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Title: New molecular tools for precision medicine in pituitary neuroendocrine tumors

Running title: Omics sciences in pituitary tumors

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Abstract

Precision, personalized, or individualized medicine in pituitary neuroendocrine tumors (PitNETs) has become a major topic in the last few years. It is based on the use of biomarkers that predictively segregate patients and give answers to clinically relevant questions that help us in the individualization of their management. It allows us to make early diagnosis, predict response to medical treatments, predict surgical outcomes and investigate new targets for therapeutic molecules. So far, substantial progress has been made in this field, although there are still not enough precise tools that can be implemented in clinical practice. One of the main reasons is the excess overlap among clustered patients, with an error probability that is not currently acceptable for clinical practice. This overlap is due to the high heterogeneity of PitNETs, which is too complex to be overcome by the classical biomarker investigation approach. A systems biology approach based on artificial intelligence techniques seems to be able to give answers to each patient individually by building mathematical models through the interaction of multiple factors, including those of omics sciences. Integrated studies of different molecular omics techniques, as well as radiomics and clinical data are necessary to understand the whole system and to finally achieve the key to obtain precise biomarkers and implement personalized medicine. In this review we have focused on describing the current advances in the area of PitNETs based on the omics sciences, that are clearly going to be the new tool for precision medicine.

Keywords: pituitary tumors, precision medicine, biomarkers, systems biology, omics

Introduction

Pituitary neuroendocrine tumors (PitNETs) are a heterogeneous group of tumors with diverse biological origin and clinical behavior, as well as different therapeutic outcomes. Substantial improvement in the scientific knowledge of PitNETs biology has been achieved over the last two decades accelerating the development of new drugs. However, up to now, the clinical approach is still based on the evaluation of the status of the tumor without implementing predictive tools enabling it to anticipate the outcome at the individual level. If this was the case, it would be possible to practice medicine more effectively, which would be unquestionably beneficial to the patient as time and money would be saved, the patient would have a better chance of being cured or, at the very least, of minimizing negative outcomes, and physicians would have help in the decision-making process when selecting a specific treatment from among several options for a given patient. Precision medicine, also known as personalized medicine, is what we refer to as the key to successfully treating patients with heterogeneous diseases for which a variety of therapy alternatives are available. The implementation of precision medicine requires robust biomarkers that allow stratify patients according to the progression of the disease, for example, the ability of a PitNET to grow and invade surrounding structures, or according to the response to a given therapeutic agent, which is typically a drug but may also be another type of treatment such as radiotherapy.

In the present paper we will review all aspects related to the implementation of precision medicine in PitNETs, both from a classic approach in which candidate biomarkers have been explored, but especially focusing on the new ways to address this issue in the era of massive data generation using state-of-the-art, high-throughput omics technologies.

Heterogeneity of pituitary tumors

PitNETs are a diverse group of tumors that vary in size, local invasiveness, and hormone production. Accordingly, these tumors have been classified as functional or non-functional based on whether they produce hormones or not. Recently, a new classification based on transcription factors has been established and identifies three main cell lineages: lactotroph, somatotroph, and thyrotroph (POU1F1/PIT1 lineage); corticotroph (TBX19/TPIT lineage); and gonadotroph (NR5A1/SF1 lineage)[1].

Functional pituitary tumors produce excessive amounts of hormones, leading to hormonal imbalances and a range of symptoms. In contrast, non-functional PitNETs do not produce

hormones and are often discovered incidentally during imaging tests for other medical conditions or are diagnosed because of symptoms due to their size and location, such as headaches, vision problems, and pituitary apoplexy. PitNETs can be further classified based on their size and invasiveness as microadenomas by convention when their size is less than 1 cm, and macroadenomas for those greater than 1 cm. Large tumors are often invasive, spread beyond the pituitary gland and may be more difficult to treat[2].

Thus, it is evident that pituitary tumors are heterogeneous, and this heterogeneity highlights the importance of individualized treatment plans that take into account the specific characteristics of each patient's tumor[3]. However, the classical approach to seek a single biomarker able to explain all the variability has not been efficient enough to bring order to this heterogeneity[4]. Thousands of molecules are involved in the pituitary tumor development in each patient. These biological determinants can belong to the genome, transcriptome, proteome or metabolome, and mutually interact to form dynamically associated molecular-network systems. These networks are also deeply involved in each individual therapeutic response. Unraveling the determinant factors driving their activation or deactivation is crucial to understand tumor heterogeneity and design personalized therapeutic strategies. A systems biology approach, rather than just focusing on individual parts, is the new and required approach to solve these questions. This point of view understands biology as a whole and recognizes that biological systems are complex and interconnected. Thus, studying them in isolation may not provide a complete understanding of their function. This approach is possible thanks to the new computational methods based on artificial intelligence and machine learning such as data mining, which are able to process the large amount of information provided by systems biology studies[5]. In that sense, integrative omics allows and calls for such an approach, that, for example, has already shown that calcium signaling pathway, cGMP-PKG (protein kinase G) signaling pathway, mammalian target of rapamycin (mTOR) signaling pathway, PI3K/AKT signaling pathway, MAPK (mitogen-activated protein kinase) signaling pathway, oxidative stress response, mitochondrial dysfunction, and cell cycle dysregulation are the most relevant pathways for tumor formation and progression and thus, may eventually act as biomarkers.

In conclusion, it can be deduced from the information currently available that heterogeneity is a distinctive feature of PitNETs that cannot be ignored. It is clear that there are various interconnected levels from which neuroendocrine tumor variability originates, ranging from cellular origin to clinical, genetic to epigenetic, functional to morphological. The complicated

interactions between the various levels of PitNETs of heterogeneity make it difficult to investigate them and find more accurate biomarkers and therapeutic targets. As a result, heterogeneity must be taken into account as a crucial element to understand tumor biology as well as for the upcoming approach and creation of therapeutic options. It is obvious that PitNETs can no longer be considered a homogeneous disease entity, and that their diagnosis, prognosis, and treatment must take this multilayered heterogeneity into account[6].

Personalized medicine based on systems biology offers an amount of possibilities as it can be applied to many different and still unexplored fields such as: a) early precision diagnosis by identifying the omics signatures of the disease, being possible to distinguish a specific disease from other similar conditions; b) prognosis, being possible to predict the outcome or the likelihood of recurrence after treatment; c) the treatment selection, helping to take clinical decisions by identifying specific altered molecular targets or pathways; and finally, d) drug development by identifying novel targets or candidates likely to respond to a particular therapy.

The challenge of personalizing medicine in pituitary tumor diseases. Which are the current biomarkers described in PitNETs for precision medicine?

Until nowadays, pituitary medicine, as well as other medical disciplines have approached treatment decision processes by using the principle of trial and error. There are some current and widely described biomarkers proposed to be used in clinical practice by some investigators although they are still in the research setting.

Loss-of-function p-53 mutations, elevated Ki-67 index and elevated mitotic count are three clearly pathological biomarkers of invasiveness and aggressive behavior in PitNETs that, when present, regular monitoring of the patient is recommended[7].

