarding the novelty of the present review, probably there are not additional original data, but a relevant number for information concerning molecular variability in DTCs in relation to the corresponding morphological aspects as well as a precise definition of the operative workflow to reveal ITH.

2. Phenotypic and Molecular Heterogeneity in FA and Follicular-Patterned Borderline Lesions

To define morphological ITH in DTTs, we need to introduce the new classification of thyroid tumors, in which some changes were introduced [30–34]. In fact, together with follicular adenoma (FA), some borderline entities were added, such as tumors with uncertain malignant potential (UMP), noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) and hyalinizing trabecular tumor (HTT) [30,32] (Figure 3). FA were defined as benign, encapsulated and non-invasive neoplasm demonstrating evidence of thyroid follicular cell differentiation without nuclear features observed in papillary thyroid carcinoma (PTC) [30]. According to the 2017 WHO classification [35], the group of encapsulated follicular-patterned UMP tumors is divided into two entities: follicular tumors with uncertain malignant potential (FT-UMP) and well-differentiated tumors with

uncertain malignant potential (WDT-UMP) (Figure 3A). FT-UMP is an encapsulated and/or well-circumscribed tumor with round nuclei that lack PTC-like features, whereas WDT-UMP presents a similar gross morphology but, well/partially developed nuclear changes of PTC and questionable capsular or vascular invasion [36,37].

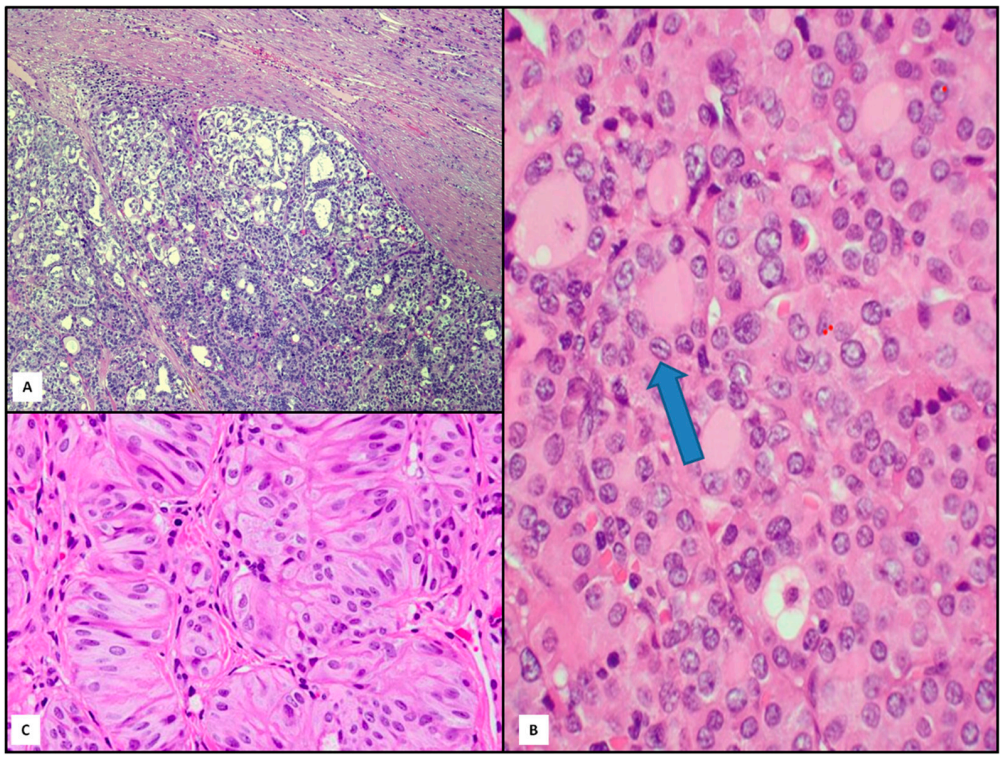

Figure 3. Histological findings of follicular-patterned borderline lesions: tumor with uncertain malignant potential (UMP) ((**A**), hematoxylin and eosin, 80×), noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) ((**B**), hematoxylin and eosin, 400×) and hyalinizing trabecular tumor (HTT) ((**C**), hematoxylin and eosin, 200×). The arrow underlines the peculiar irregular nuclear membranes.

NIFTP is a solitary encapsulated nodule displaying the following features: a complete, frequently thick, fibrous capsule delimitating the tumor from adjacent tissue, follicular growth pattern and nuclear features of PTC [38] (Figure 3B). Papillae and capsular or vascular invasion are constantly absent [38,39]. Furthermore, NIFTP may be distinguished from both FA and hyperplastic nodule by the presence of the typical nuclear changes of PTC [40–43] (Figure 3B).

Another new interesting follicular-derived borderline lesion is represented by HTT, a well circumscribed solid neoplasm without capsular/vascular invasion or invasion of thyroid tissue adjacent to the tumor. Histologically, HTT is composed of trabeculae or sometimes nests of polygonal eosinophilic large cells intermingled with thin stromal bundles. This lesion maybe associated with chronic thyroiditis, nodular goiter or PTC [44–46] (Figure 3C).

Molecular profiles concerning classical FA and follicular-patterned borderline tumors are quite different (Table 1).

Table 1. Histological and molecular heterogeneity in FA and follicular-patterned borderline lesions (H,K,N isoforms of RAS gene family).

	Mutations						
	PAX8-PPARγ	**EIF1AX**	**EZH1**	**GNAS**	**RAS**	**BRAF**	**RET/PTC**
FA	5–20%	5–10%	3%	~80%	HRAS 8%, KRAS 10%, NRAS 6%	-	-
NIFTP	5%	-	-	-	H/N/KRAS (45%)	-	-
HTT	-	-	-	-	NRAS 0%	-	47%
FT/WDT-UMP	<5%	-	-	-	HRAS 3–12%, KRAS 6–9%, NRAS 16–35%	-	-

For instance, paired box gene 8 (PAX8)-peroxisome proliferator-activated receptor-γ (PPARγ) rearrangements are detected in about 5–20% of FA, but they are absent in non-pathological thyroid parenchyma surrounding FA or in the hyperplastic nodules [47–49]. Of the other somatic genetic alterations, Eukaryotic Translation Initiation Factor 1A X-Linked (EIF1AX) gene activating mutation is found in 5–10% of FA [50,51], while telomerase reverse transcriptase (TERT) promoter mutation are very rare in genuine FA and occasionally present in FA with atypical features [50,52]. Additionally, mutations concerning Enhancer of zeste 1 polycomb repressive complex 2 subunit (EZH1) gene are detected in 3% of the FA, frequently in association with the TSH-receptor (TSHR) and/or the guanine nucleotide binding protein, alpha stimulating (GNAS) mutations, and accounting for nearly 80% of cases in some series [33,47,53]. RAS mutations exhibit different rates in FA: mutations in HRAS are detected in 8%, in NRAS in 6% and in KRAS in 10%, respectively [51,54,55]. Interestingly, RAS mutations have a higher prevalence in FA of persons living in area of iodine deficiency [56]. In UMP tumors, HRAS mutation are present in 3–12% of cases at codon 61, similarly to KRAS mutations (6–9% of cases), but less frequently than NRAS mutations (16–35% of cases) [50,51,54,55]. However, H/N/K-RAS mutations are detected in 45% of NIFTP cases, while $BRAF^{V600E}$ mutation and Rearranged during transfection (RET) fusions are absent [51,55]. Finally, HTT lacks BRAF or NRAS mutations, but it has considerable frequency of RET/PTC rearrangements (47%) similar to that encountered in PTC [55,57].

3. Phenotypic and Molecular Heterogeneity in FTC

Follicular thyroid carcinomas (FTCs), which are well-known more aggressive cancer compared with PTCs, have a prevalent histologic presentation as microfollicular or trabecular patterns, and a less frequent architecture with follicular and colloid-rich morphology [58,59]. There are also morphological rarer subtypes that are predictive of a worse prognosis, including spindle cells, clear cells, signet-ring cells, rhabdoid and insular phenotypes [60,61]. Generally, regardless of histotype, FTCs present a thin or thick fibrous capsule that contains some small vessels; consequently, capsular invasion produces an incomplete delimitation of the tumor and becomes an indicator of vascular invasion. Based on the extent of capsular/vascular invasion, FTCs may be divided into two subgroups of prognostic significance, minimally invasive FTCs (Figure 4A) and widely invasive FTCs [60,61]. In 2015 the prognostic subgroups became four: minimally invasive with capsular invasion, minimally invasive with limited [<4 vessels) vascular invasion, minimally invasive with extensive [≥4 vessels) vascular invasion and widely invasive [59,62]. Recently, the last 2017 WHO classification suggested a 3-tiered risk groups: minimally invasive [capsular invasion only), encapsulated angio-invasive and widely invasive [59–63].

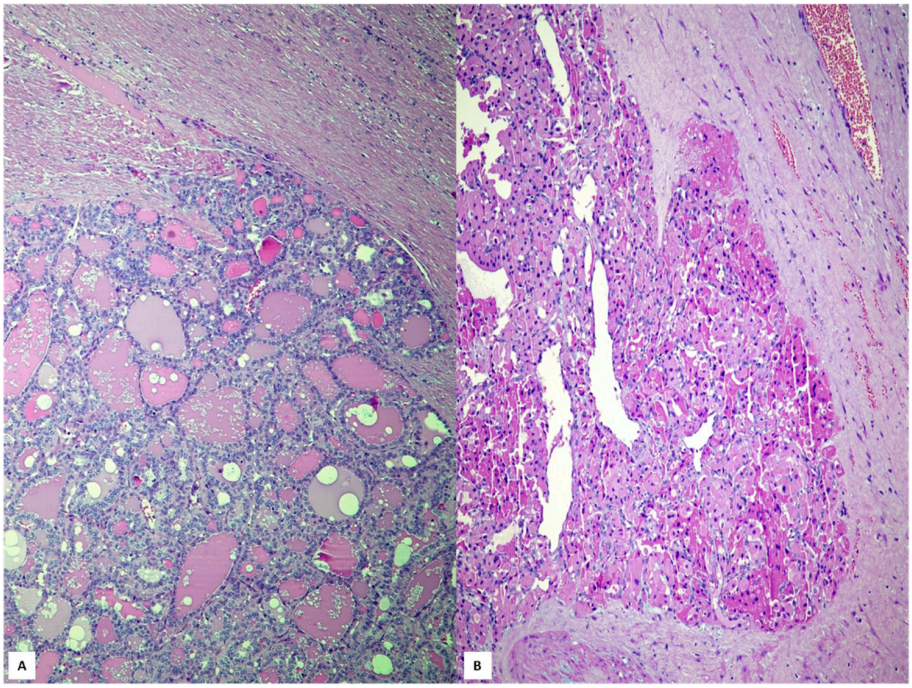

Figure 4. The follicular thyroid carcinoma with minimally invasive capsular infiltration ((**A**), hematoxylin and eosin, 80×); malignant Hürthle cell tumor characterized by an evident capsular invasion ((**B**), hematoxylin and eosin, 100×).

From a molecular perspective, the major driving mutations of FTCs are those in the RAS family of genes; for this reason, these tumors are also known as RAS-like tumors [30,51,64,65] (Table 2).

Table 2. Histological and molecular heterogeneity in FTC and HCT.

	Mutations								
	PIK3CA	**EIF1AX**	**TP53**	**PAX8-PPARγ**	**RAS**	**BRAF**	**TERTp**	**NF1**	**MADCAM-1**
FTC	10%	6%	3%	10–50%	HRAS 8%, KRAS 6%, NRAS 19%	1%	17–25%	-	-
Poorly differentiated FTC	0–15%	-	10–30%	<3%	H/N/KRAS (10–40%)	5–30%	-	-	-
HCT	-	11%	7%	<5%	KRAS 11%NRAS 6%	-	17%	7%	20%

In detail, the three concurrent somatic H/N/K-RAS mutation were detected with different percentage, 8%, 19% and 6% respectively [51]. In addition, TERT promoter (TERTp) mutations have been revealed in 25% of FTCs, which are characterized by older age of patients, larger tumor size, advanced stage (III–IV), distant metastases and disease specific mortality [52]. Several other genes, which are generally involved in Phosphoinositide 3-kinases (PI3K)/Phosphatase and tensin homolog (PTEN)/AKT pathway have been found as mutated in FTCs [66,67]. PIK3CA copy gains are encountered in FTCs (10%) in comparison to BRAF mutations (1%) [67,68]. PTEN and PIK3CA mutations as well as PIK3CA copy gains rarely coexist in FTCs, while PI3K-PTEN-AKT pathway is common in poorly differentiated and anaplastic thyroid carcinomas, suggesting their important role in tumor progression [68,69]. Moreover, in FTCs EIF1AX mutation was identified in

6% of cases related with advanced disease [67,68]. ITH was also detected in FTCs with a range of histologic aspects, namely with follicular areas coexisting with poorly differentiated ones [67]. Interestingly, in poorly differentiated aggressive FTCs are characterized by frequent mutations in p53 (10–30%), RAS (10–40%), BRAF (5–30%) [69,70], but rare PAX8/PPARγ rearrangements (7–10%) have been reported [71,72]. Nevertheless, in FTCs PAX8/PPARγ rearrangements were revealed in female and younger patients with high cellularity and invasive aspects; this positively rearranged FTC documented a lower risk for distant metastasis [50].

Even if classified as follicular-patterned tumors, Hürthle cell tumors (HCTs) present peculiar microscopic characteristics consisting in large elements with abundant eosinophilic granular cytoplasm, centrally located nuclei and prominent nucleoli [73]. The new WHO classification distinguishes benign and malignant HCTs on the basis of capsular and vascular invasion (Figure 4B), similarly to FTCs [73]. Although believed to have a poorer prognosis compared to FTCs, it was demonstrated that Hürthle cell cancer has not higher rates of recurrence and does not concentrate less radioiodine [74–76]. Nevertheless, it has been reported that somatic genomic alterations in malignant HCTs are represented by Mucosal Vascular Address in Cell Adhesion Molecule 1 (MADCAM-1) (20%), EIF1AX (11%), DAXX, PT53 (7%) and Neurofibromatosis type 1 (NF1) (7%) mutations, while no BRAF mutations and a lower rate of NRAS (9%) mutation are encountered in comparison to FTC cases [77–79] (Table 2). In addition, TERTp and KRAS mutations have been identified in 17% and 11% respectively; NRAS occurred with a lower percentage (6%) [51].

4. Phenotypic and Molecular Heterogeneity in PTC and Its Variants

As well known, PTCs are not only the most common DTCs, but also the most common malignant entity, in that they account for over 70% of all thyroid neoplasms [80]. The classical variant presents typical microscopic features (Figure 5A), such as overlapping and clearing nuclei, irregularities of nuclear membrane in papillary architecture with fibrovascolar cores, psammoma bodies and sometimes aggregates of lymphocytes. Sixteen PTC variants with different behavior have been reported so far [81–86]. After the classic variant, the most common variants are the follicular one (Figure 5B), hobnail/micropapillary (Figure 5C), Whartin-like (Figure 5D) and solid one [81–83]. In spite of this morphological variability, molecular ITH is not constantly present, even in multifocal PTCs [84–86].

Genetic ITH of PTCs was scarcely addressed so far, partly due to the relatively low number of oncogenes involved in the early stages [87–89] (Table 3). BRAF mutations have been reported in 55% of the classical phenotype with a further significant increase in more aggressive and poorly differentiated PTCs [90,91], and in up to one third of cases of the columnar-cell variant [92,93]. In addition, BRAF mutations are frequently combined with TP53, TERTp, PIK3CA, catenin β-1 (CTNNB1), epidermal growth factor receptor (EGFR), v-akt murine thymoma viral oncogene homolog 1 (AKT1) and Notch homolog-1 (NOTCH1) mutations [83,94–96]. In the hobnail variant, the mutations detected concern BRAF (25%), TP53 (55%) and NOTCH1 (5%) [83,94–96]. It is well known that mutations in BRAF and RET genes (see below) may occur both in the initial steps of carcinogenesis and in the advanced ones [97,98]. However, different foci of the same PTC may differ for their BRAF status, and such difference may also exist between a primary PTC and any of its lymph node and/or distant metastases in up to one third of cases [97–99]. For instance, $BRAF^{V600E}$ mutation may occur either *de novo* in metastasized lymph nodes, or in metastasizing mutated cells could spread from non-analyzed PTC foci of the primary tumor [100–102]. ITH in $BRAF^{V600E}$ have been also demonstrated, since only less than 50% of neoplastic elements manifested BRAF mutation [103,104]. Although, the prognostic role of $BRAF^{V600E}$ mutation is still debatable, some studies showed an association with poor outcome, extra thyroid neoplastic extension and increased recurrence risk in PTCs [50,96–98]. By contrast, PTC with low risk clinicopathological features did not exhibit $BRAF^{V600E}$ mutations [101–103].

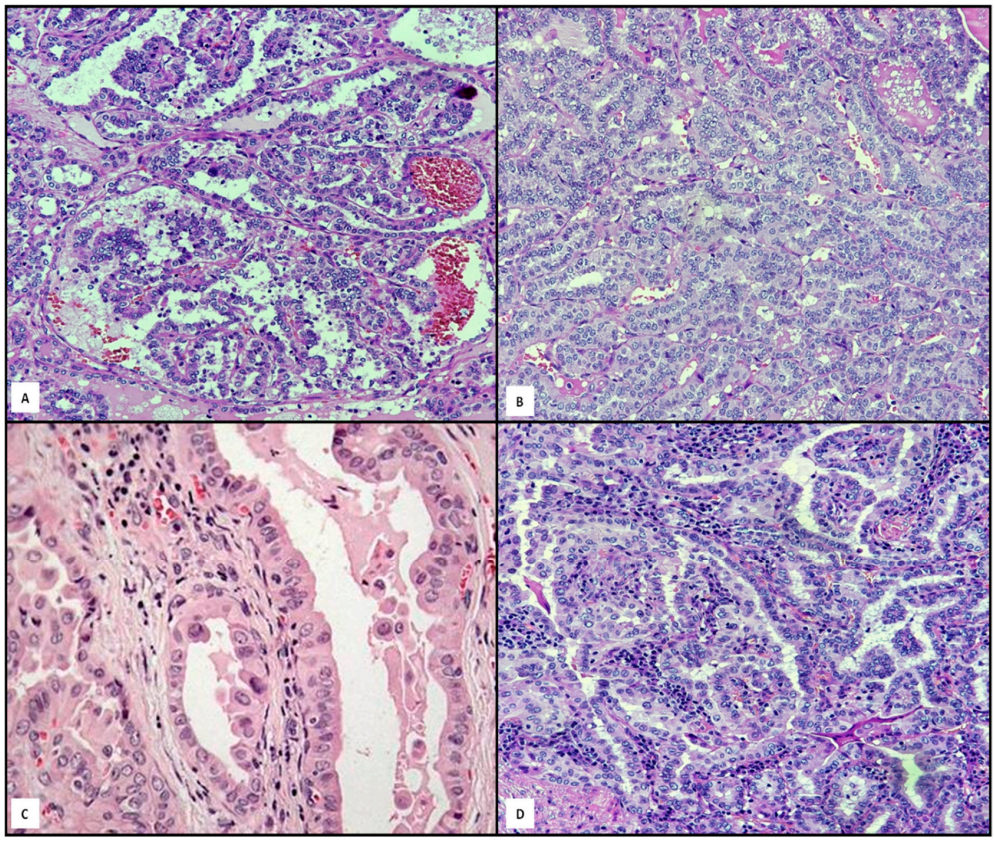

Figure 5. A gallery of some relevant variants of PTC: classical ((**A**), hematoxylin and eosin, 120×); follicular ((**B**), hematoxylin and eosin, 120×); Whartin-like ((**C**), hematoxylin and eosin, 120×); micropapillary ((**D**), hematoxylin and eosin, 160×).

