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RESEARCH

New Insights from the Expression of the Mismatch Repair System in Pituitary Neuroendocrine Tumors

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Abstract

Although defects in the mismatch repair (MMR) system have been occasionally reported in aggressive/metastatic pituitary neuroendocrine tumors (PitNETs), the potential role of MMR dysregulation in pituitary tumorigenesis is largely unknown. This study aimed to evaluate the expression of the four key MMR components in a large series of PitNETs. *MMR* gene expression was studied by RT-qPCR in 127 tumors (54 PIT1, 51 SF1, 22 TPIT), and semi-quantitative immunohistochemistry (score 0–12) in selected cases (n=46). *MSH2*/*6* and *MLH1* promoters methylation was studied in 96 tumors. Except for *MLH1*, tumor lineage of origin was the most significant factor influencing *MMR* transcripts (P=0.005, <0.001 and 0.039 for *MSH2*/*6* and *PMS2*, respectively), the highest levels being observed in SF1 tumors. Within subgroups, *MMR* transcripts were significantly lower in large/invasive PIT1 and in functioning TPIT tumors. *MSH2* promoter methylation was occasionally associated with reduced *MSH2* expression. Global loss of MSH6 (score 0), defining MMR deficiency, was observed in a single silent lactotroph PitNET, unrelated to the Lynch's syndrome. Near global loss involving MSH6, MSH2 or PMS2 (score 1) was observed in 5 tumors (1 lactotroph, 1 SF1, 3 TPIT). *MMR* mutations were excluded in 4/5 cases but 2 had LOH at *MSH2*/*MSH6* loci. Heterogeneous immunostaining for any MMR (score 2–4) was also observed in 15 cases. In conclusion, MMR deficiency was rarely observed (2.2%) but reduced MMR expression could be found, especially in functioning corticotroph and invasive lactotroph tumors. The molecular mechanisms and prognostic significance of such findings would deserve further investigation.

Keywords Pituitary neuroendocrine tumors · Genetics · Mismatch repair · Lynch syndrome

Introduction

Pituitary neuroendocrine tumors (PitNETs) are typically benign but induce significant morbidity due to endocrine dysfunction, systemic complications and/or local mass effects [1]. Only a small number become aggressive, or exceptionally metastatic [2]. PitNETs are classified according to their lineage of origin: (1) PIT1-derived (PIT1), which include functioning and silent somatotroph (GH or GH/PRL), lactotroph (PRL) and thyrotroph (TSH) tumors, (2) gonadotroph/SF1-derived (SF1) with a potential secretion of gonadotropins (FSH and/or LH), and (3) corticotroph/TPIT-derived (TPIT) with a potential secretion of ACTH [3]. Pituitary tumorigenesis involves several dysregulated molecular pathways, which may differ according to their lineage of origin [1].

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Defects in the mismatch repair (MMR) system have been occasionally reported in PitNETs. The MMR system includes four key proteins: MutS homolog 2 (MSH2), MutS homolog 6 (MSH6), MutL homolog 1 (MLH1) and post-meiotic segregation increased 2 (PMS2). MSH2/MSH6 and MLH1/PMS2 form heterodimers that are able to detect and repair small DNA alterations occurring during the cellular post-replication process, including missense mutations and small deletions or insertions [4]. In a Japanese survey of 13 aggressive/metastatic (ag/met) PitNETs treated by temozolomide (TMZ), loss of MSH6 immunostaining was the most prevalent characteristic of TMZ-resistant tumors (4/6) [5], attributed to the inability of MMR dimers to drive unrepaired cells to apoptosis [6]. Loss of MMR immunostaining and/or somatic *MMR* mutations [7–10], and occasional germline mutations in *MMR* genes [11–14] were also described in ag/met PitNETs, mostly lactotroph or corticotroph. Interestingly, *in vitro MSH2* and/or *MSH6* knockout experiments in corticotroph AtT-20ins cells supported a role for the corresponding proteins in the control of cell proliferation, whereas *MSH2/6* mRNA expression was positively correlated with tumor doubling time in non-functioning PitNETs from different lineages [15]. The same authors also found *MSH6* knockout to promote cell proliferation in somatolactotroph GH_3 cells [16].