Non-Functioning PitNETs (NF-PitNETs) are the most heterogeneous group of pituitary tumors. According to the histo-pathological classification of PitNETS[1] all three lineages and even the absence of a lineage marker are included in this clinically non-secreting group. Although they have been widely studied, it has been difficult to identify robust biomarkers with easy applicability in clinical practice. Most of the inconclusive results obtained for some of these biomarkers are probably related to the high heterogeneity of these tumors, but also to the low number of cases usually included in the studies. At the molecular level, one of the most reliable biomarkers is low E-Cadherin expression[8,9], which is concordant with a higher expression of epithelial-mesenchymal transition markers and invasiveness[10].

In acromegaly, it is well known that male younger patients, with higher levels of IGF1 and higher tumor volume present a more aggressive disease[11,12]. The T2 MRI hypointensity[11,13], the higher Somatostatin Receptor 2 (*SSTR2*) and E-cadherin expression[4] and the lower Ki-67 labeling index in relation to a more densely granulated histological pattern[14,15], and to a lower GH 2h after the acute octreotide test[16] are all markers of good prognosis and good response to first generation somatostatin receptor ligands (SRLs). In contrast, T2 MRI hyperintensity or its development during treatment seems to be a marker of good response to pasireotide[17,18].

In the case of Cushing disease, most studies have shown more inconsistent results than for acromegaly with the new available therapeutic agents, such as pasireotide, which leads to the treatment-decision to be based on clinical expertise[19]. Neither somatostatin receptors nor dopamine receptors quantification have not proved useful for prediction of treatment response in corticotropinomas so far.

Prolactinomas are in general highly responsive tumors to dopamine agonists (DA), a particularity of this PitNET type that has made the primary recommendation for treatment almost always pharmacological and not surgical. However, not all patients present the same response, either regarding hormonal control or tumor volume reduction. For those cases that do not respond to dopamine agonists in which surgical treatment is performed, a low dopamine receptors quantification has been observed in relation to different molecular mechanisms beyond this situation[20,21].

The main obstacle that does not allow the generalization of implementation of any of these potential predictive biomarkers in the clinical practice is the overlap between groups, which does not allow to adequately segregate patients according to their characteristics. This overlap is due to the aforementioned biological heterogeneity of the tumors, for which some studies have already contributed to describe it, such in the case of somatotroph tumors for prediction to SRLs response[4]. Nowadays, the way to enhance the performance of predictive markers requires the utilization of a systems biology approach and omics sciences, able to establish predictive algorithms containing multiple biomarkers. This approach is currently under development and some groups are performing investigation of pituitary biomarkers in such a way.

Which omics biomarkers for personalized medicine may be useful for PitNETs management?

In the last decade, omics technology has been intensively studied in relation to PitNETs biomarkers discovery for their application in personalized medicine. Omics technology has the potential ability to generate and analyze large amounts of biological data and to obtain specific signatures for a single patient. A biological omics signature refers to a set of biological measurements that provide information about an individual patient's disease. Based on the type of data generated, omics can be classified mainly in genomics, epigenomics, transcriptomics and proteomics.

The landscape of genomic alterations

Next-generation sequencing techniques have allowed a deep knowledge into the genome that enables us to establish a genomic signature for each patient. The genomic signature refers to a unique pattern of genetic alterations such as chromosomal aberrations, single nucleotide polymorphisms and copy number variations that is presented in each individual patient. Patients with germ-line mutations associated with PitNETs account for 5-7% of patients with PitNETs. A substantial number of genes in which germ-line mutations have been related to the pathogenesis of pituitary tumors, some causing syndromic disease while others isolated PitNETs. Some of the mutated genes associated with syndromic conditions predisposing to PitNETs are Multiple Endocrine Neoplasia Type 1 (*MEN1)*, Cyclin Dependent Kinase Inhibitor 1B (*CDKN1B)*, Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha (*PRKAR1A)*, Protein Kinase C Beta (*PRKCB*), Dicer 1, Ribonuclease III (*DICER1)*, Succinate Dehydrogenase Complex Iron Sulfur Subunit x (*SDHx)* and *MYC Associated Factor X (MAX)*, among others, while the mutated genes associated with isolated PitNETS are Aryl Hydrocarbon Receptor Interacting Protein (*AIP)* and G Protein-Coupled Receptor 101 (*GPR101*)[22] (**Table I**).

Moreover, there are also some somatic mutations already described related to the biological behavior of PitNETs[23–26]. However, according to recent large-scale sequencing studies, there are only two of them that are recurrently present and with well-known significant effects: activating mutations of G Protein Subunit α (*GNAS*) in somatotroph tumors, and mutations of Ubiquitin-Specific Protease 8 (*USP8*) in corticotroph tumors[27–31]. Other less recurrent somatic mutations are loss-of-function mutations of *MEN1* in plurihormonal PitNETs producing GH and prolactin and truncation mutations of Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1*), a glucocroticoid receptor, in corticotroph tumors[31]. Interestingly, the p.R469X

mutation of *NR3C1* has recently been associated with long-term tumor recurrence, and the potential to compromise the antiproliferative action of dexamethasone in vitro[32].

However, given the small number of recurrent mutations found, it is currently thought that the genome does not explain most of the tumorigenic mechanisms and that other types of omics data need to be investigated to explain the heterogeneity of PitNETs.

Epigenomic alterations in PitNETs

Epigenetics is the study of heritable changes in gene expression, and thus in the phenotype that are not codified in the genome. The most investigated epigenetic processes are DNA methylation and histone modifications (**Table II**). DNA methylation consists in the addition of a methyl group to DNA bases, mostly cytosines within the CpG dinucleotide, and is commonly considered a repressive mark associated with gene silencing[33,34]. Histone modifications are covalent post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation, among others[35]. These marks can affect gene expression and activate oncogenes or inactivate tumor suppressor genes without changing the DNA sequence. Epigenetic profiles can provide important information about the developmental history, environmental exposures, disease state and disease behavior of PitNETs, which is of relevance to understand the whole process of tumorigenesis and may be potentially very useful to conduct high quality precision medicine. Moreover, epigenetic modifications are reversible, which is interesting to define potential therapeutic targets[36].

In recent years, there has been an increasing interest in studying epigenetic alterations in PitNETs. First studies on DNA methylation used a candidate-gene approach and analyzed specific genes, such as genes involved in cell growth, including cell cycle regulators like Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*), Retinoblastoma Transcriptional Corepressor 1 (*RB1*) or Growth Arrest and DNA Damage 45γ (*GADD45γ*); also, components of signal transduction pathways like Ras Associated Domain Family Member 1A (*RASSF1A*); apoptotic regulators like Death-Associated Protein Kinase (*DAPK*)]; and developmental genes like Maternally Expressed 3 (*MEG3*), among many others. Remarkably, some of these genes have been identified as potential biomarkers of tumor aggressiveness, classification of PitNETs subtypes and NF-PitNETs tumor invasion[37–40].

The most recent studies on DNA methylation have investigated the methylome using DNA methylation arrays or next-generation sequencing. Duong et al.[41] performed the first genome-

wide methylome study at promoter regions and found a total of 69 genes differentially methylated in different subtypes of PitNETs compared to normal pituitary tissues. Some of them were hypermethylated in NF-PitNETs, somatotroph tumors and lactotroph tumors but not all genes showed a reduction of expression.