Table 3. Histological and molecular heterogeneity in PTC.

	Mutations							
	EIF1AX	**TP53**	**RAS**	**BRAF**	**TERT**	**RET**	**ALK**	**NOTCH1**
Classical PTC	0–5%	-	H/N/KRAS 6%	55%	5–15%	5–25%	-	-
Clear cell/solid variant PTC	-	-	H/N/KRAS 30%	>55%	-	-	5%	-
Columnar variant PTC	-	-	-	33%	-	-	-	-
Tall cell variant PTC	-	-	-	80–100%	-	-	-	-
Hobnail variant PTC	-	55%	-	25%	-	-	-	5%

Another common genetic alteration in PTCs is the RET/PTC rearrangement [105,106], which occurs in one third of cases of sporadic PTCs in adults, in half of cases of PTCs in children and young adults, mainly when lymph node metastasis and aggressive clinicopathological features were documented, similarly to NTRK rearranged PTC [107–109]. However, it has been shown that PTCs characterized by fusion oncogene (RET or NTRK) exhibited overlapping clinical behavior [109]. RET/PTC rearrangement has been also frequently observed in subjects exposed to radiation, either accidentally or therapeutically [110]. Moreover, this genetic alteration early occurs in thyroid carcinogenesis, being essentially restricted to PTCs and Hürthle cell tumors [111,112]. Finally, a low rate (1–5%) of PTCs documented ALK rearrangement in predominant follicular solid infiltrative pattern or in diffuse sclerosing variant, showing sometimes extrathyroidal extension as well as lymph node metastases [113].

As said at the beginning of this review, different methods of detection (immunohistochemistry, RT-PCR, RNA analysis after Laser Capture Microdissection) are able to detect RET/PTC rearrangements, the distribution of which may be influenced by intrinsic genetic [111]. Interestingly, Schopper et al. tested a panel of 8 cancer-related genes (BRAF, KRAS, HRAS, NRAS, EGFR, PIK3CA, KIT, and platelet-derived growth factor receptor α polypeptide [PDGFRA]) by using next-generation sequencing (NGS) in a single thyroid tumor presenting as a combination of conventional PTC with 4 variants (follicular, clear cell, columnar and poorly differentiated [112–115]. While conventional PTCs showed only a limited rate of H/N/KRAS mutation (6%), the clear cell and the follicular variants harbored KRAS mutations up to 5 times more frequently, viz. 30% and 20% respectively [114–116]. Finally, in PTCs the degree of DNA methylation is smaller than in follicular tumors (FA and FTC), and it varies according to BRAF and RAS status [117].

Despite a robust line of research, clinical implications of ITH in PTCs are questioned. Indeed, two studies demonstrated that allelic frequencies of mutated alleles are consistent with a monoclonal origin of PTCs, suggesting ITH in as many as ~10% of tumors [117,118].

5. Conclusions

ITH influences tumor progression and response to treatment, as the appearance of resistant clones due to the selection pressure of treatment may worsen the patient's prognosis. Therefore, ITH profiling can be useful to characterize thyroid cancer pathogenesis, together with the analysis of different genetic alterations associated with oncological risk. Nowadays, molecular testing in DTCs suggests a high risk for recurrence of cancer associated with $BRAF^{V600E}$, RET/PTC 1/3, ALK and NTRK fusions, while the intermediate risk may be related to $BRAF^{K601E}$, H/K/N RAS and PAX8/PPARγ (Figure 6).

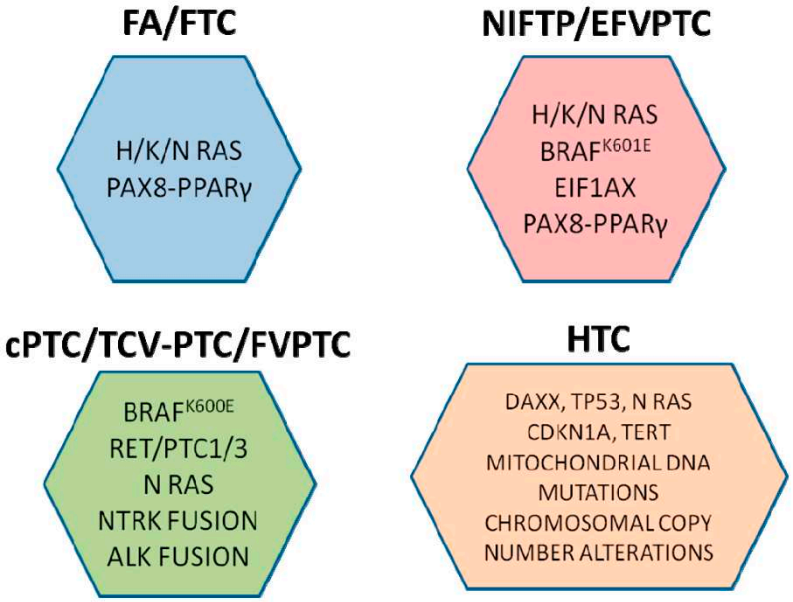

Figure 6. Synopsis showing the main biomolecular mutations in DTCs related to different histotypes.

Consequently, it may be suggested that tumor genotype is associated with peculiar phenotype; therefore, the identification of DTC morphology may be the driver to select different neoplastic portions in which molecular heterogeneity could be revealed. From this point of view, neoplastic sub-populations with different risk of recurrence or metastasis may be advantageously stratified, correctly treated and subjected to a shorter follow-up period. Recently, to measure ITH in cancer, an index has been proposed [119]; in detail, some neoplasms such as uterine carcinosarcoma, colorectal adenocarcinoma and ovarian cancer have been observed to be more heterogeneous than renal clear cell carcinoma and DTC [119].

Recent studies based on genetic analysis of thyroid tumors have brought several intriguing therapeutical personalized options for DTCs [120]. This innovative vision in which particularly targeted therapies based on specific diagnostic tests has been defined as "theranostics", in order to provide a transition from conventional to a contemporary personalized medicine [121]. The first theranostic agent has been considered as the radioiodine treatment widely used for the management of DTC. Nevertheless, about 65% of the patients with advanced thyroid disease may became radioiodine-refractory related to the sodium/iodide symporter (NIS) [120,121]. Therefore, the targeted therapy of DTC should be connected to the genetic and epigenetic alterations and signaling pathways. In detail, PPARγ agonists, HDAC inhibitors, PI3K/AKT inhibitors and MEK/ERK inhibitors, have been recommended for NIS over-expression and have caused improved iodine uptake in thyroid cancers [121]. Moreover, it was shown that Dabrafenib represents the selective inhibitor of mutated forms of *BRAF* and it can realize the radioiodine uptake in metastatic PTC $BRAF^{V600E}$-mutant iodine-refractory patients. Similarly, some molecular markers such as *p53*, *PIK3CA*, *CTNNB1* and *AKT1* may be considered indicators for an aggressive behavior of DTCs [121]. Furthermore, since fine needle aspiration cytology (FNAC) has been considered the commonly utilized morphological test, the molecular profiling may improve the diagnostic accuracy mainly in indeterminate or gray zone, furtherly supporting a personalized treatment for DTCs [122].

A better understanding of the molecular basis of thyroid cancers as well as development of more effective cancer therapies has revolutionized the treatment approach in patients with advanced thyroid cancer. Nevertheless, whether overall survival is improved with the use of these agents is still unclear. In fact, the major limitation in applying targeted therapies is their side-effects profile, as well as in the development of escape and resistance mechanisms by the tumors. Specifically, neoplastic cells may acquire resistance to the treatment by developing an escape mechanism against the targeting drugs. Consequently, most DTCs could develop resistance against targeted drugs by acquiring new mutations that result in over-activation of pathways or by induction of alternate pathways.

Nowadays, the cancer diagnosis should be assessed by a complex of information regarding to clinical, pathological, molecular and protein expression data of a specific neoplastic proliferation and its surrounding microenvironment; such an integrated system has been defined as "*tissunomics*" [123,124]. In full agreement with this approach, we contend that a systematic integration of morphology and molecular characteristics in DTC should be helpful in patient's management.

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Abbreviations

AKT1	v-akt murine thymoma viral oncogene homolog 1
DTC	differentiated thyroid carcinoma
DTT	differentiated thyroid tumor
EGFR	epidermal growth factor receptor
EIF1AX	Factor 1A X-Linked
EZH1	zeste 1 polycomb repressive complex 2 subunit
FA	follicular adenoma
FTC	follicular thyroid carcinoma
GNAS	guanine nucleotide binding protein, alpha stimulating
HTT	hyalinizing trabecular tumor
ITH	intratumoral heterogeneity
MADCAM-1	Mucosal Vascular Address in Cell Adhesion Molecule 1
NF1	Neurofibromatosis type 1
NGS	next-generation sequencing
NIFTP	noninvasive follicular thyroid neoplasm with papillary-like nuclear features
NOTCH1	Notch homolog-1
PAX-8	paired box gene 8
PDGFRA	platelet-derived growth factor receptor α polypeptide
PI3K	Phosphoinositide 3-kinases
PPARγ	peroxisome proliferator-activated receptor-γ
PTC	papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog
RET	REarranged during Transfection
H,K,NRA	isoforms of RAS gene family
TERT	telomerase reverse transcriptase
TSHR	TSH-receptor
UMP	uncertain malignant potential tumor
WDT-UMP	well-differentiated tumors with uncertain malignant potential

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Article

What Is the Optimal Duration of Adjuvant Mitotane Therapy in Adrenocortical Carcinoma? An Unanswered Question

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Abstract: A relevant issue on the treatment of adrenocortical carcinoma (ACC) concerns the optimal duration of adjuvant mitotane treatment. We tried to address this question, assessing whether a correlation exists between the duration of adjuvant mitotane treatment and recurrence-free survival (RFS) of patients with ACC. We conducted a multicenter retrospective analysis on 154 ACC patients treated for ≥12 months with adjuvant mitotane after radical surgery and who were free of disease at the mitotane stop. During a median follow-up of 38 months, 19 patients (12.3%) experienced recurrence. We calculated the RFS after mitotane (RFSAM), from the landmark time-point of mitotane discontinuation, to overcome immortal time bias. We found a wide variability in the duration of adjuvant mitotane treatment among different centers and also among patients cared for at the same center, reflecting heterogeneous practice. We did not find any survival advantage in patients treated for longer than 24 months. Moreover, the relationship between treatment duration and the

frequency of ACC recurrence was not linear after stratifying our patients in tertiles of length of adjuvant treatment. In conclusion, the present findings do not support the concept that extending adjuvant mitotane treatment over two years is beneficial for ACC patients with low to moderate risk of recurrence.

Keywords: mitotane; adjuvant treatment; adrenocortical cancer; recurrence; recurrence free survival; timing

1. Introduction

Adrenocortical carcinoma (ACC) is a rare tumor characterized by an aggressive disease course that limits long-term survival [1–3]. Disease-specific outcomes are better for patients bearing early-stage tumors that can be resected completely; however, post-operative recurrence of ACC may be considered as part of the natural history of the disease [4–8]. We have recently reported a recurrence rate of 62.5% among 152 patients with stage I to III ACC who underwent complete macroscopic resection, with a five-year recurrence-free survival rate of 38.1% [9].

The remarkable propensity of ACC towards recurrence despite complete surgical removal makes a strong case for an adjuvant therapy. Until now, the most followed adjuvant approach relied on mitotane, an old adrenolytic drug specifically approved for treatment of advanced ACC [10,11]. Use of adjuvant mitotane increased in clinical practice following the observation that adjuvant mitotane treatment was associated with prolonged recurrence-free survival (RFS) compared to surveillance without active treatment after surgery, in a retrospective study of 177 ACC patients managed at different institutions using either adjuvant mitotane or no treatment. In this cohort study, we included 47 patients followed at Italian reference centers that systematically adopted adjuvant mitotane to all radically operated ACC, and a group of 55 Italian patients and 75 German patients followed in centers not giving any post-operative treatment [12]. In this study, we showed that adjuvant mitotane treatment was associated with a significant survival advantage. Despite the retrospective nature of the study, this finding informed clinical practice, although adjuvant mitotane is not universally accepted and some experts argue against the value of this approach [13,14]. Critics of adjuvant mitotane therapy evoke the drug-related toxicity, the complexity of caring for patients on treatment, and the long duration of a treatment course [15].

The overall level of evidence available on adjuvant mitotane can be graded as low, and all recommendations are based on retrospective, non-randomized studies, plagued by potential bias and confounding [10,16]. Despite this evidence gap, adjuvant mitotane is advised for patients with ACC at high risk of recurrence in the clinical guidelines endorsed by the European Society for Endocrinology (ESE)–European Network for the Study of Adrenal Tumours (ENSAT) and by the European Society for Medical Oncology (ESMO) [10,17]. The guidelines underline that scant information is currently available on many aspects dealing with practical management of mitotane therapy. Recommendations on how to conduct adjuvant mitotane treatment are mostly based on expert opinions stemming from personal experience and practice [18]. As a consequence, care of patients treated with mitotane is heterogeneous depending on local preferences.

One of the most relevant and uncertain issues concerns the optimal duration of adjuvant mitotane treatment. The ESE-ENSAT guidelines and ESMO guidelines suggest continued use of adjuvant mitotane for at least 2 years, but not longer than 5 years [10,17]. Since no study has ever specifically addressed this issue, this recommendation is based on the observation that most recurrences of ACC occur within two years after resection, while after 5 years, the rate of recurrence is too low to justify continuation of adjuvant therapy.

What is the optimal duration of adjuvant mitotane treatment, however, remains controversial, and practice varies even among referral centers. This issue has important

consequences on both patient-centered outcomes, due to the unwanted effects of treatment and their impact on quality of life and health-care organizations, as surveillance of patients on mitotane is demanding and resource-consuming.

We tried to answer this question by organizing an international, multicentric, retrospective study aimed at assessing whether a correlation does exist between duration of adjuvant mitotane treatment and recurrence-free survival of patients with ACC.

2. Patients and Methods

2.1. Patients

We did an international, multicenter, retrospective analysis on 154 patients with ACC treated with adjuvant mitotane after radical surgery. Thirteen European centers and one center in Canada participated in the study.

To be included, patients had to meet the following inclusion criteria: age of 18 years or older at the time of diagnosis; histologically confirmed diagnosis of ACC (based on Weiss score [19]; ENSAT stage I-III [20]; R0 or Rx tumor resection, defined on the basis of a surgical report, pathology report, and post-operative imaging); treatment with adjuvant mitotane for at least 12 months following surgery; and clinical status being free of disease at the time of mitotane discontinuation. Exclusion criteria were: residual disease after resection, defined both microscopically or macroscopically (resection status, R1 or R2); patients concomitantly treated with other therapies (e.g., chemotherapy or radiotherapy); or patients experiencing ACC recurrence during adjuvant mitotane therapy. Follow-up for this study was closed in December 2017.

2.2. Methods

All data were obtained by reviewing patient history, medical records, and source documents. Data were processed by skilled and experienced personnel using specifically tailored data forms. We reported clinical and demographical characteristics, the date and type of surgery, stage at diagnosis, pathology reports (Weiss score and Ki-67 index), hormonal status, date of start and stop of mitotane treatment and reason for stops, date of recurrence and type of recurrence (single or multiple, local or distant), and the date of last follow-up or death. Date of diagnosis was defined as the date of surgery. Tumor stage was established according to the ENSAT classification (I, confined tumors $\leq$5 cm; II, confined tumors >5 cm; III, positive lymph nodes or infiltrating neighboring organs/veins without distant metastases; IV, distant metastases) [20]. Date of recurrence was defined as the date of radiological evidence of a new lesion. Patients underwent imaging follow-up (abdominal and thoracic computed tomography) every 3–4 months. Modalities of mitotane treatment, such as the initial high- or low-dose regimen, dose titration, and eventual dose changes due to toxicity were done according to local center preferences.

2.3. Statistical Analysis

The primary endpoint was to determine whether a correlation between duration of adjuvant mitotane treatment and patient survival did exist.

Frequencies and percentages were calculated for categorical data, and the median and interquartile range for continuous data. Differences in categorical variables were analyzed by means of the chi-squared test or Fisher test, as appropriate, while differences in continuous variables were analyzed by the Mann–Whitney U test. The survival curves were estimated with the Kaplan–Meyer product limit method. Recurrence-free survival (RFS) was calculated from the time of initial surgery to the first radiological evidence of recurrence. To adjust for the immortal bias due to the selection of patients who did not have recurrence on active treatment, we calculated the recurrence-free survival rate after adjuvant mitotane discontinuation (RFSAM) from the time of discontinuation of mitotane to ACC recurrence or end of follow-up. We calculated the overall survival rate after adjuvant mitotane discontinuation (OSAM) from the time of discontinuation of mitotane to the date of death. Patients who did not experience either of those events (recurrence or

death) were censored at the date of the last follow-up visit for the specific survival analysis. Cox proportional hazards regression models were fitted to determine prognostic factors on survival. The following potential predictive factors for RFS and RFSAM were investigated: patient sex and age, tumor stage, hormone secretion, Weiss score, Ki67 index, mitoses, resection status, and duration of mitotane treatment. A genetic algorithm was employed to select the variables that resulted in the best-fitted model according to AIC score [21]. Firth correction was applied to reduce the bias due to the small number of events [22]. All reported P values are two-sided. P-values of less than 0.05 were considered as statistically significant. All statistical analyses were performed using R version 4.0.2.