We therefore hypothesized that the MMR system might play a broader role in the biology of PitNETs. To clarify whether MMR defects were restricted to specific phenotypes, we investigated the somatic expression of key MMR components in a large cohort of PitNETs. Data were analysed according to bio-clinical and molecular tumor characteristics and potential translational implications are discussed.

Materials and Methods

Characteristics of Patients and Tumors

A hundred and twenty-seven PitNETs were collected at surgery. The study was approved by the Neuromed Institute Internal Review Board as part of the Biopit study (Biopit 270423) and performed according to Helsinki declarations. Written informed consent was obtained from all patients, except for a minority of archived material from patients lost to follow-up. Individual data included bio-clinical evidence of pituitary hormone hypersecretion, magnetic resonance imaging and surgical reports to define tumor volume and invasiveness, and pre-operative treatment. The pathological diagnosis was based on immunostaining for pituitary hormones and/or transcription factors (TFs) [3, 17]. Three groups were defined according to tumor lineage of origin: PIT1 (n=54), SF1/gonadotroph (n=51) and TPIT/corticotroph (n=22), each of them including functioning and silent/clinically non-functioning (NF) cases – defined by pituitary hormone immunoreactivity (IR) in the absence of bio-clinical hypersecretion –. Three *post-mortem* pituitaries and a sample of archive pituitary hypertrophy mimicking a macrotumor were included as normal pituitary (NP) controls.

RNA Extraction and Gene Expression Analyses

Surgical biopsies were collected in RNA *later* stabilization solution (Life Technologies, Segrate (MI), Italy) and stored at −80 °C until use. Total RNA was extracted with TRIzol™ (Life Technologies, Segrate (MI), Italy), 1 µg was retrotranscribed using the Wonder RT enzyme (Euroclone, Pero (MI), Italy) after DNAse treatment (New England Biolab, Pero (MI), Italy) and cDNA was amplified with Taq DNA polymerase (Life Technologies, Segrate (MI), Italy) using *GAPDH* as a housekeeping gene and pituitary TFs to exclude significant contamination by NP cells [18]. Untranscribed RNA was tested by *GAPDH*-PCR to verify successful DNAse treatment. *MMR* genes expression was evaluated on 127 PitNETs and 4 NP by Real-Time RT-qPCR using a TaqMan methodology with ready-to-use commercial probes - *MLH1* (Hs00179866_m1), *MSH2* (Hs00179887_m1), *MSH6* (Hs00264721_m1), *PMS2* (Hs00241053_m1) and *β-actin* (Hs_99999903) as a housekeeping gene - on an Applied Biosystems 7500 Fast Real-Time PCR (Life Technologies, Segrate (MI), Italy). Experiments were run at least in duplicate. *MMR* genes expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry (IHC)

Diagnostic IHC was carried out on 4-µm paraffin-embedded sections using rabbit polyclonal pre-diluted anti -PRL, -GH-, -βFSH, -βLH, -ACTH (Roche Diagnostics International, Rotkeuz, Switzerland), -PIT1 (NBP1-92273, Novus Biologicals, dil 1:500), -TPIT (AMAb91409, Atlas Antibodies, dil 1:200), -SF1 (Ab217317, Abcam, dil 1:500) primary antibodies and the Ultraview DAB detection kit (Roche Diagnostics International, Rotkeuz, Switzerland) in an automatic VENTANA (Benchmark ultra-XT IHC/ISH System).

MMR-IHC was performed on similar sections in 46 PitNETs – including 28 samples with at least one *MMR* gene expression under the median values by RT-qPCR –, using a diagnostic panel of pre-diluted monoclonal antibodies to MSH2 (clones 79H11), MSH6 (clone EP49), MLH1 (clone ES05) and PMS2 (clone EP51) and a Bond Polymer Refine DAB detection kit in an automated Bond RX staining processor (all from Leica Biosystems, Buffalo Grove,

IL). Nuclear MMR-IR was evaluated semi-quantitatively assigning individual scores (0–12) obtained multiplying positive cells (score 0–4 based on the percentage of positive cells: 0–10% = score 1; 10–50% = score 2; 50–80% = score 3; and >80% = score 4) by staining intensity (score 0–3: score 0 = absent; score 1 = faint; score 2 = moderate; score 3 = strong) [19]. Normal pituitary cells, where present, and endothelial cells or lymphocytes were used as internal reference positive controls. MMR deficiency (MMRd) was strictly defined by any loss of protein IR (score 0). Near global loss (score 1) and intermediate scores (2–4) were also considered, the latter being referred to as heterogenous protein expression [19]. Four recurrent PitNETs were then evaluated comparing samples obtained at previous or subsequent surgery.