Most of the genome-wide DNA methylation studies are focused on NF-PitNETs. Gu et al.[42] performed a global DNA methylome analysis in 6 invasive and 6 non-invasive NF-PitNETs, finding different DNA methylomes with more hypomethylated sites in the invasive tumors. Gene ontology analysis of the 307 differentially methylated genes showed that they were enriched in cell adhesion processes. In this regard, Qian et al. found the hypermethylation and downregulation of *CDH1* and *CDH13* in invasive PitNETs[43], although these results are controversial and were not validated in other studies[44]. Unlike previous studies in which only profiled DNA methylation of promoter regions was performed[41], Gu et al. showed that DNA methylation alterations were also found in gene bodies and intergenic regions. Kober et al.[45] analyzed 34 NF-PitNETs and normal pituitaries, and found different global DNA methylation signatures that may regulate cancer-related pathways. Although they found very slight differences between DNA methylomes of invasive and non-invasive NF-PitNETs, some differentially methylated genes were involved in invasiveness, such as Inositol 1,4,5-Trisphosphate 3-Kinase B (*ITPKB*) and Connector Enhancer Of Kinase Suppressor Of Ras 1 (*CNKSR1*). Hallén et al.[46] also analyzed NF-PitNETs, specifically an accurate selection of NF-PitNETs of gonadotroph lineage with 26 relapsing tumors (named reintervention group), and 17 cured-after surgery tumors (named radiologically stable group), with no pre-surgery clinical or radiological differences. They identified different methylation patterns consisting of 605 differentially methylated sites associated with clinically significant tumor growth, which showed a higher frequency of hypermethylation in the reintervention group compared with the radiologically stable group. The largest number of differentially methylated sites were detected in Nucleoporin 93 (*NUP93)* (hypermethylated) and Galectin 1 *(LGALS1)* (hypomethylated) genes. In this regard, increased expression of *LGALS1* has been associated with tumor aggressiveness in other tumors with malignant behavior[47,48]. Other interesting candidates are the Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha1 *(GABRA1)* gene, which was found to be hypomethylated in the reintervention group and has been involved in pituitary tumor pathogenesis, and the Cadherin-like and PC-Esterase Domain-Containing 1 (*CPED1*) gene, which was found to be hypomethylated in the reintervention group and has been suggested to be related with progression in other tumors[49].

Hage et al.[50] analyzed 38 patients with acromegaly and/or gigantism and found that a paradoxical increase of GH after oral glucose corresponded to a different molecular subclass of somatotropinomas with no mutations in *GNAS*, a densely granulated phenotype, high glucose-dependent insulinotropic polypeptide receptor (GIPR) expression, cytogenetic abnormalities and genome-wide DNA methylation changes. Importantly, the altered DNA methylation profile of *GIPR* gene was shown to be one of the mechanisms responsible for its increased expression.

Giuffrida et al.[51] profiled DNA methylation of 21 PitNETs comparing 11 somatotropinomas, 10 NF-PitNETS and 5 normal pituitary tissues, and identified 178 differentially methylated sites, most of them located in non-coding regions. Between these two subtypes of PitNETs, NF-PitNETs showed more hypermethylated sites than somatotropinomas in agreement with other previous studies[41,52], although invasive NF-PitNETs showed more hypomethylations than non-invasive NF-PitNETs[42]. Interestingly, they identified 3 hypermethylated genes, corresponding to Chromosome 7 Open Reading Frame 50 (*C7orf50*), G Protein Subunit Gamma 7 (*GNG7*), and BAH Domain and Coiled-Coil Containing 1 (*BAHCC1*), involved in tumorigenesis processes.

Recently, Mosella et al.[53] identified DNA methylation signatures, mainly affecting enhancer regions, that distinguished the three different PitNETs cell lineages, and thus may potentially complement conventional methods to improve the diagnostic accuracy of challenging cases of PitNETs. In this regard, Neou et al.[30] found a global DNA hypomethylation in the *POU1F1*/PIT1 lineage tumors. Interestingly, the newly documented epigenetic switch repressing the methylcytosine oxidase TET1 with completion of gonadotrophs differentiation and coinciding with the upregulation of the luteinizing hormone gene (*LHB*), may be related to the global DNA hypomethylation[54].

Some studies have also investigated histone modifications in PitNETs and their consequences in gene expression. In this regard, the Bone Morphogenetic Protein 4 (*BMP4*) gene, a growth factor driving pituitary tumorigenesis, is regulated through histone acetylation and methylation[55]. Importantly, the overexpression of the Pituitary Tumor Transforming (*PTTG1*) gene is controlled by histone acetyltransferases p300[56]. Retinoblastoma Protein-Interacting Zinc-Finger 1 (*RIZ1*), a tumor suppressor gene with a potential histone methyltransferase activity, was found overexpressed in non-invasive NF-PitNETs and correlated with global levels of some histone marks. Specifically, a lower *RIZ1* expression correlated with decreased

methylation of lysine 4 of histone 3 (H3K4) and enhanced methylation of lysine 27 of histone 3 (H3K27)[57]. Interestingly, patients with high expression of *RIZ1* presented a higher progression-free survival (52.63 ± 7.62 vs 26.06 ± 4.23 months), probably related to a direct effect on the repression of the c-MYC gene. Another study focused on EGF-Containing Fibulin-like Extracellular Matrix Protein 1 (*EFEMP1*), related to the extracellular matrix, showed that its reduced expression in PitNETs was linked to repressive histone modifications independently of the tumor subtype[58].

Additionally, some enzymes involved in the addition or removal of histone modifications are altered in PitNETs. This is the case for the histone methyltransferase Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), which has been found altered in multiple cancer types; its expression was upregulated in PitNETs while almost no expression was found in normal pituitary tissue[59]. The sirtuin (SIRT) family of histone deacetylases was also altered and showed a different expression between somatotropinomas and NF-PitNETs, so that SIRT1 was overexpressed in somatotropinomas, while SIRT3, 4 and 7 were underexpressed in NF-PitNETs[60]. Moreover, SIRT 1 and SIRT3 were related to tumor size, while no association was found with invasiveness or ki-67 index.

Transcriptomic biomarkers

Transcriptome includes protein-coding RNAs and also non-coding RNAs such as microRNAs -miRNAs-, long non-coding RNAs -lncRNAs- or circular RNAs -circRNAs-. With the implementation of microarray and RNA sequencing (RNA-seq) technologies, it has been possible to further investigate in this field and several studies have reported many potential biomarkers for prognosis, diagnosis, and treatment, thus contributing to new insights into the pathogenesis of the disease.

Many studies have been published describing differential mRNA expression patterns in all type of PitNETs, being the most represented group the NF-PitNETs. Rymuza et al.[61] reported a study of the coding-transcriptome by RNASeq of 48 somatotroph tumors. The findings showed three transcriptome subgroups with variable expression in a wide range of genes, including prognostic and GH secretion-related genes. Patients in the first group were clustered together because they lacked *GNAS* mutations, had tumors that were primarily densely granulated, and co-expressed GIPR and nuclear receptor subfamily 5 group a member 1 (NR5A1 or SF-1) proteins, similarly to the molecular subclass of somatotropinomas from patients with a

paradoxical response to oral glucose[50]. Most of the *GNAS*-mutated, densely granulated somatotroph and mixed tumors belonged to the second category, which was also the most frequent. These tumors were distinguished by their smaller size and the expression of prognosis-related genes. The third group, which primarily consisted of sparsely granulated somatotroph PitNETs, had a low *GNAS* mutation frequency, a gene expression profile associated with bad prognosis, and a higher rate of invasion and growth.