3. Results

Baseline characteristics of patients are reported in Table 1.

Table 1. Baseline characteristics of patients.

Characteristics	Valid Cases (*N*)	Values
Sex, N (%)	154	
Male		51 (33%)
Female		103 (67%)
Age at diagnosis, years	154	
Median (IQR)		45 (34–54)
Tumor stage at diagnosis, *N* (%)	154	
Stage I		14 (9%)
Stage II		110 (71%)
Stage III		30 (19%)
Hormone secretion at diagnosis, *N* (%)	113	
No		43 (38%)
Yes		70 (62%)
Glucocorticoid		41 (59%)
Androgen		21 (30%)
Aldosterone		5 (7%)
Other		3 (4%)
Tumor size, cm	148	
Median (IQR)		10 (7–15)
Ki67 at diagnosis	125	
Median (IQR)		10 (5–20)
$\leq$10%		75 (60%)
>10%		50 (40%)
Weiss at diagnosis	132	
Median (IQR)		6 (4–6)
Duration of mitotane therapy, months	154	
Median (IQR)		33 (24–59)

IQR = interquartile range.

In our series, female sex was more prevalent, and ACC presented, in most cases, as a stage II hormone-secreting tumor. The present series was skewed toward low-grade tumors with a Ki67 index of less than 10%. Median duration of adjuvant mitotane therapy was 33 months (IQR, 24–59), and median follow-up after mitotane discontinuation was 38 months (IQR, 24–61).

We stratified our patients into three groups by treatment duration (expressed in tertiles); group 1 included patients treated for 13–25 months, group 2 for 26–48 months, and group 3 for 49–143 months, respectively. Group 3 had a higher Ki67 index, longer RFS compared to groups 1 and 2, and longer RFSAM compared to group 2 (Table 2).

Table 2. Baseline characteristics of patients in different groups stratified by duration of therapy.

Characteristics	Group 1 n. 52 Patients (Treated for 13–25 Months)	Group 2 n. 51 Patients (Treated for 26–48 Months)	Group 3 n. 51 Patients (Treated for 49–143 Months)	*p*-Value
Sex, *N* (%)				0.15
Male	16 (31%)	13 (25%)	22 (43%)	
Female	36 (69%)	38 (75%)	29 (57%)	
Age at diagnosis, years				0.15
Median (IQR)	47.5 (38.5–58)	45 (32.5–53)	43 (34–51.5)	
Tumor stage at diagnosis, *N* (%)				0.44
Stage I	4 (8%)	5 (10%)	5 (10%)	
Stage II	41 (79%)	37 (72%)	32 (63%)	
Stage III	7 (13%)	9 (18%)	14 (27%)	
Hormone secretion at diagnosis, *N* (%)				0.41
No	31 (60%)	24 (47%)	29 (57%)	
Yes	21 (40%)	27 (53%)	22 (43%)	
Size tumor at diagnosis, cm				0.80
Median (IQR)	9.5 (7.2–14.5)	10.5 (7.6–14)	10 (6.7–15.5)	
Ki67 at diagnosis				0.014
Median (IQR)	10 (5–10)	10 (5–19)	15 (6–23)	
Weiss at diagnosis				0.39
Median (range)	5 (4–6)	6 (4–7)	6 (5–7)	
Recurrence, *N* (%)				**0.001**
No	47 (90%)	38 (75%)	50 (98%)	
Yes	5 (10%)	13 (25%)	1 (2%)	
RFS, months				**<0.001**
Median (IQR)	61 (49–97)	59 (48–85)	108 (90–151)	
RFSAM, months				**0.002**
Median (IQR)	38 (26–78)	22 (11–47)	35 (24–62)	
OSAM, months				0.19
Median (IQR)	44 (26–78)	37 (22–53)	35 (27–62)	

IQR = interquartile range; RFS = recurrence free survival; OSAM = overall survival after adjuvant mitotane discontinuation; RFSAM = recurrence free survival after adjuvant mitotane discontinuation. Statistically significant differences are presented in bold.

In most cases, mitotane was interrupted at the end of the scheduled period. Only in a few patients, treatment-related unwanted effects induced mitotane stop (Table 3), mainly during the second year of therapy.

Table 3. Causes of mitotane discontinuation in different groups stratified by duration of therapy.

Causes of Mitotane Discontinuation	Group 1 n. 52 Patients (Treated for 13–25 Months)	Group 2 n. 51 Patients (Treated for 26–48 Months)	Group 3 n. 51 Patients (Treated for 49–143 Months)
End of schedule	30 (57.7%)	38 (74.5%)	47 (92.2%)
Adverse effects	20 (38.5%)	8 (15.7%)	2 (3.9%)
Unattainable target level	0	2 (3.9%)	0
Severe concomitant disease	1 (1.9%)	1 (1.9%)	0
Other *	1 (1.9%)	2 (3.9%)	2 (3.9%)

* patient willing, unexpected pregnancy.

After excluding patients in which mitotane withdrawal was determined by adverse effects, we observed a wide variability in the duration of adjuvant mitotane, either among different centers or in the same center, as shown in Figure 1.

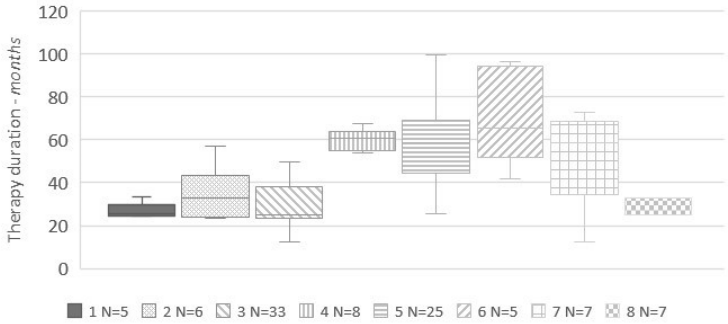

Figure 1. Distribution of the duration of adjuvant mitotane for each center (only centers with at least five patients have been included in this analysis). 1 = Berlin; 2 = Munich; 3 = Wurzburg; 4 = Florence; 5 = Orbassano; 6 = Padua; 7 = IGR (Villejuif); 8 = Montreal. N = number of patients.

In our series, 19 patients experienced recurrence after mitotane discontinuation. Recurrence types were almost equally distributed between those which were local (10 cases, 53%) and distant (9 cases, 47%); the last ones mainly in the lung (6 cases). After mitotane discontinuation, death occurred in three patients, but in only one case the death was cancer-related.

To assess where any correlation did exist between adjuvant mitotane duration and RSF, we tried different approaches. We stratified our patients by the value of 24 months' treatment duration. The comparison of the survival curves of patients treated up to 24 months vs. patients treated for a longer period, both for RFS (Figure 2) and RFSAM (Figure 3), did not show any significant difference.

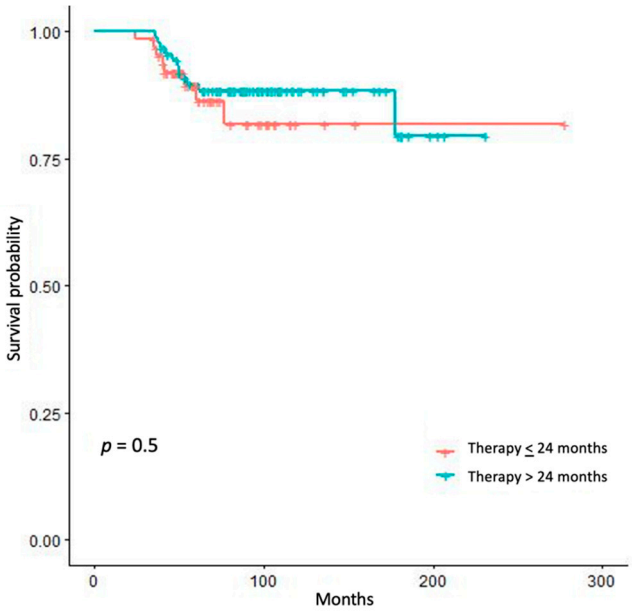

Figure 2. Kaplan–Meier estimates of recurrence-free survival (RFS) in patients treated <24 months versus patients treated >24 months.

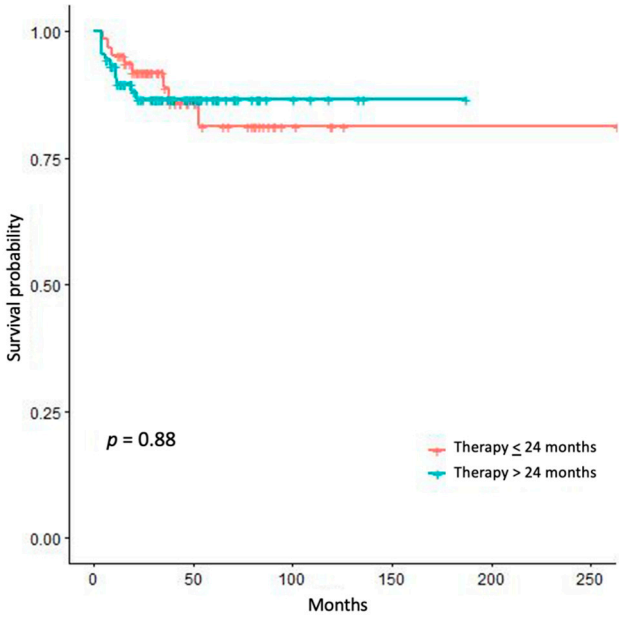

Figure 3. Kaplan–Meier estimates of recurrence free survival after adjuvant mitotane discontinuation (RFSAM) in patients treated <24 months vs. patients treated >24 months.

We performed univariate analyses, both for RFS (Table 4) and RFSAM (Table 5), without identifying any significant prognostic factors.

Table 4. Univariate analysis of predictive factors for recurrence-free survival (RFS).

Univariate Analysis	Diff	HR	95% CI		*p*
Duration of mitotane therapy $^{+}$		1.302	0.509	3.334	0.58
§ R status		0.722	0.208	2.503	0.61
‡ * Hormone secretion		1.441	0.571	3.640	0.44
* $^{\circ}$ Stage		0.917	0.272	2.526	0.87
* Tumor size	7.925	0.942	0.514	1.727	0.85
* Weiss	2.000	1.589	0.861	2.932	0.14
* Ki67%	15.000	0.805	0.426	1.521	0.50

* at diagnosis; Reference categories: $^{+}$ patients treated with mitotane ≤27 months, ‡ Secreting tumors, $^{\circ}$ Stage III, § RX.

Table 5. Univariate analysis of predictive factors for recurrence free survival after adjuvant mitotane discontinuation (RFSAM).

Univariate Analysis	Diff	HR	95% CI		*p*
Duration of mitotane therapy $^{+}$		0.894	0.354	2.257	0.812
§ R status		0.843	0.243	2.924	0.788
‡ * Hormone secretion		1.357	0.543	3.391	0.513
* $^{\circ}$ Stage		1.118	0.342	2.993	0.838
* Tumor size	7.925	0.977	0.532	1.792	0.939
* Weiss	2.000	1.766	0.957	3.260	0.069
* Ki67%	15.000	0.820	0.442	1.521	0.529

* at diagnosis; Reference categories: $^{+}$ patients treated with mitotane ≤27 months, ‡ Secreting tumors, $^{\circ}$ Stage III, § RX.

At a multivariate level, the variables' duration of adjuvant mitotane, which was modelled with a spline to account for non-linearity, sex, and Weiss were selected in the best-fitted model. Duration of adjuvant mitotane was the only statistically significant factor associated with RFS (HR 0.549, 95% CI 0.306–0.983; $p = 0.044$). The HR was calculated on a difference of 18 months in the duration of therapy, that is, 18 months' increase of adjuvant

mitotane therapy duration is associated with about 45% reduction in the hazard of RFS. No statistically significant factor resulted to be associated with RFSAM, or OSAM.

4. Discussion

The present findings do not support the concept that a longer duration of mitotane therapy (more than 2 years) is associated with a survival advantage. Since the present series was enriched with low-risk tumors, these results may be not generalizable to high-risk ACC. Although RFS was prolonged in patients treated for more than 4 years, this finding may likely be the consequence of immortal time bias.

The inclusion criteria of the study may induce an immortal bias in patients scheduled to be treated for longer periods, since these patients could not have recurred in the months prior to mitotane discontinuation, as otherwise they would have been excluded from analysis. For this reason, we primarily focused on the outcome after the end of scheduled adjuvant treatment, of whatever duration it was. We calculated the recurrence-free survival rate after mitotane (RFSAM), from the landmark time-point of mitotane discontinuation, since landmark analysis is a method used to overcome immortal time bias [23].

The duration of mitotane therapy was a factor associated to RFS but not RFSAM, and this militates against an actual benefit of prolonging adjuvant mitotane treatment. Along these lines, the breakdown of RFS by 24 months of treatment duration, which is the recommended time-length of adjuvant mitotane according to the ESE-ENSAT and ESMO guidelines [10,17], did not disclose any survival advantage of patients treated for longer. Moreover, the relationship between treatment duration and the frequency of ACC recurrence was not linear after stratifying our patients in tertiles of length of adjuvant treatment.

An interesting finding is that a large variability in the duration of adjuvant mitotane treatment does appear between different centers, and also among patients cared for at the same center. This figure reflects uncertainty on management and heterogeneous practice. However, it appears that physicians were more eager to treat patients with unfavorable prognostic factors (higher Ki-67 index) for longer periods, although our series was selected toward low-risk tumors due to the specific inclusion criteria of the study (patients who did recur on treatment were excluded). That said, a sort of "geographical" pattern does appear, since at centers in Germany, adjuvant mitotane was generally discontinued after two years, whereas in Italian centers it was usually more prolonged. In these centers, however, there was a huge variability between patients, suggesting that a tailored approach, taking into account patient preferences and biological characteristics of ACC, was followed. The heterogeneity in practice between expert centers underlines the lack of evidence on this issue since, to the best of our knowledge, this is the first study that specifically addresses the issue of the duration of adjuvant mitotane therapy. As a matter of fact, duration of adjuvant mitotane was very heterogenous between studies on this topic [14,24–28].

The main issue with a retrospective analysis of treatment duration is that in many cases, the length of adjuvant treatment is set by the timing of ACC recurrence, and not by the planned treatment schedule. In this retrospective multicenter study carried out in referral centers for ACC that are part of ENSAT, we tried to overcome this problem by including only patients treated with adjuvant mitotane for at least 12 months, in which mitotane was suspended for reasons different from recurrence of disease. Consequently, all patients were free of disease at the end of adjuvant therapy.

Recognizing that a prospective randomized trial that includes patients with ACC treated with adjuvant mitotane for different, pre-specified time lengths is the best way to define what is the optimal duration of treatment, it can be plainly accepted that such a trial is not on the horizon for the near future. Up to now, only two randomized trials have been concluded on ACC [29,30], and this outlines how it is challenging to implement a randomized trial in a rare tumor such as ACC. Therefore, a well-designed retrospective study is almost all that can be done to answer this important clinical question.

5. Conclusions

In this study, we have addressed the challenging issue of identifying the optimal duration of adjuvant mitotane therapy in a retrospective analysis. We tried to overcome the bias and confounding inherent to a retrospective analysis of treatment duration by multiple approaches. With all the disclosed limits of our study, the present findings do not support the concept that extending adjuvant mitotane treatment over two years is beneficial for patients with ACC at low risk of recurrence. Conversely, patients with ACC at high risk of recurrence were under-represented in this study, thus precluding any definitive recommendations.

Answering the question of what is the optimal duration of adjuvant mitotane is an unmet clinical need, since current practice is heterogeneous and mainly dependent on personal preferences and expertise. To the best of our knowledge, no previous study has specifically addressed this point, and the recommendation of a standard duration of adjuvant mitotane treatment of two years is not based on specific evidence. Lacking randomized studies, which will be hardly feasible in the future, the present study does provide the only evidence available on this complex issue. Finding that no obvious advantage is associated to prolonged adjuvant mitotane treatment provides some guidance for the care of patients with ACC, and sparing low-risk patients from long exposure to a toxic treatment matters for clinical practice.

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The Role of Immunohistochemical Markers for the Diagnosis and Prognosis of Adrenocortical Neoplasms

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Abstract: Adrenal cortical carcinoma (ACC) is a rare cancer with poor prognosis that needs to be distinguished from adrenocortical adenomas (ACAs). Although, the recently developed transcriptome analysis seems to be a reliable tool for the differential diagnosis of adrenocortical neoplasms, it is not widely available in clinical practice. We aim to evaluate histological and immunohistochemical markers for the distinction of ACCs from ACAs along with assessing their prognostic role. Clinical data were retrospectively analyzed from 37 patients; 24 archived, formalin-fixed, and paraffin-embedded ACC samples underwent histochemical analysis of reticulin and immunohistochemical analysis of p27, p53, Ki-67 markers and were compared with 13 ACA samples. Weiss and Helsinki scores were also considered. Kaplan−Meier and univariate Cox regression methods were implemented to identify prognostic effects. Altered reticulin pattern, Ki-67% labelling index and overexpression of p53 protein were found to be useful histopathological markers for distinguishing ACAs from ACCs. Among the studied markers, only pathological p53 nuclear protein expression was found to reach statistically significant association with poor survival and development of metastases, although in a small series of patients. In conclusion, altered reticulin pattern and p53/Ki-67 expression are useful markers for distinguishing ACCs from ACAs. Immunohistopathology alone cannot discriminate ACCs with different prognosis and it should be combined with morphological criteria and transcriptome analysis.

Keywords: adrenocortical cancer; adrenal adenomas; adrenal tumors; p53; p27; ki-67; reticulin

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1. Introduction

Adrenocortical carcinoma (ACC) is a highly aggressive malignancy with an estimated worldwide prevalence of 4–12 cases per million adults and a five-year survival rate ranging from 16 to 38% [1,2]. Although several different scoring systems have been proposed to assess the malignant potential in adrenocortical neoplasms, the Weiss score remains the most utilized tool in distinguishing benign from malignant adrenocortical neoplasms [3]. This score counts nine histopathologic criteria: eosinophilic ("dark") cytoplasm in more than 75% of tumor cells, a "patternless" diffuse architecture, necrosis, nuclear atypia, mitotic index above 5 per 50 high-power fields, atypical mitoses, sinusoidal, venous, and capsular invasion [4]. An adrenocortical neoplasm is classified as malignant when it meets three or more of these criteria [5]. However, the distinction of noninvasive low-grade ACC with a low Weiss score from adrenocortical adenoma (ACA) poses a diagnostic

challenge especially in small-sized and purely localized lesions and in large tumors without invasive features or cellular atypia in which well-differentiated cells resemble those seen in ACAs [2,6].