Molecular Studies

Tumor DNA (tDNA) was extracted using a Quick-DNA miniprep plus Kit (Zymo Research distributed by Euroclone, Pero (MI), Italy). Genomic DNA (gDNA) was extracted using a MagCore® Genomic DNA whole blood kit (RBC Bioscience Corp. Brussels, Belgium). Both were used after appropriate quality controls.

Sanger Sequencing

Somatic hotspot mutations in *GNAS1*, *SF3B1* and *USP8/USP48* were searched for in 24 somatotroph, 14 lactotroph and 16 corticotroph PitNETs, respectively. Primers and conditions for PCR amplification were already reported [20–22] or modified from the literature [23–25] and are available in Supplemental Table 1. PCR products obtained from 200 ng tDNA with a high fidelity AmpliTaq Gold (Life Technologies, Segrate (MI), Italy) were purified from a 1.2% agarose gel (Norgen Biotek kit, distributed by SIAL, Rome, Italy) and subjected to Sanger sequencing with the Brillant Dye Terminator (Resnova, Rome, Italy) on a 3500 Genetic-Analyzer (Life Technologies, Segregate (MI), Italy).

Methylation-Specific PCR (MS-PCR)

The methylation pattern of *MMR* gene promoters was assessed by MS-PCR in 96 PitNETs and two NP samples. MS-PCR primers and conditions were retrieved from the literature for *MLH1* [26] and home-designed for *MSH2*/*MSH6* on template sequences containing CpG islands spanning the promoter and first exon involved in gene expression regulation [27, 28] (Supplemental Table 1). *PMS2* methylation was not analysed due to the absence of functional studies. One µg of tDNA was treated by sodium bisulfite (SB) using the EZ DNA Methylation-Gold Kit, including fully methylated (M) and unmethylated (U) control DNAs in each experiment (Zymo Research, distributed by Euroclone, Pero (MI), Italy). MS-PCR was performed on 100 ng of SB-treated tDNA using a hot start DNA Platinum Taq (Life Technologies, Segrate (MI), Italy), in conditions ensuring the specificity and a comparable efficiency of M/U alleles amplification. MS-PCR products were run on a 2.5% agarose gel, stained with ethidium bromide, visualized on Chemidoc (Biorad, Milan, Italy) and analysed semi-quantitatively using the ImageJ Software 1.52a after background removal (https://imagej.net). Samples were classified as fully methylated (M: 90–100%), fully unmethylated (U: 90–100%) or partially methylated (M/U).

NGS DNA Sequencing

MMR gene sequencing was proposed in the presence of very low MMR-IR (score 0–1) and performed on gDNA in patients who agreed for LS screening or on tDNA. Experiments were performed on Illumina NextSeq 550 System (San Diego, CA) using: (1) for gDNA (n=3), the SOPHiA DDM™ Hereditary Cancer Solution – covering the coding regions and splicing junctions of genes associated with LS (the four key *MMR* genes and *EPCAM*) – and SOPHiA DDM software for annotation, alignment of sequence on the reference genome GRCh38 and selection of potentially pathogenic variants, (2) for tDNA, (n=2) the OncoDEEP panel – targeting relevant genes involved in tumorigenesis including *MMR* and *TP53*, as well as regions associated with loss of heterozygosity (LOH) in tumor suppressor genes. Each variant was classified according to the 2015 ACMG-AMP guidelines as Pathogenic (P/class 5), Likely Pathogenic (LP/class 4), Variant of Uncertain Significance (VUS/class 3), Likely Benign (LB/class 2) and Benign (B/class 1).