Transcriptomic studies could be used also to infer the different cell populations that form PitNETs. Zhou et al.[62] presented a comprehensive analysis of their immunological characteristics trying to predict the potential implications of immunotherapy as a therapy in PitNETs. Their computational inference of immune infiltrates showed correlation with the clinical characteristics of the patient. Somatotropinomas had greater B cell and CD8+ T cell infiltration than other PitNETs. Increased PD-1/PD-L1 expression and greater immune infiltration were linked to tumor development. Cancer/Testis Antigen 2 (*CTAG2*) and TSPY Like 6 (*TSPYL6*) were identified to be promising immunotherapeutic targets in somatotropinomas and NF-PitNETs, respectively, by analysis of cancer-testis antigen expression and CD8+ T-cell abundance.

Chen et al.[63] focused their research on ACTH-producing pituitary tumors. They performed RNAseq to investigate transcriptional dysregulation in Cushing's Syndrome. They found that Achaete-Scute Family BHLH Transcription Factor 1 (*ASCL1*), a pioneer TF, was overexpressed in both *USP8*-mutant and WT tumors. Further experiments showed that ASCL1 promoted hormone overproduction and tumorigenesis, directly regulating POMC, suggesting ASCL1 as a possible target. Additionally, ASCL1 overexpression was associated with larger tumor volume and higher hormone secretion.

Zhang et al.[64] also studied corticotroph tumors but with a single-cell RNAseq (scRNAseq) approach. They compared 5 silent corticotroph tumors vs. 5 functioning corticotroph tumors. The findings indicated that silent tumors exhibited differences in their tumor cell populations compared to functional ones. When compared to active corticotroph tumors, a number of transcripts for genes involved in the structural organization of secretory vesicles, tight junctions and hormone processing peptidases were decreased in silent corticotroph tumors. Silent corticotroph tumors had various characteristics of epithelial-to-mesenchymal transition (EMT), with increased expression of mesenchymal genes and the loss of transcripts that control hormone synthesis and secretion. Additionally, differences in stromal cells were observed, with

fibroblasts in functional tumors participating in extracellular matrix organization and inflammation, while vascular smooth muscle cells and pericyte stromal cell populations from silent corticotroph tumors also showed plasticity in their mesenchymal characteristics.

Regarding NF-PitNETs, Falch et al.[65] analyzed the transcriptome of 8 non-functioning gonadotroph tumors distinguishing among fast and slow-growing tumors according to the tumor volume doubling time median. They were able to identify 350 differentially expressed genes (282 genes upregulated and 68 downregulated in the fast group). Among them, they selected 40 genes for RT-qPCR validation in another 20 gonadotroph adenomas. Of those, 11 genes that presented a higher expression in fast-growing tumors were related to the tumor volume doubling median time, distinguishing a total of 6 genes that were related to epithelial-mesenchymal transition [Sperm Associated Antigen 9 (*SPAG9)*, SKI like proto-oncogen *(SKIL)*, Metadherin *(MTDH)*, Hook Microtubule Tethering Protein 1 *(HOOK1)*, CCR4-Not Transcription Complex Subunit 6-Like (*CNOT6L)* and Protein Kinase CAMP-Activated Catalytic Subunit Beta *(PRKACB*)].

These studies also are useful to describe different models of tumor-tissue and to investigate the effects of SRLs and DA on tumor cells. Saksis et al.[66] examined the transcriptomic profile of GH-producing PitNETs with and without SRLs treatment, primary tumors derived from them and GH3 cells treated with SRLs line. They observed distinct changes in GH-related pathways, inner cell signaling, ion transport, cell adhesion, and extracellular matrix patterns in GH-producing PitNETs and that medical treatment exerted different effects on transcriptome profile among different groups. This heterogeneity depending on the model highlights the importance of selecting the correct model system for PitNET studies. Some authors overcomed the heterogeneity and found that Cyclin Dependent Kinase 4 (*CDK4*) could be a target to inhibit proliferation in somatotropinomas. Moreover, Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*) predicted the insensitivity to the CDK4 inhibitor, palbociclib[67]. Some authors suggested, using the same methodology, to target Potassium Voltage-Gated Channel Subfamily A Regulatory Beta Subunit 2 (*KCNAB2*) to inhibit GH secretion[68]. A similar methodology is applied by Jian et al.[69] to characterize DA resistance in PitNETs, in this case using the cell line MMQ as well as GH3 as a model. They found NIMA Related Kinase 2 (*NEK2*) to be key in the resistance to dopamine agonists and to be overexpressed in resistant prolactinomas.

miRNAs are small, single-stranded, non-coding RNA molecules considered essential for gene expression regulation. Several miRNAs have been proposed as promising biomarkers for

PitNETs. Their overexpression or downregulation has been related to differential protein expression and to tumor cell proliferation, migration, invasion and apoptosis[70–72], and moreover they have been also described as potential therapeutic targets for invasive PitNETS[73,74]. There is a recent study that investigates the implications of miRNAs in the resistance to SRLs[75]. They analyzed 5 acromegaly-controlled patients vs. 5 non-controlled patients under SRLs treatment, and they found 59 differentially expressed miRNAs. MiR-181a-5p and miR-181b-5p were downregulated, while miR-383-5p was upregulated in the non-controlled group. An additional set of 22 samples together with the previous 10 were used to validate the results. miR-383-5p showed a remarkable ability for SRLs non-response prediction, obtaining predictive values of response in receiver operating characteristic (ROC) curve of 84.3% (NPV) and 84.5% (PPV), which means that MiR-181a-5p is a strong candidate to be used as a biomarker for prediction in SRLs treatment response in the clinical practice.

Unlike miRNAs, lncRNAs are a heterogeneous group of non-coding RNA that includes all intracellular RNAs with more than 200 base pairs long. They can be cytoplasmic or intranuclear and are involved not only in transcription and mRNA traduction regulation, but they also act as miRNAs sponges (molecules with ability to create loss-of-function phenotypes for miRNA families) and as a guide for chromatin modifiers[76]. Microarray studies have identified differential expressed lncRNAs as potentially involved in PitNETs development with well-established protein pathways. We highlight the LncRNA *H19* that inhibits tumor cell proliferation via mTORC1[77] and that has been proposed as a potential therapeutic agent administered via exosome[78]; the overexpression of LncRNA *RPSAP52* that has been identified as an oncogenesis enhancer via *HMGA1* and *HMGA2*[79]; LncRNA IFNG-AS1 that functions as an oncogene in PitNETs, increasing the expression of the epithelial splicing regulatory protein 2 (*ESRP2*)[80]; LncRNA *CCAT2* that promotes carcinogenesis via Pituitary tumor transforming gene 1 (*PTTG1*)[81]; and LncRNA *MEG3* that acts as a tumor suppressor and its inactivation/downregulation contributes to the development of NF-PitNETS[82] among others (**table III**).