In addition, intratumoral morphologic, proliferative, and molecular heterogeneity have been recognized in these adrenocortical neoplasms. Microscopic regions with low-grade proliferative features can be encountered in high-grade ACCs, and low-grade ACCs can contain areas indistinguishable from ACAs [7,8]. Furthermore, recent observations also suggest the possibility of adenoma−carcinoma progression in some adenomas although this needs to be confirmed [9–12]. In the last decade there has been enormous progress in our understanding of the molecular biology of adrenocortical neoplasms [13–15]. The development of genomics has led to a new classification of ACC by two independent international cohorts; one from the European Network for the Study of Adrenal Tumors (ENSAT) network [14] in Europe and the other from the Cancer Genome Atlas [8] consortium in America, Europe and Australia, with two distinct molecular subgroups, C1A and C1B associated with poor (5-year survival rate of 20%) and good prognosis (5-year survival rate of 91%), respectively [7,16,17]. The C1B group is characterized by low mutation rate, and a very low incidence of mutations of the main driver genes of ACC whereas the C1A group is characterized by high mutation rate and driver gene alterations. This group is further divided into a subgroup of aggressive tumors showing hypermethylation at the level of the CpG islands located in the promoter of genes ("CIMP phenotype").

However, this transcriptome analysis is still not widely available making it necessary to utilize currently readily available histochemical and immunohistochemical markers for the distinction of adrenocortical neoplasms and their prognosis.

Several immunohistochemical markers have been proposed to improve the histological recognition of malignancy and eventually obtain a more precise characterization of these histologically characterized "grey zones" [16,18–20]. In order to provide prognostic biomarkers for the evaluation of surgical samples with adrenocortical neoplasms, we investigated the role of altered reticulin framework, a fast and cheap technique with high interobserver reproducibility, as well as of proteins involved in cell proliferation and mitotic spindle regulation such as Ki-67, p53, and p27 in a surgical series of benign and malignant adrenocortical neoplasms. We also studied their association with the clinical prognosis of patients with ACCs including progression-free survival (PFS) and overall survival (OS).

2. Materials and Methods

2.1. Samples and Clinicopathologic Parameters

We identified 65 consecutively treated patients with histologically confirmed ACCs (n = 35) and ACAs (n = 30) from the Endocrine Unit of the Laiko General Hospital. Paraffin-embedded blocks were available for 37 of these patients (24 ACCs and 13 ACAs). All surgical samples were reviewed by two experienced pathologists blinded to clinical history or outcome. The protocol of this study was approved by the institutional Research Ethics Board of the National and Kapodistrian University of Athens.

In all cases investigated, three consecutive 4 μm thick tissue sections were obtained from a representative neutral buffered formalin-fixed, paraffin-embedded sample. ACAs and ACCs were defined grossly and microscopically following the criteria and the nomenclature system of pathological features proposed by Weiss et al. [3]. All primary malignant adrenal tumors reviewed as part of this study demonstrated three or more of the histopathologic criteria needed for the diagnosis of ACC [3].

Markers of adrenal cortical differentiation (Steroidogenic Factor 1 (SF)-1, Melan-A, calretinin, alpha-inhibin, and synaptophysin) were applied at the time of the diagnostic workup of each neoplasm. All adrenal cortical neoplasms were classified according to the universal diagnostic criteria endorsed by the WHO classifications including the modified Weiss criteria as well as the Lin−Weiss−Bisceglia criteria [21,22]. The mitotic grade was assessed based on mitotic count in 50 high power fields (HPF) from high mitotic density

areas in all samples. ACCs displaying up to 20 mitotic figures per 50 HPF were classified as low-grade carcinomas, whereas those exceeding 20 mitotic figures per 50 HPF were recorded as high-grade carcinomas [23,24]. Vascular invasion was defined by tumor cells invading through a vessel wall and/or intravascular tumor cells admixed with thrombus was recorded in all ACCs.

The available follow-up clinical information was reviewed to determine the status of disease including relapse and mortality rate, distant metastasis, PFS and OS.

2.2. Histochemistry and Immunohistochemistry

Each section series was stained with different methods:

Hematoxylin-Eosin (HE) to confirm the diagnosis of adrenal nodular lesions.

Monoclonal antibodies against Ki-67 (clone MIB-1, DAKO), p53 (Mouse clone DO-7, DAKO) and p27 (Mouse clone SX53G8, DAKO).

Formalin-fixed paraffin-embedded tissue sections (4 μm) were dewaxed in 5 changes of xylene and rehydrated through graded alcohols. Negative and positive control tissues were selected based on manufacturer recommendations (p27) as well as previous publications where these antibodies were applied (p53 and Ki-67%) [25]. Multiple control experiments were undertaken to optimize each antibody. Endogenous peroxidase was blocked with 3% hydrogen peroxide. For the p27 (clone SX53G8, DAKO) and p53 (clone DO-7, DAKO) immunohistochemistry the BOND Polymer Refine Detection System (Leica Biosystems) was used which contains a peroxide block, postprimary, polymer reagent, DAB chromogen and hematoxylin counterstain all ready-to-use for the automated BOND system. The Ki-67 (clone MIB-1, DAKO) immunohistochemistry was performed in an automated stainer (Ventana Benchmark).

Tissue microarray assays (TMA) blocks were subjected to Gordon-Sweet Silver histochemistry in order to reveal the reticulin framework in all tumors. The loss of reticulin network was scored as follows: score 1: no loss of reticulin framework; score 2: minimal loss (<25%) of reticulin framework; score 3: focal loss (25% to 50%) of reticulin framework; and score 4: obvious loss (>50%) of reticulin framework. Qualitative pattern changes on the reticulin framework were also documented [26].

For the evaluation of the Ki -67 proliferation index we calculated the percentage of positive cells by manual count of the hot spot area which always contained at least 500 cells. Weakly stained nuclei were also counted [27].

For the evaluation of p53 the recently suggested tripartite interpretation guide was used where an "overexpressed or no expression (all or nothing)" nuclear staining pattern was highly predictive of an underlying TP53 mutation while a normal/wild type pattern was not. The distribution of nuclear staining in a "wild type" pattern ranged from a few positive cells to almost all ("high" wild type staining due to high proliferation) cells staining, but with variable intensity with a few nuclei stained strongly. Overexpression defined as nuclear staining in at least 50% of tumor cell nuclei while overexpression in at least >80% was considered strongly associated with TP53 mutations [28,29].

For p27/kip-1 protein there was a qualitative and quantitative evaluation of the nuclear expression of the protein (analyzed by the pathologists) in the tumor cells where the intensity of the expression was determined as 0 (no expression), 1 (weak expression), 2 (moderate expression) and 3 (strong expression) and the percentage of tumor cells expressed p27/kip-1 was scored as 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), 4 (76–100%). The percentage of positive nuclei of cells was calculated in more than 1000 cells of five successive and representative high-power fields ($\times$400 magnification microscope). The immunoreactive score (IRS) was applied to determine the final staining score by multiplication of the intensity score and the distribution score [30].

2.3. Statistical Analysis

All the data are reported as median (range) for continuous parameters and proportions for categorical variables. Differences between patients with ACCs and ACAs were assessed

using Mann−Whitney test for continuous variables and Fisher's exact test for categorical variables. For correlation analysis we used Spearman's rank correlation coefficient test. The diagnostic accuracy of the markers was evaluated using the receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) was used to measure how well a marker can distinguish ACCs and ACAs. Based on the AUC, the test was considered excellent between 0.90 and 1.00; good between 0.80 and 0.90; fair between 0.70 and 0.80; and poor between 0.60 and 0.70, and the test was considered to have failed if the value was below 0.60 [31]. The Kaplan–Meier method was used to evaluate the PFS and survival of ACC patients. Univariate Cox proportional hazards models were used to examine the association between histopathological and immunohistochemical characteristics and the main end points of our study (PFS and OS). No multivariate analyses were performed because of the small number of cases. Statistical significance was set at 0.05 and all computation were made using PRISM 7.

3. Results

3.1. Clinical and Morphological Features of ACC and ACA Patients

In this study, we retrospectively examined 24 ACCs (median size = 10 cm, range 1–24 cm) and 13 ACAs (median size = 5 cm, range 1.3–9 cm) samples obtained from the archival files of 37 patients submitted to adrenal surgery (Table 1). The median age was 54.5 (21–76) and 63.5 (38–71) years for the 24 ACCs (14 females) and the 13 ACAs (9 females) patients, respectively. The 45.8% of the ACCs (3 cortisol-secreting, 2 aldosterone-secreting, 6 both cortisol and androgen secreting) compared to 61.5% of the ACAs (7 cortisol-secreting, 1 aldosterone-secreting) were functional.

Table 1. Comparisons of the clinical, histopathological and immunohistochemical characteristics of the adrenocortical neoplasms.

Characteristics	ACCs (n = 24)	ACAs (n = 13)	p-Value
Clinical characteristics			
Age (median(min−max)), ys	54.5(21–76)	63.5(38–71)	0.119
Sex (Female, n(%))	14(58.3)	9(69.2)	0.724
Size (median(min-max)), mm	10(1–24)	5(1.3–9)	0.002
Functionality (n(%))	11(45.8)	8(61.5)	0.495
Histopathological characteristics (median(min-max))			
Weiss	6(4–9)	0(0–1)	0.001
Helsinki	28(10–56)	3(1–5)	<0.001
Reticulin score	4(3–4)	0(0–1)	<0.001
Immunohistochemical characteristics			
Ki-67% (median (min-max))	23.5(15–45)	3(1–5)	<0.001
p27 (IRS) (median(min-max))	12(4–12)	8(8–12)	0.121
p53 (pathological, n(%))	15(88.2)	0(0)	<0.001
p53 (WT vs. overexpression 20–49% vs. overexpression ≥50%, n(%))	2(11.)/8(47.1)/7(41.2)	All wild	<0.001

Abbreviations: ACC: adrenal cortical carcinoma; ACA: adrenocortical adenoma; IRS: immunoreactive score; WT: wild type.

Nine ACCs were classified as ENSAT stage 1–2 and 15 ACC as ENSAT stage 3–4. Five out of 24 ACC patients (20.8%) exhibited in computed (CT) or magnetic resonance (MRI) imaging distant metastases at diagnosis (2 patients had pulmonary, one liver, one both pulmonary and liver metastases and the last atrial thrombus) (Table 2).

Table 2. Clinical and histopathological characteristics of ACC.

Clinical Characteristics	
Stage (median (min-max))	3 (1–4)
1 or 2 (*n*(%))	9 (37.5)
3 or 4 (*n* (%))	15 (62.5)
Metastatic at presentation (*n* (%))	5 (20.8)
Duration of mitotane treatment (median (min-max)), months	9.8 (4.6–36.9)
PFS (median (min-max)), months	6.57 (1.93–19.7)
Rate of PD (*n* (%))	13 (54.2)
Mortality (median (min-max)), months	11.4 (5.63–27.5)
Mortality rate (*n* (%))	8 (33.3)
Follow-up (median (min-max)), months	18.4 (2.13–101.9)
Histopathological Characteristics	
Capsular invasion (*n* (%))	19 (79.2)
Vascular invasion (*n* (%))	11 (45.8)
Necrosis (*n* (%))	23 (95.8)
Mitoses >20 per 50 HPF (*n* (%))	11 (45.8)
Atypical mitoses (*n* (%))	21 (87.5)

Abbreviations: ACC: adrenal cortical carcinoma; PFS: progression free survival; PD: progression disease; HPF: high-power fields.

Eight out of 24 ACC patients died (mortality rate 33.3%) at a median time of 18.4 (2.13–101.9) months from diagnosis. Thirteen (54.2%) patients relapsed or developed disease progression with a median PFS of 6.57(1.93–19.7) months during a median follow-up of 18.4 months (Table 2). Functionality in ACC patients was significantly associated with relapse ($p = 0.001$) and increased mortality ($p = 0.043$) (Table 3). All patients were treated with mitotane that was initiated either immediate after surgery for ENSAT stages 3,4 ($n = 13$) and for stages 1,2 ($n = 5$) or during relapse (for stages 3,4, ($n = 3$) and for stages 1,2, ($n = 3$). The Kaplan−Meier survival curve for PFS and OS of all patients with ACCs is shown in (Figures S1 and S2). Univariate Cox regression analysis showed a significant association between PFS and functionality ($p = 0.003$), and PFS and ENSAT Stage ($p = 0.047$).

Table 3. Association of clinical, histopathological and immunohistopathological markers with prognostic factors.

Characteristics	No Relapse	Relapse	*p*-Value	No Death	Death	*p*-Value
Clinical						
Size	9(3.5–15.2)	10(1–24)	0.417	9.5(3.5–24)	11(1–16)	0.759
Functionality (*n* (%))	1(9.09)	10(76.9)	0.001	5(31.3)	6(75)	0.043
Histopathological						
Weiss (median (min-max))	6(4–8)	6(4–9)	0.434	6(4–8)	6.5(6–9)	0.481
Helsinki (median (min-max))	30(10–56)	28(20–53)	0.619	28(10–56)	33(23–53)	0.426
Capsular invasion (*n* (%))	7(63.6)	12(92.3)	0.142	12(75)	7(87.5)	0.631
Vascular invasion (*n* (%))	4(36.4)	7(53.9)	0.444	6(37.5)	5(62.5)	0.390
Nuclear atypia (*n* (%))	10(90.9)	13(100)	0.458	15(93.8)	8(100)	0.999
Mitoses >20 per 50HPF (*n* (%))	3(27.3)	8(61.5)	0.123	7(43.8)	4(50)	0.999
Reticulin (score 4, *n* (%))	5(55.6)	4(44.4)	0.999	6(50)	3(50)	0.999

Table 3. *Cont.*

Characteristics	No Relapse	Relapse	*p*-Value	No Death	Death	*p*-Value
Immunohistopathological (median(min-max))						
Ki-67%	23.5(15–40)	25(15–45)	0.668	23.5(15–40)	25(15–45)	0.785
p27([IRS)	12(8–12)	9(4–12)	0.175	12(6–12)	9(4–12)	0.302
p53 (pathological, *n* (%))	7(87.5)	8(88.9)	0.999	9(81.8)	6(100)	0.515
p53 (WT/overexpression 21–50%/≥ 50%, *n* (%))	1(12.5)/6(75)/1(12.5)	1(11.1)/2(22.2)/6(66.7)	0.057	2(18.2)/8(72.7)/1(9.1)	0(0)/0(0)/6(100)	0.001

Abbreviations: ACC: adrenal cortical carcinoma; HPF: high-power fields; IRS: immunoreactive score; WT: wild type.

3.2. Expression of Histochemical and Immunohistochemical Markers in Adrenocortical Neoplasms

All ACC samples possessed 3 or more of the Weiss criteria (median score 6, ranging from 4 to 9) compared to 0 (median score 0, ranging 0–1) for all ACAs. Median Helsinki score was 28 (10–56) for ACCs and 3 (1–5) for ACAs ($p < 0.001$) samples (Table 1). The Weiss and Helsinki scores had statistically significant positive correlation ($r^2 = 0.33$, $p = 0.007$). Neither Weiss nor Helsinki were found to be significantly associated with PFS or OS.

The median reticulin scores were 4(3–4) and 0(0–1) for ACC and ACA samples, respectively, ($p < 0.001$) (Table 1). Eight out of 13 (62%) ACA samples displayed an intact reticulin framework (median score:0) (Figure 1A) whereas only 5 (38%) ACA samples presented a very focal minimal loss of reticulin (median score:1) (Figure 1B). All ACCs studied (18 samples) had an abnormal reticulin framework; 9 (50%) ACC samples showed highly disrupted architecture of the pericellular reticulin pattern (median score:3) whereas the other 9 ACC (50%) samples had a complete loss of reticulin pattern (median score: 4).

The nuclear expression of the proliferation marker Ki-67 labeling index was significantly higher for ACC compared with ACA samples (23.5% (15–45 for ACC vs. 3% (1–5) for ACA ($p = 0.001$)) (Table 1, Figure 1C). The cut off of Ki-67 > 5% exhibited a 92.3% sensitivity and 95.4% specificity for the distinction of ACC from ACA samples with high accuracy (AUC = 99%, $p = 0.009$).

Abnormal p53 protein expression in immunostaining was noted in 88.2% (15/17) of all ACC samples whereas wild type expression was described in only 11.8% (2/17) of them. All ACA samples showed wild type expression of p53 ($p < 0.001$) (Table 1, Figure 1E). Nine patients (8 with pathological expression of p53) had stage 3 or 4 and 8 (7 with pathological expression of p53) had stage 1or 2.

Eight ACAs showed moderate p27 expression and 5 strong whereas in ACCs all but 2 showed strong expression (Figure 1G–H). No statistically significant differences in the expression of the markers studied were observed concerning the secretory component of these neoplasms (functional versus nonfunctional). The schematic presentation of immunohistochemical markers' expression in adrenocortical neoplasms is shown in Figure 2.

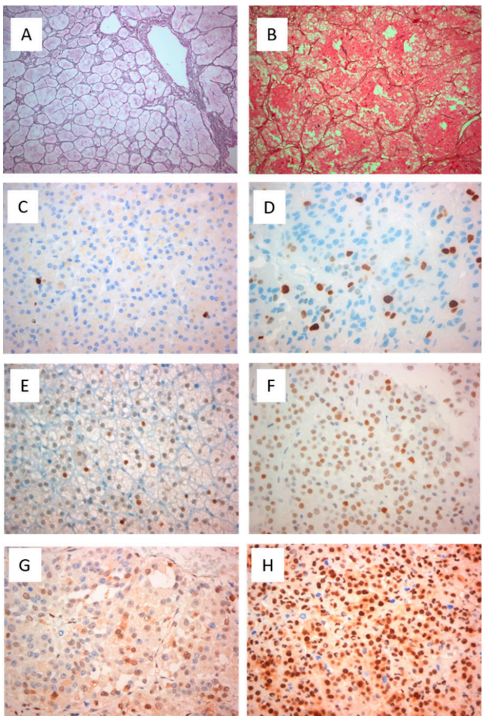

Figure 1. Histochemical and immunohistochemical expression of reticulin, Ki-67%, p53 and p27 in adrenocortical neoplasms. Intact reticulin framework with the characteristic acinar pattern in adrenocortical adenoma (ACA) (×200) (**A**). Highly disrupted architecture and loss of the reticulin framework in adrenocortical carcinoma (ACC)(×400) (**B**). Increase of positive (brown stained) nuclei in Ki-67 immunostaining in ACA (**C**) compared to ACC (**D**). Strong nuclear expression (brown stained nuclei) of p53 protein in a few tumor cells in ACA (wild type pattern) (×400) (**E**), Strong nuclear expression of p53 protein in >80% of tumor cells in ACC (overexpression) (×400) (**F**). Moderate nuclear staining of p27 protein in ACA (×400) (**G**). Strong nuclear staining of p27 protein in the majority of cells in ACC (×400) (**H**).