Statistical Analyses

Data were analysed using Jamovi 2.2.5 (https://www.jamovi.org) and GraphPad Prism v10.5.0 (Adalta software, Arezzo, Italy) software. Continuous variables were expressed as median [range] and analysed by the non-parametric Mann–Whitney (two groups-analysis), Kruskal–Wallis (multiple comparisons) and Spearman's rank correlation tests. Categorical values were compared by the Chi-square test. P<0.05 was considered significant.

Results

Patients and Tumors Characteristics

The median age of patients (65 F, 62 M) at surgery was 51 yrs [16–83]. Patients were pre-operatively diagnosed with clinically non-functioning (NF) tumors (*n*=63), acromegaly (*n*=31), Cushing's disease (CD, *n*=14), prolactinoma (*n*=13), central hyperthyroidism (*n*=3) or functioning gonadotroph tumors (*n*=3). Data are summarized in Table 1 according to tumor lineage of origin. Significant differences were found in patients demographics (*P*<0.001 for age and sex). Patients with PIT1 tumors were the youngest (*P*<0.001 *vs* SF1 and TPIT). Microtumors – all functional – were a minority (*n*=9). SF1 tumors were the largest (*P*<0.001 *vs* PIT1-, *P*=0.012 *vs* TPIT- tumors). Similar proportions of invasive or recurrent tumors were present in each group (around 50% and 10%, respectively).

MMR Genes Expression in PitNETs

Significant positive correlations between the expression of the four genes of interest were observed on the whole series (*P*<0.001, r=0.51–0.79) (Table 2), suggesting variable degrees of activation of the *MMR* system. Fig.1 illustrates the most relevant findings: 1) median *MSH2/MLH1* transcripts were up to 10-folds higher than that of their partners *MSH6*/*PMS2*; 2) a normal/high *MMR* expression was observed in most PitNETs compared to NP; 3) except for *MLH1*, significant differences in *MMR* genes expression were found according to tumor lineage of origin (P<0.001, P=0.005 and P=0.039 for *MSH6, MSH2* and *PMS2*, respectively). In this series, SF1 tumors had significantly higher *MMR* transcripts than NP (P=0.007, P=0.014 and P=0.004 for *MSH2, MSH6* and *PMS2,* respectively), TPIT- (P<0.001 for *MSH2*/*MSH6*, P=0.013 for *PMS2*) and PIT1- (P<0.001 for *MSH6* only) tumors. In contrast, *PMS2* transcripts were significantly higher in PIT1 tumors *vs* NP and TPIT tumors (P=0.006 and P=0.028, respectively).

Overall, no significant differences appeared according to patients' sex or macroscopic tumor characteristics or in recurrent *vs* non-recurrent cases. However, a significantly lower expression of *MSH2* was found in tumors from young patients (≤30 yrs) (*P*=0.022 *vs* >30 yrs). *MSH6* expression was also significantly lower in clinically functioning cases (*P*<0.001 *vs* NF). Because age and functional status varied according to tumor lineage of origin, lineage-specific peculiarities were further analysed. The analysis of transcriptional data obtained on the whole series and in lineage subgroups is detailed in Supplemental Table 2.

Table 1 Bio-clinical characteristics of patients and PitNETs according to tumor lineage of origin

	All	PIT1	SF1	TPIT	*P*
Number of patients	127	54	51	22	
Sex (F/M)	65/62	26/28	20/31	19/3	<0.001
Age (yrs)	51.0 [16–83]	39.0 [16–74]	60 [36–83]	53.5 [26–78]	<0.001
Young ≤30 yrs	17 (13.4%)	16 (29.6%)	0 (0%)	1 (4.5%)	<0.001
Functioning tumors	64 (50.4%)	47 (87%)	3 (5.9%)	14 (63.6%)	<0.001
Maximal diameter (mm)	25.5 [5–60]	23 [8–47]	30 [16–60]	23 [5–42]	<0.001
Volume (m/Ma/G)	9/100/18	3/47/4	0/40/11	6/13/3	<0.001
Invasive	64/125 (51.2%)	26/53 (49.1%)	27/51 (52.9%)	11/21 (52.4%)	ns
Recurrent	13 (10.2%)	5 (9.3%)	6 (11.8%)	2 (9.1%)	ns

Data are expressed in median [range]; P values refer to comparisons between the three groups of PitNETs (Kruskall-Wallis for continuous values, Chi-square test for percentages).