CircRNAs are intracellular single-stranded, covalently closed RNA molecules derived from exonic sequences by alternative mRNA splicing. They are resistant to exonuclease digestion and their presence is very stable across different species with a tissue or developmental-stage-specific expression. They play an important role as microRNA sponges and as regulators of splicing, transcription, RNA-binding protein sponges and protein/peptide translators[89]. There

are a limited number of studies reported in PitNETs, which mainly investigate the circRNA expression profiles for invasive compared to non-invasive PitNETs and for somatotropic PitNETs compared to normal pituitary glands. Hu et al.[90] described that Hsa_circRNA_405761 and hsa_circRNA_000992 were significantly upregulated in invasive NF-PitNETs, while hsa_circRNA_102598 and hsa_circRNA_102597 were significantly downregulated. They could demonstrate that hsa_circRNA_102597 was significantly correlated with tumor diameter and Knosp grade and that it was able to accurately differentiate invasive from non-invasive NFPAs as well as predict tumor progression/recurrence (AUC=0.783). Zhang et al.[91] investigated circVPS13C; which expression is upregulated in high-risk NF-PitNETs. Du et al.[92] identified hsa_circ_0001368 that was upregulated in somatotroph tumors and correlated with the invasiveness and serum GH levels. Finally, Xiong et al.[93] described that rno_circ_0001004 regulate GH synthesis and cell proliferation acting as a novel sponge for miR-709.

Potential proteomics biomarkers

The proteome corresponds to the totality of proteins in a sample (the entire organism, a tissue or a fraction of them) and is a reflection of the coding transcriptome. Its study allows accurate functional inferences from gene expression data at the moment of the sample collection. Moreover, it can be analyzed in accessible tissues such as plasma or urine, which implies an added value to its investigation and application. Its study became possible with the introduction of the quantitative mass spectrometry (MS) technology and its advances. There are many aspects that we can study from proteins: their identification, quantification, location, activity, post-translational modifications, structural composition, biological functions and protein interactions, and it also has many potential applications in personalized medicine, biomarkers discovery and drug discovery among others.

There are not yet many studies using MS-proteomic analysis on PitNETs. In 2003 Zhan et al. generated the first reference map of a human PiTNET proteome using two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF MS and (LC-ESI-Q-IT MS) mass spectrometry[94]. They mainly identified pituitary hormones, cellular signaling molecules and proteins involved in structural organization, transport and defense. In 2010 Liu et al.[95] studied prolactinomas by immuno-laser capture microdissection (LCM) coupled with MS, identifying more than 2000 proteins, and in 2011, conducted a similar analysis on lactotrophs identifying more than 1600 proteins[96]. These studies established extensive proteomic databases of normal and tumoral lactotroph cells. The proteome analysis of different NF-PitNETs subtypes

performed by Cheng et al.[97] using TMT-6-plex labeling followed by LC-MS/MS showed that the overlapped proteins were enriched in several pathways including focal adhesion, cGMP-PKG pathway and platelet activation signaling pathways, and proposed that the overlapped proteins could be potential biomarkers.

More importantly from a clinical point of view, other works have shown that the dysregulation of different proteins of Notch and Wnt signaling pathways in NF-PitNETs[98] and in prolactinomas[99]. Invasive PitNETs have also shown different protein expression in different studies. Wang et al.[100] demonstrated that ECM-receptor interaction, focal adhesion, and PI3K-Akt signaling pathways were significantly associated with tumor invasiveness and aggressiveness. In addition, these findings offered the scientific evidence to in-depth understand molecular characteristics of FSH-positive NF-PitNETs, and effectively stratify these post-surgery patients for personalized prognostic assessment and targeted treatment. In this regard, Zhang et al.[101] also performed a quantitative proteomic analysis on tissue samples from patients with invasive and non-invasive pituitary adenomas. By integrating the differential proteins with data on invasion and EMT, they identified 46 EMT-related differential proteins. Among these, solute carrier family 2 member 1 (*SLC2A1*) was found to be significantly upregulated in invasive pituitary adenoma. *SLC2A1* showed a strong correlation with the invasiveness of the tumor and was associated with EMT-related functions and pathways. Experimental validation confirmed the significant upregulation of *SLC2A1* in invasive pituitary adenoma. Additionally, other researchers focused specifically on the kinome combining gene expression and proteomics[102]. They found distinctive expression patterns of kinase-encoding genes in the different PitNET lineages. The study suggests that the kinome of PitNET can be classified into distinct groups based on the driving transcription factor and highlights the potential of these complexes as molecular therapy targets.

Moreover, Chen et al.[103] focused on invasive somatotropinomas: they collected 10 invasive and 9 noninvasive somatotroph PitNETs. The proteomic analysis revealed distinct patterns and identified several pathways associated with tumor proliferation, migration, and invasion. In particular, high expression of cathepsin Z (*CTSZ*) was found in invasive PitNETs and was positively correlated with invasive and growth parameters. The researchers validated these findings in vitro using Ctsz-overexpressing GH3 cells, which showed increased proliferation, invasion, and migration. Regarding somatotropinomas, Li et al.[104] focused on changes in hGH isoforms using proteomics analysis. The researchers identify specific proteoform patterns

associated with pituitary adenomas and suggest their potential as biomarkers. Moreover, Tang et al.[105] explored using quantitative proteomics the molecular characteristics of different types of granulated somatotroph adenomas. By analyzing protein expression patterns, the researchers identify distinct molecular features specific to different subtypes of granulated somatotroph adenomas.

On the other hand, post-translational modifications, such as protein ubiquitination, nitration and phosphorylation, have also been studied. A reduced ubiquitination of the 14-3-3 zeta/delta protein in NF-PitNETs may end with its upregulation, suggesting a contributing role in disease progression[106]. Another study that investigated the effect of protein nitration in NF-PitNETs found the presence of 9 nitroproteins and 3 proteins that interacted with nitroproteins that were not present in normal pituitary gland, suggesting a possible role in the pituitary adenomas developmen[107]. The alterations in acetylated protein profiles and acetylation-mediated molecular pathways have also been studied in NF-PitNETs. Wen et al.[108] identified 296 acetylated proteins with 517 acetylation sites, the majority of which were significantly down-acetylated in NF-PitNETs. These proteins were involved in cellular processes and signaling pathways such as metabolism, translation, cell adhesion, and oxidative stress. Additionally, there is a study that integrates phosphoproteomics and transcriptomics data in invasive and non-invasive NF-PitNETs. They found 130 proteins differentially phosphorylated that corresponded to 130 differentially expressed genes, all of them involved in multiple biological processes representing potential predictive/prognostic markers in NF-PitNETs[109]. Finally, another study focused on phosphoproteomics found the phosphorylation of β-catenin at Serine552 to be related to the invasion and recurrence of NF-PitNETs[110].

What is known about integrating multi-omics data in pitNETs?

It is obvious that molecules from different omics levels are interconnected. In a disease stage, dysregulations that occur at some levels have an impact on others, affecting all omics profiles. Thus, if we are looking for a systems biology integrative approach, it is of utmost importance that the studies interrelate different omics signatures as far as possible. Most of the studies which we have included in the present review have performed mono-omic analysis and supervised comparisons based on disputable clinical and morphological criteria. However, there are already some studies that do integrate multi-omics data.