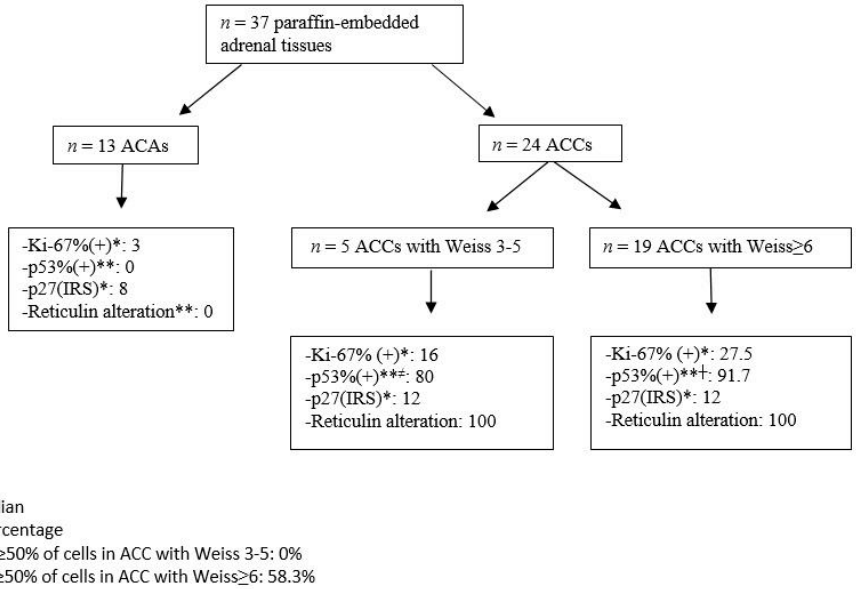

Figure 2. Expression of the Ki-67%, p53, p27 immunohistochemical nuclear markers as well as reticulin expression in adrenocortical neoplasms. All ACA cases exhibited Ki-67% labeling index ≤ 5% (median: 3%) and expressed the wild type p53 expression. No altered reticulin pattern was observed and median IRIS of p27% staining was 8. In all ACC cases Weiss was higher than 3, with altered reticulin pattern and similar IRS of p27 staining. However, those with relatively lower Weiss 3–5, exhibited lower median Ki-67% labeling index and p53 staining compared with ACC cases with Weiss scores greater than 6. Abbreviations: ACA: adrenocortical adenoma; ACC: adrenal cortical carcinoma; IRS: immunoreactive score; WT: wild type

3.3. Prognostic Role of the Histopathological Markers in ACC Patients

All ACC samples with altered reticulin pattern had concomitant necrosis and/or capsular/vascular invasion and/or increased mitotic activity (>5/50 HPF). Thus, using the reticulin algorithm all ACCs would be diagnosed as malignant (significant positive correlation with Weiss score, $r^2 = 0.72$ $p < 0.0001$). Ki-67 labeling index was positively associated with a mitotic count >5/50 HPF ($r^2 = 0.16$, $p = 0.049$) and a Weiss score ($r^2 = 0.31$, $p = 0.007$) but not with vascular or capsular invasion. The p53 expression was also significantly correlated with mitotic count (>5/50 HPF) ($p = 0.02$), but not with capsular or vascular invasion. No statistically significant difference was noted between high-grade and low-grade ACCs in respect to reticulin pattern, p27 staining ($p = 0.366$), p53 staining ($p = 0.485$) and Ki-67 labeling index ($p= 0.074$) although in the last case it reached statistical significance and high grade ACCs had higher median (min-max) Ki-67 labeling index (30% (15–45)) compared with low-grade ACCs (16% (15–45)).

Neither Ki-67 labeling index, p27 expression nor reticulin pattern score were found to be significantly associated with PFS or OS (Table S2). However, p53 abnormal expression ($\geq$ 50% of positive cells) was statistically significantly higher in metastatic ACC samples compared to nonmetastatic ACC samples ($p = 0.035$) as well as in nonsurvivors compared to survivors ($p < 0.001$) (Table S1, Table 3). A cut off of 23% of the percentage of p53-immunostaining positive cells was significantly associated with PFS (AUC 82%, $p = 0.0013$, sensitivity = 71%, specificity 100%).

4. Discussion

In the current study, among the studied markers, altered reticulin pattern, Ki-67% labeling index value and abnormal nuclear expression of p53 protein were found to be statistically significant histopathological and molecular markers for distinguishing malignant from benign adrenal neoplasms although in a small series of patients. In contrast, p27 expression was not found to be a significant marker for the distinction of benign from malignant adrenal cortical neoplasms. However, only the pathological p53 nuclear protein expression was found to have a prognostic role since it was significantly correlated with mortality rate, PFS and metastatic status. The reticulin algorithm defines malignancy through an altered reticulin framework associated with one of the three following parameters: necrosis, high mitotic rate, and vascular invasion for the diagnosis of ACCs [18,19,32,33]. The "reticulin" diagnostic algorithm has been proposed, based on the observation that the tumoral reticulin framework (highlighted by reticulin silver-based histochemical staining) is consistently disrupted in malignant cases but only in a small subset of benign cases. Several case series on ACCs have confirmed the usefulness of the reticulin algorithm for the distinction of ACCs from ACAs [2,19,32–34], although without showing any correlation with prognosis in these patients [6,35]. Our series confirmed the quantitative loss of reticulin framework as a significant finding, distinguishing ACCs and ACAs, showing also a significant positive correlation with Weiss score without being significantly associated with prognosis.

Adrenal cortical malignancy is a proliferation-driven malignancy and this study demonstrated significantly increased expression levels of markers related to cell proliferation such as Ki-67 and p53 in ACCs compared with ACAs. Nuclear expression of the proliferative marker Ki-67 was also significantly correlated with mitotic activity and Weiss score. We have also demonstrated with high accuracy that a Ki-67 labeling index cut-off value > 5% (92.3% sensitivity and 95.4% specificity) could discriminate ACCs from ACAs, confirming the existing data of the literature [9,10,36]. However, this series failed to confirm the current literature and showed no statistically significant association of Ki-67% with PFS and OS, probably due to the small number of samples and the short follow-up period. In line with these data in the literature, there are also some studies that have not confirmed a significant association of Ki-67% with either OS [37] or disease-free survival [38] although in the last study Ki-67% was found to be an independent prognostic factor for OS. Moreover, although the practical utility of Ki-67% staining was indisputable and confirmed in

many studies, there are also studies supporting the idea that it is hard to set a diagnostic threshold that is mainly attributed to possible interobserver variations [39].

The cell cycle regulation molecular marker p53 encoding a protein that promotes DNA repair, was present in almost all ACC (15/17) and absent in ACA samples. The half-life and expression of p53 protein is low and therefore undetectable by immunohistochemistry [6,40,41]. Aberrant nuclear immunohistochemical staining for p53 in ACC samples varies in the literature from 5% to 60% [42,43]. In adult sporadic ACCs, about one quarter of tumors harbor somatic TP53 mutations [43–45], and more than a half harbor loss of heterozygosity at the TP53 locus [13,46]. Previous studies, have shown that ACAs had significantly lower levels of immunohistochemical p53 nuclear expression than ACCs [6], whereas others have failed to show such a difference [47]. Moreover, it has also been reported that high-grade ACCs exhibit higher p53 expression than low-grade ones, a finding consistent with the enrichment of TP53 mutations in high grade carcinomas [9,48].

Transcriptome studies have led to further understanding of the role of p53 in sporadic ACCs. Indeed, TP53 mutated tumors are enriched in a subgroup of ACCs identified by unsupervised clustering of the tumors [17]. Finally, genes positively regulated by p53 such as RRM2B, TP53INP1 and MDM2, were found to be downregulated in this subgroup. The present series confirmed the aberrant nuclear immunohistochemical expression of p53 in ACCs compared to ACAs, although 11.8% of the ACCs showed wild type expression of p53 as all ACAs. Abnormal expression of p53 was the only marker in our series of adrenal neoplasms that showed to have a prognostic role since it was associated with increased mortality rate and the presence of metastases, whereas a cut off of 23% of the percentage of p53-immunostaining positive cells was significantly associated with PFS. Although in molecular analysis the prognostic role of the abnormal p53 expression in ACC is clear [17], immunohistochemical data on the prognostic role of p53 are rather conflicting. Several studies have failed to show a prognostic role of p53 protein [6,42,49,50], whereas others have shown that patients with abnormal p53 staining tended to have higher grade and stage ACCs tumors, increased relapse rates and poorer disease-free survival [46,51].

The protein encoded by p27 (CDKN1B) is another cell cycle regulator marker, that when it is upregulated, results in cell cycle arrest and apoptosis [52]. Our study found no statistical difference of p27 expression between ACCs and ACAs. Accordingly, two previous studies [50,53] have failed to show that p27 could be used as an immunohistochemical markers for distinguishing ACCs from ACAs. However, a more recent study showed that p27 staining was significantly higher in ACCs compared with ACAs, using an automated method of analysis with a high diagnostic accuracy of 7.23% as the best cut-off value [47]. This study had the novelty to use an automated method of analysis in contrast to previous studies which were carried out by direct observation by the researchers [50,53].

All ACC samples exhibited positive staining for Ki-67, p53 and p27 except for two ACC samples that were p53 negative and p27 and Ki-67 positive. In contrast to p53 staining which was found normal in all ACAs (wild type), Ki-67 and p27 were positive in all ACAs. The overexpression of p27 in ACC samples is somewhat contradictory. Several explanations have been proposed, either that adrenal cancer cells develop a tolerance to this inhibitor of cell cycle progression, suggesting that p27 could be present but inactive to arrest the cell cycle, or that p27 gene is mutated resulting in a modified p27 protein [47,54].

Our study has several limitations, that are mainly related to its retrospective nature and the number of samples analyzed. The relatively limited number of samples did not allow a more fruitful statistical analysis that, along with the short follow-up period, may have affected the identification of the prognostic role of the immunohistochemical biomarkers in these tumors. We anticipate that the inclusion of more patients may provide the additional power to reach meaningful clinical findings.

In conclusion, Ki-67, p53 as well as abnormal reticulin pattern, but not p27 expression, could be used to define malignancy in adrenocortical neoplasms and differentiate ACCs from ACAs. Furthermore, p53 expression was significantly associated with increased mortality, metastatic status and lower PFS. However, the small number of patients did

not allow a more robust conclusion; perhaps if confirmed in larger studies it may offer a diagnostic/prognostic tool available in everyday clinical practice. Immunohistopathology alone cannot fully discriminate ACCs with poor prognosis from those with good prognosis and it should be combined with further morphological criteria and recently developed transcriptome analysis which have shown clear differences between adenomas and high- or low-grade carcinomas.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-4426/11/3/208/s1, Table S1: Association of clinical, histopathological and immunohistopathological markers with prognostic factors, Table S2: Univariate Cox regression analysis for risk factors associated with PFS and OS in 24 patients with ACC, Figure S1. Kaplan−Meier curve for PFS of patients with ACC, Figure S2. Kaplan−Meier curve for OS of patients with ACC.

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Case Report

Immunotherapy in Corticotroph and Lactotroph Aggressive Tumors and Carcinomas: Two Case Reports and a Review of the Literature

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Abstract: Once temozolomide has failed, no other treatment is recommended for pituitary carcinomas and aggressive pituitary tumors. Recently, the use of immune checkpoint inhibitors (ICIs) has raised hope, but so far, only one corticotroph carcinoma and one aggressive corticotroph tumor treated with immunotherapies have been reported in the literature. Here, we present two cases, one corticotroph carcinoma and one aggressive prolactinoma (the first one reported in the literature) treated with ipilimumab (1 mg/kg) and nivolumab (3 mg/kg) every three weeks, followed by maintenance treatment with nivolumab (3 mg/kg every 2 weeks) in the case of the corticotroph carcinoma, and we compare them with the two previously reported cases. Patient #1 presented a biochemical partial response (plasma ACTH decreased from 13,813 to 841 pg/mL) and dissociated radiological response to the combined ipilimumab and nivolumab—the pituitary mass decreased from 37 × 32 × 41 to 29 × 23 × 42 mm, and the pre-existing liver metastases decreased in size (the largest one from 45 to 14 mm) or disappeared, while a new 11-mm liver metastasis appeared. The maintenance nivolumab (21 cycles) resulted in a stable disease for the initial liver metastases, and in progressive disease for the newly appeared metastasis (effectively treated with radiofrequency ablation) and the pituitary mass. Patient #2 presented radiological and biochemical progressive disease after two cycles of ICIs—the pituitary mass increased from 38 × 42 × 26 to 53 × 57 × 44 mm, and the prolactin levels increased from 4410 to 9840 ng/mL. In conclusion, ICIs represent a promising therapeutic option for aggressive pituitary tumors and carcinomas. The identification of subgroups of responders will be key.

Keywords: immune checkpoint inhibitors (ICIs); ipilimumab; nivolumab; prolactinoma; Cushing's disease; aggressive pituitary tumor; aggressive PitNET; aggressive pituitary adenoma; pituitary carcinoma

1. Introduction

The vast majority of pituitary adenomas, recently renamed pituitary neuroendocrine tumors (PitNETs) [1,2], are benign and are easily treatable with already established therapeutic options—surgery; conventional medical treatments, including somatostatin receptor ligands and dopamine receptor type 2 agonists; and sometimes radiotherapy (RT) [3]. However, PitNETs may also prove to be aggressive and, very rarely, to metastasize, in the latter case being called pituitary carcinomas [1]. Both pituitary carcinomas and aggressive PitNETs lead to increased morbidity and mortality, and are very difficult to manage. The European Society of Endocrinology (ESE) guidelines on aggressive PitNETs and pituitary carcinomas recommend temozolomide, an oral alkylating agent, to be used after the failure of surgery, conventional medical treatments, and radiotherapy [3]. Unfortunately, in the ESE survey, the largest series on the use of temozolomide, this treatment led to a partial or complete radiological response in only 37% of cases, and to stable disease in 33% of cases. Moreover, after treatment ceased, progressive disease was noted in 25, 40, and 48% of patients initially responding to treatment with a complete response, partial response, and stable disease, respectively [4], and a second course of temozolomide proved to be effective only in rare cases [4–6]. Once temozolomide has failed, no other treatment option is formally recommended, because of a lack of sufficient evidence regarding efficacy in aggressive PitNETs, including carcinomas. Recently, the use of immune checkpoint inhibitors (ICIs) has raised hope, but so far only two cases of corticotroph tumors (one carcinoma treated with combined ipilimumab, an anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and nivolumab, an anti-programmed cell death protein 1 (PD-1), which showed a partial response [7], and one aggressive corticotroph tumor treated with pembrolizumab alone, an anti-PD-1, which showed progressive disease [8]) are reported in the literature.

Here, we report two cases. First, we present the response of a functioning corticotroph carcinoma to a different dosing regimen of combined immunotherapy with ipilimumab and nivolumab than the one already reported in literature (chosen based on a better tolerance with comparable efficacy of this regimen in other cancers [9–11]). Second, we present the first reported case of an aggressive prolactinoma treated with immunotherapy.

2. Patients and Methods

Here, we present a case series describing the clinical management of one corticotroph carcinoma and one aggressive prolactinoma, with an emphasis on the effect of immunotherapy with ipilimumab (1 mg/kg every 3 weeks) and nivolumab (3 mg/kg every 3 weeks), followed by maintenance treatment with nivolumab alone in the case of the corticotroph carcinoma (3 mg/kg every 2 weeks). The cases were treated in two different French centers, Lille and Limoges, where they had been followed since 2001 and 2012, respectively. For both cases, the indication of immunotherapy was validated after a tumor board multidisciplinary discussion conducted at the national level (*HYPOCare* multidisciplinary reunion conducted by the French Reference Center for Rare Pituitary Diseases *HYPO*). Clinical, radiological, and pathological data were collected retrospectively from the patients' medical records. All of the hormonal assays, immunohistochemistry, and methylation analyses were performed with commercially available kits. The study is in accordance with the Ethics Committee of Lille University Hospital and Limoges University Hospital. Informed consent was obtained from each patient after a full explanation of the purpose and nature of all of the procedures used, as well as for publication of the article along with accompanying images.

3. Patient #1

A 42-year-old female presented in 2001 with headaches, diabetes, hypertension, and obesity, and was diagnosed with Cushing's disease based on an elevated urinary free cortisol (UFC) level of 212 µg/24 h (N 20-110), elevated morning plasma adrenocorticotropic hormone (ACTH) of 115 pg/mL (N < 46), and invasive pituitary tumor measuring 25 × 23 × 20 mm. The first transsphenoidal surgery (TSS), which was subtotal as a result of left cavernous sinus invasion, revealed a tumor with a Ki-67 index of 2%, absence of mitosis, and negative p53; therefore, it was classified as a grade 2a corticotroph tumor.

The regrowth of the tumor residue required multiple additional treatments between 2003 and 2017, as shown in Figure 1A, which shows the evolution of the plasma ACTH under the different treatments—fractionated RT (50 Gy in 28 fractions) in 2003; second TSS (rare mitosis) followed by Gamma knife radiosurgery (25 Gy) in 2007; 10 cycles of temozolomide 250–270 mg/day, 5 days every 4 to 5 weeks in 2009-2010 (with initial biochemical complete response and radiological partial response, but with relapse 3 years after treatment ceased; side effects: thrombocytopenia); three additional cycles of temozolomide 250 mg/day, 5 days every 5 weeks in 2013 (stopped due to radiological progressive disease and thrombocytopenia); third TSS (Ki-67 index 5%, 5 mitoses/10 high-power fields (HPFs), and positive p53–2%) in 2013; pasireotide 0.9 mg twice daily for 4 months in 2015 (ineffective); cabergoline 2 mg/week for 5 months in 2015-2016 (ineffective); hydroxyurea 1500 mg/day for 3 months in 2016–2017 (ineffective and causing pancytopenia); and fractionated RT (45 Gy in 25 fractions) in 2017.