Salomon et al.[29] studied in 2018 the exome, transcriptome and methylome of 37 PitNETs including GH-secreting (n=17), ACTH/silent/secreting (n=10/3) and endocrine-inactive (n=18) tumors. Supporting the previous mono-omics findings, exome analysis confirmed that recurrent single nucleotide and small somatic mutations were infrequent, but they identified an increase in copy number variations (CNV). Interestingly and in agreement with previously reported studies, DNA methylation alterations could explain much better the disease etiology and evolution as it demonstrates a well-separate clustering of each specific secretion type. A global hypomethylation in somatotroph tumors in comparison with ACTH-corticotroph tumors was described and silent-corticotroph tumors clearly clustered with the ACTH-secreting tumors rather than with non-functioning tumors suggesting that the same pharmacological treatment may be effective. The integrated omics analysis concluded that promoter hypomethylation induces overexpression of (Somatostatin Receptor 5 (*SSTR5*) and *GH2* in somatotroph tumors and the overexpression of *POMC* in corticotroph ACTH-secreting tumors. This expression was independent from the *USP8* mutation. There was no correlation between *GNAS* expression and the state mutated/wildtype, illustrating that its expression may be independent of its mutation status and warrants further investigation.

Long et al.[111] in 2019 presented the first omics meta-analysis to comprehensive analyze nine sets of documented NF-PitNETs omics data, including transcriptomic and proteomic, quantitative data, mapping protein and protein nitration data with mapping protein, protein nitration and phosphorylation control data. A total of 42 hub-molecule panels and 9 canonical-pathway panels were identified to be significantly associated with tumorigenesis. Four important molecular-network systems, including PI3K/AKT, mTOR, Wnt, and ERK/MAPK pathway-systems were confirmed in NFPAs and nineteen high-frequency hub-molecules were also validated. Moreover, mTOR and Wnt pathways were validated through Western blot analysis, identifying a decreased expression of *PRAS40* and increased phosphorylation levels p-PRAS 40 in mTOR pathway, and identifying a decreased expression for GSK-3b and increased phosphorylation levels of GSK-3b as well as increased expression level of b-catenin in Wnt pathway.

Moreover, Neou et al. published in 2020 a comprehensive multi-omics study that pretended a pangenomic classification of PitNETs[30]. They performed the study of the genome (exome; n=83 and RNAseq; n=134), chromosome alterations (n=86), miRNAseq (n=111) and methylome (n=86) and applied unsupervised clusterings according to omics information of 134

PitNETs with well identified histological, secretory pattern, aggressiveness and clinical characterization information with the aim to provide public extensive molecular data from a single set of PitNETs. There were no differences among histological subtypes according to the exome study. They only confirmed 2 mutated genes in >5% of PitNETs: *USP8* and *GNAS*, as already described and pointing to the epigenetic signature as more relevant for tumor characterization. The number of chromosome alterations varied according to secretion type and there were more aberrations found in hyperfunctioning tumors than in NF-PitNETs, but there was no correlation with aggressiveness. Dopamine Receptor 2 (DRD2) showed the highest expression in lactotroph PitNETs and a variable expression in somatotroph tumors, with a higher expression in *GNAs* mutated PitNETs, proposing a potential value of *GNAS* as a response predictor factor. The unsupervised clustering of PitNETs according to their miRNAome identified four groups strongly associated with tumoral secretion. The unsupervised hierarchical clustering of PitNETs based on their methylome profile identified three groups associated with tumor type and secretion, and it was a parallel classification to the six groups generated with the unsupervised transcriptome classification. *USP8*- and *GNAS*-mutated PitNETs were associated with the specific transcriptome subgroups (t1-Corticotrophs and t6-Somatotrophs respectively), with specific signatures distinct from those of their wild-type counterparts. The integration of multi-omics data highlights a strong relationship between the WHO 2017 lineage's PitNETs classification, and the omics signatures identified: POU1/Pit1 lineage (thyrotroph tumors) was the better characterized group. This group presented a characteristic epigenetic signature consisting in a global DNA hypomethylation, mainly in "open sea" DNA inversely correlated with the demethylating enzyme TET methylcytosine dioxygenase 2 (*TET2*) highly expressed and associated to chromosomal instability. In T-Pit lineage (corticotroph tumors), tumors were divided in 3 groups depending on mutated vs. wild type *USP8* mutation and dedifferentiation to gonadotroph tumors (silent corticotroph PitNETs). *USP8* wild type appeared to be more aggressive and *USP8* mutated showed fewer sinus invasion, limited epithelial-mesenchymal transition transcriptome signature, higher Somatostatin Receptor 5 (*SSTR5*) expression and low mRNA level of O-6-methylguanine-DNA methyltransferase (MGMT); which may have relevant clinical implications in medical response treatment. Most of the other PitNETs were equally identified with both WHO 2017 and transcriptome classifications. However, this molecular approach detects some discrepancies with the WHO 2017 classification regarding the gonadotroph group. Null-cell PitNETs are included in the transcriptome gonadotroph group even if they do not express Steroidogenic Factor (*SF1*). Silent corticotroph PitNETs displayed both

corticotroph and gonadotroph transcriptome signatures; this last confirmed by the expression of gonadotroph marker GATA3 by immunohistochemistry. *SF1* was found expressed in somatotroph GNAs wild type tumors, questioning the specificity of *SF1* as a marker of gonadotroph lineage.

Another important multi omic study, including 200 PitNETs, was the one performed by Zhang et al.[112] using transcriptomics, genomics and proteomics. The results revealed that GNAS copy number gain could be used as a diagnostic marker for hyperproliferation of the PIT1 lineage. Through proteomics-based classification, seven distinct clusters of PitNETs were identified, with a subgroup showing higher expression of EMT markers, suggesting increased invasiveness. The study also identified potential therapeutic targets, such as Cyclin Dependent Kinase 6 (*CDK6*), TWIST family bHLH transcription factor 1 (*TWIST1)*, Epidermal Growth Factor Receptor (*EGFR*), and Vascular Endothelial Growth Factor (VEGFR2), specific to different clusters. Furthermore, immune subtyping analysis uncovered associations between alterations in the JAK1-STAT1-PDL1 axis and immune exhaustion, as well as changes in the JAK3-STAT6-FOS/JUN axis and immune infiltration, indicating the potential application of immunotherapy in PitNETs. These findings were validated in an independent cohort of 750 PitNET patients.

Through proteomics, transcriptomics and genomics analysis, Yamato et al.[113] also found GNAS mutations to be a key factor in somatotropinoma biology. Proteomics analysis revealed that GNAS mutation influenced the expression of various proteins, particularly those involved in the GPCR pathway, which plays a role in GH secretion and cell proliferation. However, SSTR2 did not show differential expression between WT and mutated GNAS patients. The study further identified several proteins, including ATP2A2, ARID5B, WWC3, SERINC1, and ZFAND3, that were correlated with GH change rate or tumor volume change rate in response to octreotide.

Despite sequencing the transcriptome and the genome of NF-PitNETs, somatotroph and lactotroph tumors, Chen et al.[114] focused on the clinical behavior of lactotroph PitNETs. They found widespread genomic copy number amplifications in some prolactinomas. Classifying these tumors according to CNVs, they found that tumors with high CNVs had increased prolactin production, dopamine resistance and higher proliferative capacity. This might be caused by some key genes with copy number amplification that results in transcriptional activation, such as *BCAT1*.