In November 2018, the patient was admitted for asthenia, weight loss (10 kg), accentuation of melanoderma, right ptosis, and diplopia due to right third and sixth cranial nerve palsy. The plasma ACTH varied between 3957 and 11,191 pg/mL, compared with 398 pg/mL in May 2018. The pituitary computed tomography (CT) showed the global stability of the pituitary mass compared with the pituitary magnetic resonance imaging (MRI) performed in February 2018. The ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT showed an intense uptake by the pituitary mass, the skull (corresponding to one lytic occipital lesion), and three hepatic lesions; there was also a suspected T2 vertebral lesion. The abdominopelvic CT showed five hepatic lesions, with maximal diameters of 31, 22, 12, 7, and 5 mm. A liver biopsy was performed and showed the presence of a corticotroph tumor metastasis (immunohistochemistry: Ki-67 index 10%, positive p53–7%, and absent programmed death ligand 1 (PD-L1) expression), confirming the diagnosis of pituitary carcinoma. The pituitary and thoraco-abdominopelvic CT repeated in February 2020 showed the progression of the pituitary mass (anteroposterior diameter: 37 mm vs. 32 mm) and of the two largest liver metastases (maximal diameters of 45, 30, 12, 7, and 6 mm). The evolution of the pituitary mass and of the liver metastases is presented in Figure 1B,C, respectively.

Immunotherapy with ipilimumab (1 mg/kg) and nivolumab (3 mg/kg) every 3 weeks was initiated in February 2019, and was administered for five cycles. A very rapid and significant decrease of plasma ACTH was observed after the first cycle, from 13,813 to 1036 pg/mL. The plasma ACTH continued to further decrease (nadir of 549 pg/mL after three cycles) and remained < 1000 pg/mL during the five cycles of combined immunotherapy. Regarding the radiological response, after three and four cycles, the pituitary mass decreased in size from 37 × 32 × 41 mm to 29 × 23 × 42 mm, while the five known liver metastases decreased in size from 45 to 14 mm, from 30 to 13 mm, from 12 to 5 mm, or were not visible anymore (for the smallest ones). The ^{18}F-FDG-PET/CT showed a very positive metabolic response for the known metastases and the disappearance of the suspect T2 vertebral lesion, but the appearance of a new focal liver uptake was noted, corresponding to a new 11 mm lesion on the CT (described only afterwards on the CT). A concomitant improvement of melanoderma and right ptosis were observed, the diplopia disappeared, and the body weight stabilized. The patient did not report any side effects from the combined immunotherapy other than asthenia on the day of administration.

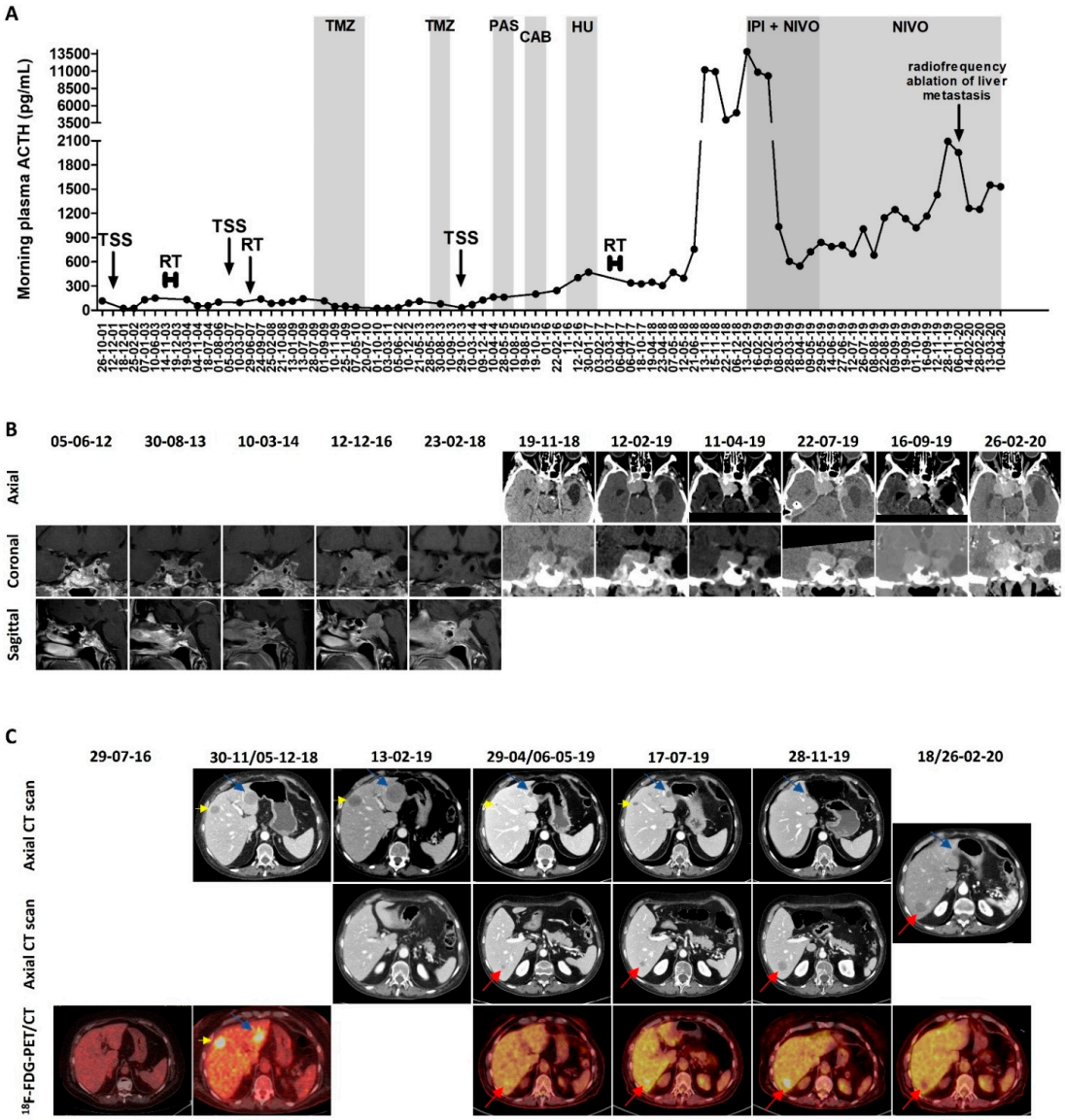

Figure 1. 18-year follow-up of a corticotroph carcinoma (patient #1), from diagnosis to the present day. (**A**) Evolution of morning plasma adrenocorticotropic hormone (ACTH) levels under the different treatments. Every dot represents an individual measurement. (**B**) Radiological evolution of the pituitary mass as seen on the magnetic resonance imaging until February 2018 (contrast-enhanced T1-weighted images) and on the pituitary computed tomography from November 2018. (**C**) Radiological and metabolic evolution of the liver metastases. An ^{18}F-fluorodeoxyglucose (FDG)-PET/CT performed on 29 July 2016 shows the absence of liver metastases. The blue and yellow arrows indicate the two largest liver metastases that appeared in November 2018. The metastasis indicated by the yellow arrow is not visible at every time point in this figure, but was still present, measuring 30 mm in February 2019, 13 mm in April 2019, 11 mm in November 2019, and 8 mm in February 2020. The red arrow indicates the liver metastasis that appeared after four cycles of combined immunotherapy. Abbreviations: TSS—transsphenoidal surgery; RT—radiotherapy; TMZ—temozolomide; PAS—pasireotide; CAB—cabergoline; HU—hydroxyurea; IPI—ipilimumab; NIVO—nivolumab; CT—computed tomography; PET—positron emission tomography.

Since May 2019, the patient received maintenance treatment with nivolumab alone (3 mg/kg every 2 weeks). After the fourth cycle of nivolumab, the right ptosis worsened again, concomitantly with a progressive increase of plasma ACTH (1147 pg/mL, 1248 pg/mL, 1431 pg/mL, and 2094 pg/mL after

the fourth, seventh, eleventh, and twelfth cycle in November 2019, respectively). The increase in the pituitary mass (from 29 × 23 × 42 to 29 × 25 × 45 mm in July 2019) was initially considered to be non-significant, however the pituitary CT performed in February 2020 confirmed the local progression (38 × 28 × 54 mm). Regarding the liver metastases, in November 2019, the thoraco-abdominopelvic CT showed the progression of the liver metastasis that became visible after the start of the immunotherapy (22 mm vs. 14 mm in July and 11 mm in May), whereas the other liver metastases were stable, measuring 12, 11, and 4 mm. Radiofrequency ablation of this liver metastasis was performed in January 2020, and 1-2 months later, the concurrent stigma of the radiofrequency ablation on the CT scan, a complete metabolic response of the hepatic lesion on the ^{18}F-FDG-PET/CT, and a decrease of plasma ACTH were observed. The three other liver metastases remained stable, measuring 12, 8, and 4 mm. At the last follow-up in April 2020, the plasma ACTH was stable (1530 pg/mL) in comparison with March, but higher than in February (1250 pg/mL). The patient died four days later of an unknown cause. As potential side-effects of nivolumab, the patient reported asthenia, anorexia, and progressive weight loss (5 kg).

Of note, for the hypercortisolism, the patient also received ketoconazole and mitotane (before the biochemical remission induced by the 10 cycles of temozolomide), and after relapse, metyrapone 1000-3000 mg/day from August 2015 to December 2018 (side-effects: abdominal pain, hair loss, and hirsutism), and mitotane up to 2000 mg/day since February 2017 (side-effects: anorexia and vertigo).

4. Patient #2

A 60-year-old male presented in August 2012 with visual disturbances and bitemporal hemianopsia, and was diagnosed with a prolactinoma based on the presence of an invasive pituitary tumor measuring 36 × 31 × 26 mm, and a prolactin level of 5130 ng/mL (N 2.30-14.7). Figure 2A presents the evolution of the prolactin levels under the different treatments, while Figure 2B presents the evolution of the pituitary mass.

Cabergoline 0.5 mg × 3/week was started, and then increased to 0.5 mg daily from January 2013, enabling a decrease of prolactin to 1330 ng/mL, concurrent with a 50% decrease in tumor volume in March 2013. Cabergoline was further increased and maintained at 1 mg daily from June 2013. Despite an initial further decrease in the tumor volume and recovery of visual signs (nadir prolactin level of 787 ng/mL), the patient presented with the recurrence of the left temporal hemianopsia in December 2015. The pituitary MRI showed rapid tumor regrowth between July and December 2015 (from 7 × 19 × 17 mm to 30 × 25 × 25 mm). The TSS performed in January 2016 was subtotal as a result of left cavernous sinus invasion, and revealed a proliferative tumor (Ki-67 index of 10%, 5 mitosis/10 HPFs), and was therefore classified as a grade 2b prolactinoma. Adjuvant fractionated RT (50.4 Gy in 30 fractions) was performed in 2016.

Following the first TSS, the patient was kept on 1 mg of cabergoline daily. At the end of 2018, the patient presented again with rapid tumor progression, with optic chiasm compression, for which a second TSS was performed in January 2019 (Ki-67 index 10–11%, 1 mitosis/10 HPFs). After surgery, cabergoline was further increased to a maximum of 10.5 mg/week and pasireotide 0.6–0.9 mg twice daily was administered for two months (ineffective and resulting in QT interval prolongation). The administration of temozolomide was decided, but due to the rapid tumor progression with a worsening of the visual field, decreasing visual acuity, and involvement of the left third cranial nerve, before starting temozolomide, a third TSS was performed in June 2019 (Ki-67 index 25%, >20 mitosis/10 HPFs). Neither the ^{18}F-FDG PET/CT performed in June 2019 nor the medullary MRI performed in August 2019 found any metastasis.

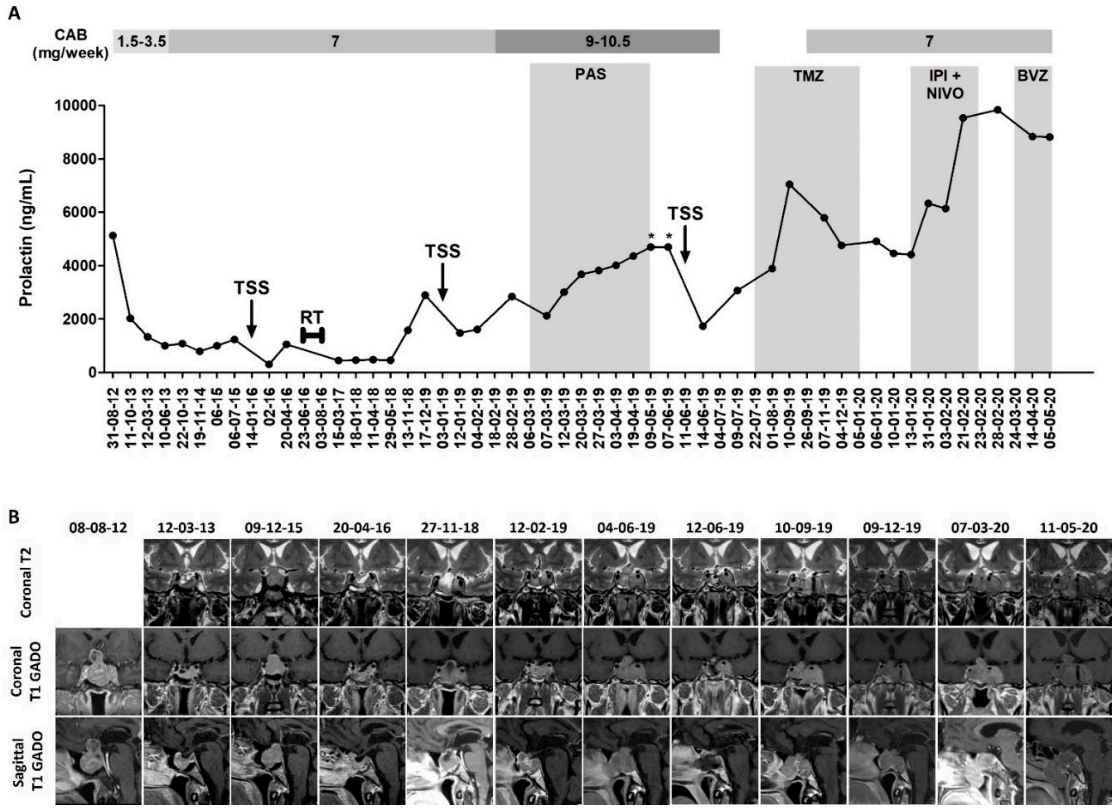

Figure 2. Eight-year follow-up of an aggressive prolactinoma (patient #2), from diagnosis to the present day. (**A**) Evolution of the prolactin levels under the different treatments. Every dot represents an individual measurement. * ≥4700 ng/mL (the upper limit of the respective prolactin assay). (**B**) Radiological evolution of the pituitary mass as seen on the magnetic resonance imaging. Abbreviations: CAB—cabergoline; TSS—transsphenoidal surgery; RT—radiotherapy; PAS—pasireotide; TMZ—temozolomide; IPI—ipilimumab; NIVO—nivolumab; BVZ—bevacizumab; T2—T2-weighted image; T1 GADO—contrast-enhanced T1-weighted image.

The cabergoline was interrupted in July 2019, being considered ineffective given the rapid progression despite very high doses, and six cycles of 220–290 mg of temozolomide per day for 5 out of 28 days were administered starting at the end of July 2019. The tumor progressed under temozolomide, with worsening of the left cavernous sinus syndrome (diplopia, ptosis, mydriasis, and trigeminal neuralgia, for which methylprednisolone up to 80 mg daily was administered), a significant increase in the tumor size seen on the MRI performed in September 2019 (39 × 43 × 27 mm compared with 20 × 20 × 23 in June 2019), and a concurrent significant increase in prolactin levels. Then, 1 mg of cabergoline daily was reintroduced, enabling a decrease in prolactin levels, which, nevertheless, remained higher compared with the moment when the temozolomide was started. An improvement in the left cavernous sinus syndrome was also noted (possibly due to the methylprednisolone as well), but with the MRI performed in December 2019 not showing any regression in the pituitary mass (38 × 42 × 26 mm).

Given the inefficacy of temozolomide, the administration of immunotherapy with ipilimumab (1 mg/kg) and nivolumab (3 mg/kg) every three weeks was decided. The administration of methylprednisolone was progressively decreased, and stopped at the end of December 2019 in order to start the combined immunotherapy, while cabergoline was maintained at 1 mg daily. Unfortunately, the combined immunotherapy resulted in rapid progression, with the prolactin levels increasing from 4410 ng/mL before initiation to 9840 ng/mL after the first two cycles, and with the size of the pituitary tumor increasing from 38 × 42 × 26 mm in December 2019 to 53 × 57 × 44 mm in March 2020. Left

ptosis also reoccurred. After the two cycles, the patient also presented severe side-effects (grade 3–4 diarrhea, and grade 1 nausea and vomiting), and therefore the combined immunotherapy was stopped. Starting at the end of March, 15 mg/kg of bevacizumab was administered every three weeks, with, so far, a stabilization of prolactin levels (~8830 ng/mL in April and May 2020), a stable disease shown in the MRI, and good tolerance (minimal epistaxis and grade 1 hypertension).

Of note, the somatostatin receptor type 5 (SST5) immunohistochemistry performed in January 2020 on the tumor block from January 2019 showed the absence of staining, which may partly explain the lack of an effect of pasireotide [12]. At the same time, as a potential marker of response to temozolomide, O6-methylguanine-DNA methyltransferase (MGMT) immunohistochemistry was performed, but was not interpretable, while the methylation analysis revealed a weak methylation of the MGMT promoter (average between 9 and 12%). On the tumor block from June 2019, genetic analysis was performed, as follows: (1) next-generation DNA-sequencing in January 2020—FoundationOne®companion diagnostic (CDx; Foundation Medicine, Inc., Cambridge, MA, USA)—revealing the absence of alterations with a known clinical and/or therapeutic impact (absence of microsatellite instability, low mutational burden (1 mutation/Mb), amplification of the *HGF* gene, deletion of the *CDKN2A/B* gene, mutation (p.Ser514*) of the *BCORL1* gene, mutation (p.Asn2fs*52) of the *FLCN* gene, and mutation (p.Lys666Glu) of the *SF3B1* gene), and (2) next-generation RNA-sequencing in March 2020—Archer® FusionPlex® CTL Panel (ArcherDX, Inc., Boulder, CO, USA)—revealing the absence of mutations or of fusion transcripts.

5. Discussion

ICIs block the inhibitory signals of T lymphocyte function and/or activation, and, more precisely, they block CTLA-4 or PD-1, which are found on T lymphocytes or its ligand, programmed death ligand 1 (PD-L1), which is found on antigen-presenting cells and on tumor cells. These inhibitory signals would otherwise enable tumors to evade immune response. By blocking them, ICIs enhance T lymphocyte function and reactivate antitumor immune responses [13–15].

Recent studies on the tumor microenvironment of PitNETs [16] have demonstrated in these tumors, and especially in functioning PitNETs, the presence of tumor-infiltrating lymphocytes [13,17,18] and of PD-L1, a potential biomarker of response to ICIs [13,17]. These findings have raised the hope that ICIs might be effective in these tumors. ICIs might be especially promising for patients who previously received conventional chemotherapies, such as temozolomide, because the administration of conventional chemotherapies can induce somatic hypermutations that will render ICIs more effective [7]. A recent in silico analysis of the immune tumor microenvironment of PitNETs also revealed that functioning corticotroph tumors had higher CD8+ T lymphocyte infiltration than somatotroph, lactotroph, thyreotroph, and non-functioning PitNETs, suggesting that functioning corticotroph tumors may be more amenable to ICIs than other PitNET subtypes [19].

So far, only two cases of corticotroph tumors have been treated with ICIs [7,8]. Table 1 summarizes their findings, together with the findings from our two cases. The two cases that showed a partial response were both carcinomas that had been previously treated with multiple conventional chemotherapies (including multiple courses), in comparison with the two cases that resulted in progressive disease, which were both aggressive pituitary tumors that had been previously treated with only one course of temozolomide. Although we did not perform genetic analyses before the start of the ICIs (and neither did the authors of the corticotroph tumor that showed progressive disease), a higher mutational burden would be expected in the two responders, and the liver metastasis of the responsive corticotroph carcinoma reported in the literature did indeed show somatic hypermutations [7]. However, it is worth mentioning that the aggressive corticotroph tumor that showed the progressive disease had a mismatch-repair deficiency (as did the liver metastasis from the other reported case), which, in other cancers, is associated with responsiveness to anti-PD-1 therapy [8]. Other than a less important mutational burden, additional possible reasons for ICI inefficacy in this case consists of using monotherapy instead of combined ICIs (less effective) and

of the presence of high levels of cortisol during ICI administration, given that glucocorticoids have immunomodulating effects (the hypercortisolism was controlled in the other two cases). Moreover, this tumor did not express PD-L1 in the immunohistochemistry, but the predictive value of the PD-L1 expression still needs to be proven in pituitary tumors, especially given the fact that its predictive value has not been consistent across studies in other cancers [8]. Importantly, the liver metastasis that was biopsied in patient #1 did not express PD-L1 either, but proved to have an excellent response to ICIs, as did the liver metastasis from the other corticotroph carcinoma, which also only had <1% positive PD-L1 staining [7]. Therefore, the lack of PD-L1 expression should not preclude the indication of immunotherapy. Interestingly, both in our case and in the case of the corticotroph carcinoma previously reported [7], the liver metastases showed a greater decrease in size compared with the pituitary masses, which may be at least partly explained by a different composition of the tumor microenvironment between the liver and the pituitary (as two different anatomic sites), but also between primary and secondary lesions in general. In addition, given the rapid and significant biochemical response seen in the two responders (an ~10-fold decrease in ACTH levels within 1 week [7], and after the first cycle in patient #1), the biochemical response might prove to be an early and easy-to-perform marker of response to ICIs.

As limited experience is available on the use of ICIs in pituitary tumors, we think that response to treatment should be evaluated on a case-by-case basis. Given the regression of the pituitary mass and of all five pre-existing liver metastases, we consider patient #1 to be a responder to combined ipilimumab and nivolumab, instead of classifying the response as progressive disease based on the new liver metastasis that appeared after the four cycles of combined immunotherapy, as we could have done based on the iRECIST guidelines [20]. Although this last metastasis continued to grow on maintenance nivolumab and was finally treated by radiofrequency ablation, and the pituitary mass slowly progressed, nivolumab alone continued to be effective on the rest of the metastases. Therefore, in the case of a dissociate response (which might reflect a distinct evolution of the genetic/epigenetic landscape or of the tumor microenvironment), in which only one or very few of the lesions progress, the use of complementary local therapies such as radiofrequency ablation or surgery might be tried in combination with continued immunotherapy, instead of considering the immunotherapy ineffective and ceasing it. Additionally, the reintroduction of double immunotherapy might also prove useful in the future.

Moreover, besides a complete/partial response, stable disease, and progressive disease, one should be aware of two additional patterns of tumor response when using immunotherapy, namely: pseudoprogression (i.e., apparent increase in the tumor burden initially, followed by delayed tumor shrinkage, which could lead to the premature cessation of effective immunotherapy) [20,21] and hyperprogressive disease (i.e., accelerated tumor progression following the introduction of ICIs) [22,23]. Hyperprogressive disease was shown to be associated with older age—in a study including 131 patients treated with anti-PD-1/PD-L1 therapy, 7 out of 36 patients ≥65 years old (19%) were classified as having hyperprogressive disease, compared with 5 out of the 95 patients ≤64 years old (5%), $p = 0.01$, of which only one patient was <55 years old [22]. Patient #2 from the current study, who was 68 years old when immunotherapy was introduced, presented with rapid progression following ICIs initiation, but it is difficult to say whether it was the cessation of temozolomide, the natural history of the disease, or the introduction of ICIs that led to this evolution.

Table 1. Cases of pituitary carcinomas and aggressive pituitary neuroendocrine tumors treated with immunotherapy.

Ref.	Sex and Age [1]	Tumor Type	Previous Tumor-Directed Treatments	Immunotherapy		
				Posology	Response	Side Effects
[7]	**F, 41**	**Corticotroph carcinoma**	NS × 2, RT, NS × 2, pasireotide, cabergoline, TMZ + capecitabine × 2 (4 and 2 cycles), etoposide + carboplatin (2 cycles), RT	IPI (anti-CTLA-4) 3 mg/kg + NIVO (anti-PD-1) 1 mg/kg every 3 weeks (5 cycles)	R: partial response: 59% ↘ in primary tumor volume and 92% ↘ in main liver metastasis volume B: partial response: plasma ACTH ↘ from 45,550 to 66 pg/mL	Fever (40 °C), mild transaminitis, possibly hypophysitis
				Maintenance NIVO	R: "continues to respond" B: stable disease: plasma ACTH of 59 pg/mL at the 6-month follow-up	No additional immunologic side effects
[8]	M, 66	Aggressive corticotroph tumor	NS × 2, RT, TMZ for 2 years, pasireotide, NS	Pembrolizumab (anti-PD-1) 200 mg flat dose (4 cycles)	R: progressive disease, i.e., ≥ 20% ↗ in the sum of diameters B: progressive disease: plasma ACTH ↗ from 269 to 544 ng/L	None
	F, 60	Corticotroph carcinoma	NS, RT, NS, RT, TMZ × 2 (10 and 3 cycles), NS, pasireotide, cabergoline, hydroxyurea, RT	IPI 1 mg/kg + NIVO 3 mg/kg every 3 weeks (5 cycles)	R: partial response: primary tumor ↘ from 37 × 32 × 41 to 29 × 23 × 42 mm; the 5 liver metastases ↘ (the 2 largest ones from 45 to 14 mm and from 30 to 13 mm) or disappeared + a new 11 mm liver metastasis appeared B: partial response: plasma ACTH ↘ from 13,813 to 841 pg/mL	Asthenia on the day of administration
				Maintenance NIVO 3 mg/kg every 2 weeks (21 cycles)	R: stable disease for the initial 5 liver metastases + progressive disease of the newly appeared metastasis and of the pituitary mass B: progressive disease: plasma ACTH ↗ from 841 to 1954 pg/mL after 14 cycles	Possibly asthenia, anorexia, and progressive weight loss
	M, 68	Aggressive prolactinoma	Cabergoline (ongoing), NS, RT, NS, pasireotide, NS, TMZ (6 cycles)	IPI 1 mg/kg + NIVO 3 mg/kg every 3 weeks (2 cycles) + cabergoline 1 mg/day	R: progressive disease: ↗ from 38 × 42 × 26 to 53 × 57 × 44 mm B: progressive disease: prolactin levels ↗ from 4410 to 9840 ng/mL	Grade 3-4 diarrhea, grade 1 nausea and vomiting

[1] The age of the patient when immunotherapy first started. Abbreviations: Ref.—reference; F—female; M—male; NS—neurosurgery; RT—radiotherapy for the primary tumor; TMZ—temozolomide; IPI—ipilimumab; CTLA-4—cytotoxic T-lymphocyte-associated protein 4; NIVO—nivolumab; PD-1—programmed cell death protein 1; R—radiological; B—biochemical; ACTH—adrenocorticotropic hormone.

Regarding the side-effects of ICIs, combined immunotherapy, besides being more effective, also increases treatment-related adverse events [17]. In the four pituitary cases reported so far, the most frequently affected was the gastrointestinal tract, with patient #2 from the current study being the only one experiencing severe treatment-related adverse events. Regarding ICIs-related endocrinopathies, both of our patients had already substituted central hypothyroidism at the time immunotherapy started (for which the replacement therapy was maintained) and had type 2 diabetes mellitus (which remained well controlled during immunotherapy). No new endocrinopathies were diagnosed during immunotherapy.

6. Conclusions

So far, there is not enough data to enable any formal conclusion on the efficacy of ICIs in pituitary carcinomas and aggressive PitNETs, or on which subgroups of patients will prove to respond, but ICIs appear to be a promising therapeutic option. At the moment, there are two clinical trials running on combined ipilimumab and nivolumab, namely: NCT04042753, a clinical trial dedicated to pituitary carcinomas and aggressive PitNETs; and NCT02834013, a basket trial that also accepts pituitary carcinomas. Hopefully, their results will provide valuable information on the efficacy of ICIs in these tumors. At the same time, potential leads for the improvement of ICI efficacy emerging from studies on other cancers, such as the combination of ICIs with drugs targeting angiogenesis [24,25] or with radiotherapy [26,27], will potentially be transferable to pituitary carcinomas and aggressive pituitary tumors as well and will result in better outcomes for the patients treated with such therapies.

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Correlation between F18-FDG PET/CT Imaging and BRAF V600E Genetic Mutation for the Early Assessment of Treatment Response in Papillary Thyroid Cancers

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Abstract: In thyroid neoplastic pathology, the BRAF V600E mutation is shown to be involved in the oncogenesis of papillary thyroid cancer and its subtypes. The purpose of this study is to evaluate the correlation between the mutation of the BRAF V600E oncogene and the pathological standardized uptake values (SUV) at the F18-fluorodeoxyglucose (F18-FDG) positron emission tomography/computed tomography (PET/CT) evaluation, for a group of 20 patients with radically treated (total thyroidectomy and radioiodine therapy) papillary thyroid cancer, with subclinical persistent disease, at 6 months after the initial treatment. We analyzed the correlations between the values of SUV and the presence of the BRAF mutation as well with other prognostic factors such as stage, age, specific tumor markers (thyroglobulin and anti-thyroglobulin), extrathyroid extension, the presence of metastatic lymph nodes or distant metastasis. The value of SUV in the case of BRAF+ (positive) patients was higher than in the negative ones, but without statistical significance, thus, the values of the SUV cannot be a predictable factor for the presence of the genetic mutation. There was a statistically significant correlation in BRAF+ subgroup between the SUV values and the positive resection limit following surgery, showing a higher SUV value in the PET/CT evaluation. No correlation was observed between the aforementioned prognostic factors involved in papillary thyroid cancer and the BRAF V600E mutation.

Keywords: papillary thyroid cancer; SUV PET/CT; BRAF V600E

1. Introduction

The BRAF mutation is associated with the oncogenesis of several tumors as well as colon cancer, melanoma and thyroid cancer [1]. In thyroid neoplastic pathology, the BRAF V600E mutation is shown to be involved in the oncogenesis of papillary thyroid cancer (PTC) and its subtypes [2]. Activation of the mutation in the mitogen-activated protein kinase (MAPK) pathway where the BRAF V600E gene is included, leads to the non-controlling tumor proliferation and thus, to the phenomenon of cell de-differentiation. Therefore, the presence of the mutation is associated with aggressive forms of the disease, the presence of an unfavorable prognostic factor and in some cases with the loss of avidity of tumor for the I-131 treatment [3,4]. Taking into account this scenario, the treatment option might be changed. One of these options is systemic therapy with tyrosine kinase inhibitors;

numerous studies and clinical trials are ongoing on this topic, some of them with positive results and improvement of the long-term survival rate. In order to identify the cases with a high degree of aggressiveness and tumor de-differentiation, the correlation between gene mutation and the increased rate of carbohydrate metabolism assessed by positron emission tomography/computed tomography (PET/CT) hybrid imaging using F18-fluorodoxyglucose (F18-FDG) as a tracer, was studied [5–9].

The purpose of this study is to evaluate the correlation between the mutation of the BRAF V600E oncogene and the pathological standardized uptake values (SUV) at the PET/CT evaluation, for a group of patients with papillary thyroid cancer with persistent subclinical disease at 6 months after the initial radical treatment, consisting of surgery and radioiodine (I-131). We analyzed the correlations between the value of SUV and the presence of the BRAF mutation as well with other prognostic factors such as stage, age, extrathyroid extension, the presence of lymph nodes or distant metastasis, in order to identify those patients with aggressive outcomes and to personalize their therapies.

2. Patients and Method

2.1. Patients

This study includes 20 patients diagnosed with pure forms of papillary thyroid carcinoma, selected from a number of 663 new cases of thyroid carcinoma evaluated during 2019 in the Endocrine Tumor and Nuclear Medicine Department of the "Prof. Dr. Ion Chiricuţă" Institute of Oncology, a tertiary center, representing 3% of all treated patients. All patients were investigated, treated and evaluated uniformly in the same institution, a fact that suggests a unitary approach of this pathology.

All patients were evaluated after 6 months from the initial therapy during the monitoring program; the cases with persistent disease after the initial therapy were referred to F18-FDG PET/CT scans. The persistent disease was defined as follows: clinical, thyroid ultrasound and whole body I-131 scan all negatives, but with detectable serologic thyroglobulin (Tg) and/or pathologic levels of antibodies anti-thyroglobulin (anti-Tg) determined in stimulated conditions (elevated thyroid stimulating hormone-TSH).

The 20 selected patients with positive PET/CT scans were further investigated for BRAF V600E mutation. These patients had mean ± standard deviation (SD) age of 58.6 ± 13.35 years old, with a minimum 22 and maximum 74 years of age; there were 8 males and 12 females, with a ratio of 2:3. They had been radically treated with total thyroidectomy +/- lymphadenectomy, where this was necessary, and treated with one single administration of I-131; the mean ± SD I-131 activity was 82.2 ± 37.6 mCi (3.4 ± 1.39 GBq); all patients followed suppressive doses of Levothyroxine.

2.2. Genetic Analysis

The processing of samples for analysis of the BRAF V600E mutation was carried out within the Laboratory of Functional Genomics, Proteomics and Experimental Pathology of the same institution. For the isolation of genomic DNA, 5 sections of 10 μm thick paraffinated tissues were performed, which subsequently underwent the macrodissection technique. Genomic DNA was extracted using the Purelink Genomic DNA Mini kit (Invitrogen). For the quantification reaction of the BRAF V600E mutation through the real-time polymerase chain reaction (PCR) technique, it is essential to use a sufficient concentration of integrated DNA. As a result, the isolated DNA was quantitatively analyzed using the NanoDrop ®ND-1000 Thermo Scientific spectrophotometer, which determines the concentration and purity of the isolated genetic material. Thus, DNA can be measured directly in aqueous solutions by recording the absorption of that molecule at wavelengths of 230, 260, 280 nm, respectively, determining the difference between the amount of light transmitted to the sample and that received. In the case of pure DNA samples, the ratio $A_{260/}A_{230}$ must be within the 1.8–2.2 range, and in the case of the ratio $A_{260/A}/A_{280}$ the value of approximately 1.8 is accepted. The detection of the BRAF V600E mutation in the samples studied was undertaken by the real-time PCR technique using the IVD EntroGen BRAF Codon 600 Mutation Analysis kit II and the real-time PCR LightCycler 480

Roche. A concentration of DNA samples of 20 ng/µl was used for the PCR reaction. The evaluation of amplification products was carried out by reading two fluorochromes: FAM (483–533) for the BRAF V600E mutation and VIC (523–568) for internal control.

2.3. Positron Emission Tomography/Computed Tomography (PET/CT) Imaging

Images were acquired with a GE Optima 560 PET/CT; F18-FDG PET/CT studies were performed after a period of fasting of 6 hours; the blood glucose levels, between 70 mg/dl and 150 mg/dl were measured before the F18-FDG injection; injected doses of 185–600 MBq F18-FDG were calculated accordingly to the patient's weight and to the EANM procedure guidelines for tumor imaging, version 2.0 and the examination was performed after an uptake period of 60–90 minutes. CT images, with a slice thickness of 3.75 mm, were acquired using a low-dose protocol (100–120 Kv, 50–100 auto mA, index noise of 20%) in order to reduce the irradiation dose for patients. PET/CT images were evaluated by a team formed by a nuclear medicine physician and a radiologist. For all PET/CT studies, SUVlbm (the standardized uptake value lean body mass) was used as a semi-quantitative parameter for the F18-F18-FDG uptake calculation respecting a standard protocol on the work station (Volumetrix for PET/CT). All abnormal F18-FDG findings were correlated with neck ultrasound, serological tumor marker levels, and clinical examination of the patients.

2.4. Serologic Analysis

Serum thyroglobulin (Tg) and thyroglobulin antibody (anti-Tg) measurements were carried out using immunochemical methods with electrochemoluminescence detection, Roche kits, and Cobas instruments (both from Roche Diagnostics, Basel, Switzerland), in the same accredited laboratory. Samples for Tg quantitation were taken after thyroid hormone withdrawal of ≥2 weeks with a TSH rise to 40 mIU/L. The Tg assay had a lower limit of detection of <0.04 µg/L, an intra-run coefficient of variation (CV) of 1.8%, and an inter-run CV of 3.0%. The TgAb assay had an intra-run CV of 5.6% and an inter-run CV of 8.7%. anti-Tg determinations <115 IU/mL were considered to be negative.