On the other hand, Wang et al.[115] focused on the chromatin accessibility and its regulatory effect in the expression in GH-secreting tumors. They identified differentially expressed genes in somatotroìnomas compared to normal pituitary tissues. They concluded that Somatostatin Receptor 1 (*SSTR1)*, Wnt Family Member 5B (*WNT5B)*, and Growth Hormone Releasing Hormone Receptor (*GHRHR)* may have their expression levels significantly upregulated as a result of the enhanced chromatin accessibility at promoter-TSS regions or distal regulatory elements. The upregulated differentially expressed genes were involved in hormone-related signaling pathways, while the downregulated, were enriched in focal adhesion pathways. The researchers found that the downregulated genes were associated with hypo-accessible chromatin regions. These regions contained motifs for critical regulatory factors like CCCTC-Binding Factor (*CTCF*), Regulatory Factor X2 (*Rfx2*), and splicing factor 1 (*SF1*), suggesting that the decreased chromatin accessibility could hinder the DNA-binding ability of transcription factors and lead to the downregulation of critical genes associated with growth hormone-producing pituitary tumors. This study provides insights into the regulatory network of acromegaly and its implications for tumor formation and growth.

Finally, the most recent studies take advantage of single-cell genomics. In Cui et al.[116], the authors used RNA sequencing (scRNA-seq) and single-cell whole-genome sequencing (scWGS) techniques to analyze tumor samples obtained from different patients with PitNETs. By examining the gene expression profiles of individual cells within the tumors, they aimed to identify distinct cell populations and understand their underlying genetic features. The study identified several distinct cell clusters within PitNETs, including hormone-secreting cell types and non-secreting cell types. The authors also discovered specific gene expression signatures associated with different cell populations, shedding light on the cellular heterogeneity within PitNETs. Additionally, the researchers performed scWGS to investigate the genomic alterations present in PitNETs. They identified at single cell level recurrent genetic mutations and copy number variations in genes that are known to be involved in PitNET development, such as *MEN1*, *AIP*, and *USP8*.

In another article, Asuzu et al.[117] use sc-RNAseq in Cushing's disease to characterize a subpopulation of proliferating, terminally differentiated corticotroph cells. Studying the tumor's heterogeneity allowed the investigators to identify a population of proliferating cells that may be responsible for tumorigenesis. Moreover, they found recurrent promoter hypomethylation and transcriptional upregulation of Phorbol-12-Myristate-13-Acetate-

Induced Protein 1 (*PMAIP1*) (encoding pro-apoptotic BH3-only bcl-2 protein noxa) but paradoxical noxa downregulation. Also, they recognized apoptosis escape through noxa degradation by the proteasome as an oncogenic pathway in Cushing's disease. Selective proteasomal inhibition prevented the development of noxa and induced apoptosis in human primary cells, suggesting that it may be useful in treating human Cushing's disease.

Which are omics limitations in PitNETs clinical studies?

The use of omics studies in PitNETs is a promising and stimulating field from which we can expect interesting advances in the next coming years. However, at present we still do not have enough evidence to draw many conclusions. Most of the studies are exploratory and retrospective, with a variable number of patients, mostly insufficient for subgroup analysis and probably including too heterogeneous tumors, which hinders the extraction of consistent conclusions and the replication of the results in other independent cohorts. The datasets that include all the omics and reliable clinical information that some groups are publishing will be essential for validation and to deepen this knowledge, as these studies are very expensive and unaffordable for most research groups. Lastly, the black box of machine learning methods will also be a difficulty that has to be overcomed. Currently there are different algorithms available being used by each research group with no evidence-based recommendations yet on how to explain the mathematical models; in the near future this will certainly interfere with the results comparison among the studies. Finally, more multi-omics studies integrating clinical, radiological and molecular disorders are required to really understand the systems biology of PitNETs. Therefore, it will be essential to work in a multidisciplinary team with the integrated expertise of clinicians, radiologists, molecular pathologists and biologists, and obviously, also computer engineers.

Conclusions

At present, clinically relevant questions such as the identification of potentially aggressive tumors and the prediction of the medical treatment response remain unsolved challenges for personalized medicine research. However, all these studies presented above are a step towards uncovering the complexity of the individual molecular mechanisms involved in PitNETs pathophysiology and represent a first and important step towards a three-dimensional whole-tumor approach with radiomics. A combination of both omics and clinical data will be the key

to finally identify really precise biomarkers, able to correctly segregate patients and make precision medicine a reality.

Conflict of interests

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this manuscript.

Authorship and contributorship

MPD conceived and designed the work. MMP performed the information research and wrote the manuscript; JG, EV, JP, MJ and MPD revised the manuscript and contributed to the last version of the paper. All authors approved the last version to be published.

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Table I. Main mutations in PitNETs.

Classification		Gene	Mechanism	Clinical presentation
Germline mutations	**Syndromic mutations**	**MEN1**	Reduced expression of the tumor suppressor nuclear protein menin	MEN1 Syndrome. PitNETs in 30-40% of them (lactotroph > non-functioning > somatotroph PitNETs)
		CDKN1B	Loss-of-function mutation of *CDK1B* and reduced expression of *p27* tumor suppressor gene	MEN4 Syndrome. PitNETs in 37% of them. (Unknown subtype classification)
		GNAS	Constitutive activation of the receptor Gsα and increase of cAMP levels	Mc Cune-Albright Syndrome. 20% of them present excess of GH secretion, but only some of them will present a GH-secreting PitNET. (Somatotroph PitNETs > somatotroph hyperplasia)
		PRKAR1A	Loss-of-Function of PKA1α regulatory subunit and increase of cAMP levels	Carney Complex Syndrome. 75% of them present excess of GH secretion but only 10-15% present somatotroph tumors (Hyperplasia >> Somatotroph >> Lactotroph PitNETs)
		PRKCB	Duplication of PRKCB and increase of cAMP levels	
		DICER1	Loss-of-function of DICER that prevents the microRNA precursors' maturation	Dicer Syndrome. <1% present pituitary blastomas with adrenocorticotropic secretion
		SDX MAX	Loss-Of-Function of SDX with impairment of the electron transfer chain and metabolites accumulation such as HIF1α that induces VEGF upregulation	Pheochromocytoma/Paraganglioma with Pituitary Adenoma Syndrome. 0.3% present PitNETs (lactotroph or somatotroph > non-functioning PitNETs)
	Isolated PitNETs	**AIP**	Loss-of-function of AIP cochaperone protein, a probable tumor suppressor gene with multiple targets in different vital pathways	Family Isolated Pituitary Tumors (somatotroph/ somatolactotroph >> lactotroph >> non-functioning PitNETs)
		GPR101	Overexpression of GPR101, a G-protein coupled receptor. Unknown function	X-Linked Acrogigantism (Somatotroph PitNETs > somatotroph hyperplasia)
Somatic mutations		**GNAS**	Constitutive activation of the receptor Gsα and increase cAMP levels	Somatrotroph PitNETs
		USP8	Increased EGFR signaling	Corticotroph PitNETs
		MEN1	Loss-of-function of tumor suppressor menin	Plurihormonal PitNETs (somatolactotroph)

	NR3C1	Variant forms of glucocorticoid receptor. Unknown pathway	Corticotroph PitNETs

Table note: Mutations can be germline and somatic. Germline mutations have been divided in two groups associated with syndromic mutations and isolated PitNETs. Adapted from Barry S and Korbonits M; Update on the Genetics of Pituitary Tumors. *Endocrinol Metab Clin N Am* 49: 433–452, 2020[22].

Table II. Classification and main conclusions of the epigenomic studies focuses on DNA methylation and histone modifications in PitNETs.

Classification	Compared groups	Conclusions	Reference
DNA methylation	7 GH- vs. 6 ACTH- vs. 6 PRL- vs. 13 NF-PitNETs vs. 4 normal pituitary glands	They found hypermethylated genes in all PitNETs types compared to normal pituitary glands, but not all those genes showed a reduction of the expression.	Duong *et al.* 2012[41]
	6 invasive vs. 6 non-invasive NF-PitNETs	Cluster analysis of differentially methylated CpGs demonstrated a complete distinction between invasive and noninvasive NF-PitNETs. Invasive tumors presented more hypomethylated sites than non-invasive NF-PitNETs; most of them related with genes involved in cell adhesion process.	Gu *et al.* 2016[42]
	34 NF-PitNETs vs. normal pituitary grands. (+75 NF-PitNETs for validation)	NF-PitNETs exhibited distinct global DNA methylation profile as compared to normal pituitary gland. A promoter hypermethylation and a decreased expression of *SFN, STAT5A, DUSP1, PTPRE* and *FGFR2* genes was found in NF-PitNETs. Invasive tumors presented very slightly different methylation profiles. Among those differences, *ITPKB* was upregulated and *CNKSR1* was downregulated in invasive PitNETs.	Kober *et al.* 2018[45]
	41 GH- PitNETs	32% of patients expressed *GIPR*. Among them, none of them presented *GNAS*-mutated. These tumors exhibited a hypermethylator phenotype compared with *GNAS*-mutated that resulted in a hypermethylation of the *GIPR* gene body. In some cases, *GIPR* overexpression is due to gene CNVs.	Hage *et al.* 2019[50]
	177 PitNETs vs. 20 normal pituitary glands. (+86 PitNETs for validation)	Methylation signatures, mainly affecting enhancer regions, clustered three different PitNETs cell lineages.	Mosella *et al.* 2021[53]
	11 GH- vs. 10 NF-PitNETs vs. 5 normal pituitary glands	NF presented more hypermethylated sites than GH-PitNETs. *C7orf50*, *GNG* and *BAHCC1* were identifyed as hypermethylated genes involved in tumorigenesis processes.	Giuffrida *et al*. 2022[51]
	26 relapsing NF- vs. 17 cured-after surgery NF-PitNETs	More hypermethylated sites were found in the relapsing NF-PitNETs compared with the cured-after surgery group. *LGALS1*, hypomethylated; *GABRA1*, hypermethylated and *CPED1*, hypomethylated; were related with tumor aggressiveness and pathogenesis.	Hallén *et al.* 2022[46]
Histone modification	In vitro studies realized with 293T human embryonic kidney epithelial cells	The overexpression of *PTTG1* gene is controlled by histone acetyltransferase p300.	Li *et al.* 2009[56]
	In vitro studies realized with somatotroph, corticotroph and lactotroph lineage cell models. 5 GH-	*BMP4* is overexpressed in prolactinomas and underexpressed in the rest of PitNETs compared to normal pituitary gland.	Yacqub-Usman *et al.* 2012[55]

	vs. 7 ACTH- vs. 9 PRL- vs. 14 NF-PitNETs	Differences are not associated with methylation status but with differential histone acetylation and methylation.	
	6 GH- vs. 7 ACTH- vs. 7 PRL- vs. 13 NF-PitNETs vs. 4 normal pituitary glands	PitNETs showed reduced *EFEMP1* expression compared with normal pitutiary, due to repressive histone modifications.	Duong *et al.* 2013[58]
	165 PitNETs vs. 19 normal pituitary glands	The histone methyltransferase EZH2 was upregulated in PitNETs while almost no expression was found in normal pituitary tissue.	Schult *et al.* 2015[59]
	50 invasive vs. 53 non-invasive tumors (23 PRL-, 23 GH-, 57 NF-PitNETs)	Different histone methylation patterns were found between invasive and non-invasive PitNETs. Downregulation of *RIZ1* correlated with hypomethylation of H3K4 and hypermethylation of H3K27 in non-invasive PitNETs. Patients with high expression of *RIZ1* presented a highest average progression-free survival.	Xue *et al.* 2017[57]
	37 GH- vs. 31 NF-PitNETs	*SIRT* family of histone deacetylases, a group of proteins involved in regulation of longevity, showed different expression between GH- and NF-PitNETs. SIRT1 was overexpressed in GH-PitNETs, while SIRT3, 4 and 7 were underexpressed in NF-PitNETs	Grande *et al.* 2018[60]

Table III. Characteristics and pathophysiology of the described Long Non-Coding RNAs (Lnc-RNA) involved in PitNETs.

LncRNA	Oncogene/ Tumor suppressor	Physiological function	Altered expression in PitNETs	Pathogenic pathway involved	Reference
MEG3	Tumor suppressor	Cell cycle detention at G1 phase	Inactivated/ downregulated	P53 inhibition	Chunharojrith *et al.* 2015[82]
C5orf66-AS1	Tumor suppressor	Inhibits cell viability and cell invasion	Downregulated	Unknown	Yu *et al.* 2017[83]
H19	Tumor suppressor	Inhibits tumor cells proliferation	Downregulated	mTORc1 inhibition	Wu *et al.* 2018[77]
CCAT2	Oncogene	Regulates adenoma cells proliferation, migration, and invasion	Upregulated	PTTG1 enhancement	Fu *et al.* 2018[81]
IFNG-AS1	Oncogene	Promotes cell proliferation, invasion, and migration and inhibits apoptosis	Upregulated	ESRP2 enhancement	Lu *et al.* 2018[80]
AFAP1-AS1	Oncogene	Promotes cell proliferation and inhibits apoptosis	Upregulated	Downregulation of PTEN and enhancement of PI3K/AKT	Tang *et al.* 2018[84]
CLRN1-AS1	Tumor suppressor	Suppresses cell proliferation, promotes apoptosis, and inhibits autophagy	Downregulated	Wnt/β-catenin inhibition	Wang *et al.* 2019[85]
RPSAP52	Oncogene	Promotes cell growth by enhancing the G1-S transition of the cell cycle	Upregulated	HMGA2 and HMGA1 enhancement	D'Angelo *et al.* 2019[79]
SNHG7	Oncogene	Inhibits apoptosis and promotes cell migration and invasion	Upregulated	Upregulation of miR-449a; with unknown pathways	Yue *et al.* 2021[86]
PCAT6	Oncogene	Cell viability, migration, invasion, proliferation	Upregulated	Downregulation of miR-139-3p and consequently enhancement of BRD4	Zhao *et al.* 2021[87]
LINC00473	Oncogene	Promotes cell proliferation	Upregulated	LINC00473 inhibits miR-502-3p increasing KMT5A expression, and, finally, stimulating cyclin D1 and CDK2 expression.	Li et al. 2021[88]