All patients signed the general institutional informed consent both for therapies and diagnostic procedures and for the possible use of anonymized data for scientific reports. The diagnosis protocol and decision criteria are highlighted in Figure 1.

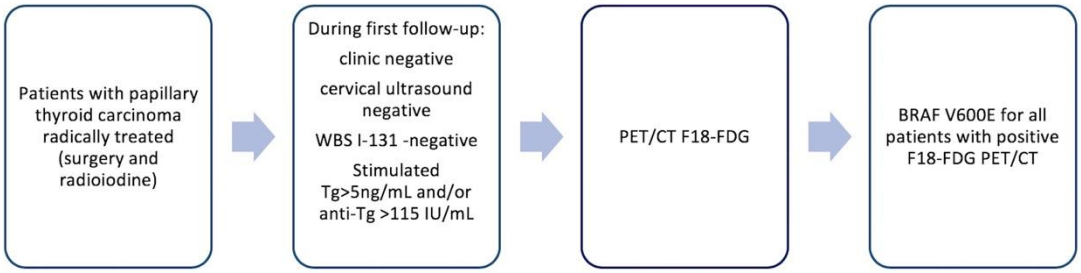

Figure 1. Diagnostic sequence for patients included in the study.

2.5. Statistics

Data are presented as means ± standard deviations (SD), proportions, or both. Subgroup comparisons regarding characteristics and correlations were made using the Student's *t*-test, Shapiro–Wilk test for uniform data and Spearman test for correlations. Statistical analyses were made using GraphPad Prism 6.0; $P \leq 0.05$ was deemed to be significant.

3. Results

Considering previous published data [10] the number of patients with DTC and positive F18-FDG PET/CT scans represents 38%. For all patients with positive scans, we performed the BRAF mutation. Following the analysis of the BRAF V600E mutation, among the 20 patients, 10 of them had a positive

result (50%), 9 a negative result (45%), and in one case the analysis was inconclusive. The distribution of SUVlbm Max values in the patient group was between Min and Max 2.5–21.57 with a mean ± SD of 8.25 ± 6.32 and is represented in Figure 2.

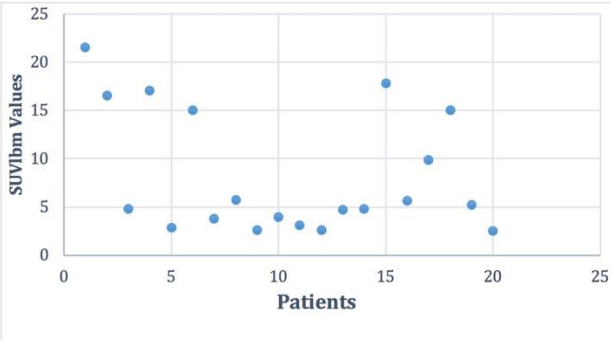

Figure 2. The distribution of the SUVlbm (standardized uptake value lean body mass) Max values in the studied cohort.

The mean ± SD SUVlbm value for BRAF+ (positive) patients was 8.99 ± 7.24 and it was higher than in the group BRAF– (negative) patients, where mean ± SD was 7.99 ± SD 5.64.

The PET/CT evaluation reports detected pathological uptake of the tracer with suspicion of persistent disease as follows: latero-cervical lymph nodes in 8 cases (40%); thyroid bed in 3 cases (15%); both in the thyroid bed and at the cervical lymph node level in 2 cases (10%), and with suspicion of distant secondary metastases in lungs in 2 cases (10%), bones in 2 cases (10%), lung and bone in 1 case (5%) and mediastinum in 2 cases (10%). After distributing patients by stage and establishing a cut-off value of 55 years for the variable age, 75% of patients were over 55 years old; 7 patients (35%) were in advanced stages III and IVB. Prognostic factors, such as tumor stage, presence of metastatic lymph nodes or distant metastases, extrathyroid extension (EET), venous invasion (V), lymphatic invasion (L), and resection limit (R) were analyzed in Table 1.

Table 1. Characteristics of patients.

No	*Sex*	*BRAF status*	*SUV*	*Age yo*	*T*	*N*	*M*	*L*	*V*	*R*	*EET*	*Stage*
1	M	+	21.57	69	T4a	N1b	0	1	1	1	Yes	III
2	M	+	16.57	72	T3b	N1b	0	1	0	1	Yes	II
3	M	-	4.8	53	T3b	N1b	0	0	1	1	Yes	II
4	F	-	17.09	62	T2	N1b	0	1	0	0	Yes	II
5	F	+	2.86	58	T3b	N1b	0	0	0	0	Yes	II
6	F	+	15	60	T4a	N1b	0	1	1	1	Yes	III
7	F	-	3.76	39	T3a	N1b	0	0	0	0	Yes	I
8	M	+	5.7	41	T3b	N1b	0	1	0	1	Yes	I
9	M	+	2.6	63	T1a	N0	0	0	0	0	Yes	I
10.	F	-	3.94	61	T2	N1b	0	1	0	1	Yes	II
11.	F	N/a	3.11	62	T2	N1b	0	0	0	0	No	I
12.	F	+	2.56	66	T4a	N1b	0	0	0	1	Yes	III
13.	M	-	4.68	22	T3a	N1b	0	1	1	1	Yes	I
14.	F	-	4.82	71	T3b	N0	0	0	1	0	Yes	II
15.	F	-	17.78	72	T3b	N1b	1	1	1	1	Yes	IVB
16.	M	+	5.61	41	T2	N1a	0	1	1	0	No	I
17.	F	-	9.86	62	T3a	N1b	0	0	0	0	No	II
18.	F	+	15	59	T2	N0	1	0	0	1	No	IVB
19.	M	-	5.25	65	T3a	N0	1	1	1	0	Yes	IVB
20.	F	+	2.5	74	T1b	N0	1	0	0	0	Yes	IVB

SUV—standardized uptake value; T—tumor size; N—status lymph nodes; M—metastatic status; L—lymphatic invasion present or not; venous invasion present or not; R—resection margins; EET—extrathyroid extension; N/a—not available.

To analyze the statistical differences, between the BRAF+ and BRAF– subgroups and the prognostic factors, we performed the Student *t*-test; and the results are shown in Table 2.

Table 2. Determination P in BRAF+ vs. BRAF–.

Parameter	SUV	Age	T	N	M	L	V	R	Stage	Tg	Anti-Tg	EET
Student *t*-test (P)	0.74	0.55	0.25	0.55	0.91	0.82	0.28	0.52	0.73	0.93	0.84	0.61

SUV—standardized uptake value; T—tumor size; N—status lymph node; M—metastatic status; L—lymphatic invasion present or not; V—venous invasion present or not; R—resection margins; EET—extrathyroid extension; Tg—thyroglobulin; anti-Tg—anti-thyroglobulin antibodies.

In Table 2 we observe that none of the prognostic factors express a statistically significant difference between the two subgroups BRAF+ and BRAF–. The difference between SUV values is not statistically significant: ***P = 0.74 > 0.05;***

Regarding the specific tumor marker (Tg and anti-Tg) the Tg mean values ± SD in BRAF+ and BRAF– patients' subgroups do not correlate statistically significantly: Tg 78.93 ± 142.6, respectively 74.03 ± 105.66; ***P = 0.93 > 0.05.*** With pathologic values of anti-Tg in the subgroup BRAF+ we had only 2 patients (4002 IU/mL and 181.7 IU/mL), and in the subgroup BRAF– only 1 patient with pathological values (anti-Tg – 917.4Iu/mL); also in this analysis ***P = 0.84 > 0.05*** and shows no statistical significance.

Correlations of the BRAF variable were made with prognostic factors to verify the existence of correlations between them and the status of the genetic mutation (Table 3). After performing the correlation coefficients, we observed that the patient subgroup with BRAF V600E mutation did not have any statistically significant correlation with the variables studied, so the prognostic factors were not influenced by the status of the genetic mutation.

Table 3. Analysis of the correlation between **BRAF** status and the prognostic factors.

Variable	Correlation Coefficient	P	Interpretation
SUV	0.080	0.743	Low-intensity positive correlation, statistically insignificant
Age	0.148	0.543	Low-intensity positive correlation, statistically insignificant
T	0.270	0.262	Low-intensity positive correlation, statistically insignificant
N	–0.145	0.552	Low-intensity negative correlation, statistically insignificant
M	–0.027	0.911	Low-intensity negative correlation, statistically insignificant
L	–0.055	0.821	Low-intensity negative correlation, statistically insignificant
V	–0.258	0.285	Low-intensity negative correlation, statistically insignificant
R	0.155	0524	Low-intensity positive correlation, statistically insignificant
EET	–0.121	0.619	Low-intensity negative correlation, statistically insignificant
Stage	0.082	0.737	Low-intensity positive correlation, statistically insignificant
Tg	0.020	0.933	Low-intensity positive correlation, statistically insignificant
Anti-Tg	–0.049	0.839	Low-intensity negative correlation, statistically insignificant

SUV—standardized uptake value; T—tumor size; N—status lymph node; M—metastatic status; L—lymphatic invasion present or not; V—venous invasion present or not; R—resection margins; EET—extrathyroid extension; Tg—thyroglobulin; anti-Tg—anti-thyroglobulin antibodies.

Following the correlation between the stage and the rest of the prognostic factors, the results are presented in Table 4.

A positive, statistically significant, medium-intensity correlation was identified between stage and age, so in the study group, patients with older age had a more advanced stage of the disease.

In the BRAF+ patients' subgroup, we assessed the correlation of SUV values with the prognostic variables (Table 5).

Table 4. Analysis of the correlation between the **stage** of the disease and prognostic factors.

Variable	Correlation Coefficient	*p* Value	Interpretation
SUV	0.367	0.122	Low-intensity positive correlation, statistically insignificant
BRAF V600E	0.082	0.737	Low-intensity positive correlation, statistically insignificant
Age	*0.653*	*0.002*	***Positive medium-intensity, statistically significant correlation***
T	−0.147	0.547	Low-intensity negative correlation, statistically insignificant
N	−0.307	0.200	Low-intensity negative correlation, statistically insignificant
M	*0.806*	*0.001*	***Positive correlation of strong intensity, statistically significant***
L	−0.015	0.95	Low-intensity negative correlation, statistically insignificant
V	0.145	0.551	Low-intensity positive correlation, statistically insignificant
A	0.180	0.460	Low-intensity positive correlation, statistically insignificant
EET	−0.007	0.977	Low-intensity negative correlation, statistically insignificant
Tg	0.131	0.592	Low-intensity positive correlation, statistically insignificant
Anti-Tg	0.115	0.639	Low-intensity positive correlation, statistically insignificant

SUV—standardized uptake value; T—tumor size; N—status lymph node; M—metastatic status; L—lymphatic invasion present or not; V—venous invasion present or not; R—resection margins; EET—extrathyroid extension; Tg—thyroglobulin; anti-Tg—anti-thyroglobulin antibodies.

Table 5. Analysis of the correlation between the SUV and prognostic factors in the subgroup of patients with BRAF+.

Variable	Correlation Coefficient	*p*	Interpretation
Age	0.251	0.483	Low-intensity positive correlation, statistically insignificant
T	0.128	0.722	Low-intensity positive correlation, statistically insignificant
N	0.277	0.437	Low-intensity positive correlation, statistically insignificant
M	−0.017	0.96	Low-intensity negative correlation, statistically insignificant
L	0.566	0.087	Positive medium-intensity, statistically insignificant correlation
V	0.482	0.157	Low-intensity positive correlation, statistically insignificant
R	*0.666*	*0.035*	***Positive medium-intensity, statistically significant correlation***
EET	−0.095	0.793	Low-intensity negative correlation, statistically insignificant
Stage	0.316	0.372	Low-intensity positive correlation, statistically insignificant
Tg	0.025	0.943	Low-intensity positive correlation, statistically insignificant
Anti-Tg	−0.300	0.399	Low-intensity negative correlation, statistically insignificant

SUV—standardized uptake value; T—tumor size; N—status lymph node; M—metastatic status; L—lymphatic invasion present or not; V—venous invasion present or not; R—resection margins; EET—extrathyroid extension; Tg—thyroglobulin; anti-Tg—anti-thyroglobulin antibodies; *P*-statistically significant <0.05.

Based on statistical analysis, in the BRAF+ subgroup we can say that the value of the SUV was statistically significant only by the resection limit. Thus, BRAF+ patients with a positive post-surgical resection limit had a higher SUV.

4. Discussion

It is well known that differentiated thyroid cancer, especially the papillary type, is a neoplastic pathology that generally has a very good prognosis, but there are also a few cases with less favorable evolution. The cases with serological persistent disease after the initial therapy (detectable Tg and antiTg) is a controversial issue. There is no consensus for the long-term clinical management of patients with DTC who have an elevated serum Tg or anti-Tg [11] and that is why the research in this field is open. In the present study we tried to focus on a cohort of patients with early serological persistent disease of classic papillary thyroid cancer, evaluated by F18-FDG PET/CT and BRAF mutation. According to published data, the subclinical persistent disease occurs in less than 15% and the positive scans for this group represents less than 40% [6,10]. From this group we selected the cases for mutation analysis. So, despite the large number of thyroid cancer patients, the low number of subjects taken in by the study was justified by the multiple criteria for selection and this is a limitation of our study.

In the case of thyroid carcinomas arising from the follicle cells, the mutation of the BRAF and RAS genes are the most common; the BRAF gene mutation was considered present especially in papillary thyroid carcinomas [2]. The presence of this genetic mutation is associated with aggressive cases of thyroid carcinomas, with a low response rate to the iodine treatment and an unfavorable prognosis.

Analysis of the BRAF V600E mutation in patients with differentiated thyroid neoplasia is not undertaken routinely, because differentiated thyroid cancer (DTC), especially the papillary form, has a high rate of response to treatment. In some prospective studies published to date on the analysis of BRAF V600E mutation in patients with papillary thyroid carcinoma, the determination of the mutation was largely undertaken at the time of the total thyroidectomy surgical act or with the surgical reintervention for tumor relapse [12,13].

The PET/CT evaluation was carried out due to the suspicion of aggressive patterns of initial histology, tumor relapse based on elevated Tg values, as well as Anti-Tg values. Negative scanning after administration of I-131 correlated with positive values of tumor markers or their increase in dynamics, revealing an incomplete or even absent response to treatment and imposing a PET/CT evaluation. The contribution of the determination of BRAF V600E in these patients, and how the therapeutic behavior changes, is intensively debated and studied. Following genetic testing for the BRAF V600E mutation in the study group, in $n = 10$ patients (50%) the presence of the genetic mutation was identified; in 9 cases (45%) we found a negative result and in one patient the result was inconclusive due to insufficient DNA material. Among the selected patients with serological persistent disease after initial therapy at 6 months, only two patients had pathologic values of stimulated anti-Tg and had positive PET/CT scan and BRAF+; the statistical analysis showed that the values represent low-intensity negative correlation, which was statistically insignificant. Anti-Tg is considered a surrogate marker in PTC [13], thus the role in the definition of persistent disease should not be overlooked.

We have correlated the BRAF mutation and prognostic factors such as age, sex, stage, presence of lymph node or distant metastases, presence of vascular or lymphatic invasion; no correlations between the presence of genetic mutation and the rest of the prognostic factors were revealed following statistical analysis. A similar result was also published for a population group in Korea, where the authors did not find any correlation between BRAF and the prognostic factors studied [14]. Also, a literature review and a multicenter study conducted on a group of patients in Italy concluded that the only association with the presence of the mutation was the advanced age of patients without the existence of any other correlation with a low prognosis in aggressive cases [9].

The two subgroups of BRAF+ and BRAF– patients were compared and no statistically significant differences were observed. More attention was paid to the SUV variable, since a significant positive correlation between the two variables is often associated. In our case, the value of SUVlbm Max for BRAF+ patients was higher, but without a statistically significant difference thus, the value of the SUV cannot be a predictable factor for the presence of the genetic mutation. In a work published in 2017, on a group of 107 patients the authors concluded that there was a correlation between the value of the SUV and the presence of the BRAF mutation, but in the more advanced tumor it was also correlated with the presence of its extrathyroid extensions and venous invasion [5,15]. The meta-analysis of Santhanam et al. [15] shows that the presence of the BRAF V600E mutation in PTC is related to a higher F18-FDG avidity and is associated with higher SUV uptake values compared to BRAF V600E mutation negative status. All 12 studies taken in the meta-analysis had a significant variation of different tumour types: follicular neoplasm, classic papillary thyroid cancer, poorly differentiated thyroid cancer, tall cell variant etc. The results of this meta-analysis compared with the present research which focused only on classic papillary forms, might be different due to this histological subtype of variation.

We also looked at how the SUV value was correlated with the rest of the prognostic variables. In the group of 20 patients, we identified a statistically significant correlation between the SUV value and the positive resection limit. In this way, patients with a positive resection limit following surgery had a higher SUV value following the PET/CT evaluation.

It is known that in the case of differentiated thyroid cancer the increased age is associated with a higher probability of death, this being influenced by the stage of the disease; therefore, in the present study, a positive correlation with statistical signification between the patient's age and stage was revealed, which translates into the fact that patients taken into the study at a more advanced stage of the disease were older [16].

The number of cases with classic PTC and non-favorable outcome are very low; despite this clinical scenario the role of BRAF status is to select those who would benefit from a personalized approach as regards the treatment option, after PET/CT evaluation and determination of BRAF mutation. In our study the surgical intervention was addressed in 10 patients and systemic therapy with tyrosine kinase inhibitors (TKI) was addressed for 5 patients, 2 of them also having the BRAF mutation present. In fact, the evaluation of genetic mutations is applicable in the process of development of therapies targeting the production pathway [17–19]. In some studies, a better response rate to treatment has been observed in BARF+ patients, leading to the development of studies only for this category of patients [20–24]. So, even if we focus only on few patients, selecting their best personalized treatment option is important. Patients require careful monitoring during treatment, and not least monitoring of the response to treatment through serial PET/CT evaluations.

5. Conclusions

The presence of BRAF V600E is associated with an increased risk of tumor relapse and a lack of response to iodine therapy. The value of SUV in the case of BRAF+ (positive) patients was higher than in the negative ones, but without statistical significance, thus, the value of the SUV cannot be a predictable factor for the presence of the genetic mutation. There was a statistically significant correlation between the SUV value and the positive resection limit following surgery, showing a higher SUV value in the PET/CT evaluation. No correlation was observed between the prognostic factors involved in papillary thyroid cancer and the BRAF V600E mutation.

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