Abbreviations: F female, G giant tumors, M male, Ma macrotumor, m microtumor, ns non-significant.

Table 2 Correlation matrix between *MMR* genes expression by RT-qPCR analysis

		MSH2 mRNA	*MSH6* mRNA	*MLH1* mRNA	*PMS2* mRNA
***MSH2* mRNA**	*rho*	—			
	P-value	—			
***MSH6* mRNA**	*rho*	0.563	—		
	P-value	<0.001	—		
***MLH1* mRNA**	*rho*	0.778	0.510	—	
	P-value	<0.001	<0.001	—	
***PMS2* mRNA**	*rho*	0.793	0.560	0.771	—
	P-value	<0.001	<0.001	<0.001	—

Legend: Correlations were determined according to Spearman's test.

PIT1 PitNETs No significant differences in *MMR* transcripts were found according to patient demographics or between functioning and silent/NF tumors. As illustrated in Fig. 2, *MMR* transcripts were significantly lower in invasive *vs* non-invasive tumors (*P*=0.005 for *MSH2 and PMS2*, *P*=0.002 for *MLH1*, *P*=0.012 for *MSH6*, Fig. 2A). Except for *MSH6*, a significant inverse correlation was also found between *MMR* transcripts and maximal tumor diameter (*r*=−0.518, *P*<0.001; *r*=−0.461, *P*=0.001; *r*=−0.417, *P*=0.004 for *MLH1*, *MSH2* and *PMS2*, respectively, Fig. 2B). Additional significant differences appeared according to the functional phenotype (*P*=0.038, *P*=0.033 and *P*=0.036 for *MSH2*, *MLH1 and PMS2*, respectively, Fig. 2C).

Five somatotroph tumors had a somatic *GNAS1* mutation (Arg201Cys C>T) but *MMR* genes expression was unremarkable; no hotspot *SF3B1* mutation was found in lactotroph tumors.

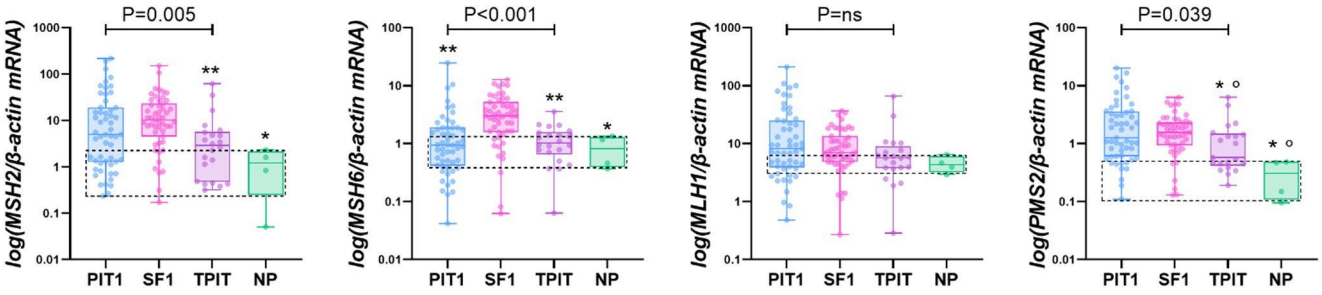

Fig. 1 *MMR* genes expression in 127 PitNETs according to their lineage of origin and in normal pituitaries. *Legend:* Data were obtained by RT-qPCR. Box plots represent median values and 10th-90th percentiles. NP normal pituitaries, ns non-significant. P values refer to 3-groups comparisons within PitNETs (Kruskall-Wallis test). Symbols refer to significant differences between PitNETs subgroups and/or *vs* NP by 2-groups comparisons (Mann-Whitney test): * P≤0.014 *vs* SF1, **P<0.001 *vs* SF1, °P<0.028 *vs* PIT1

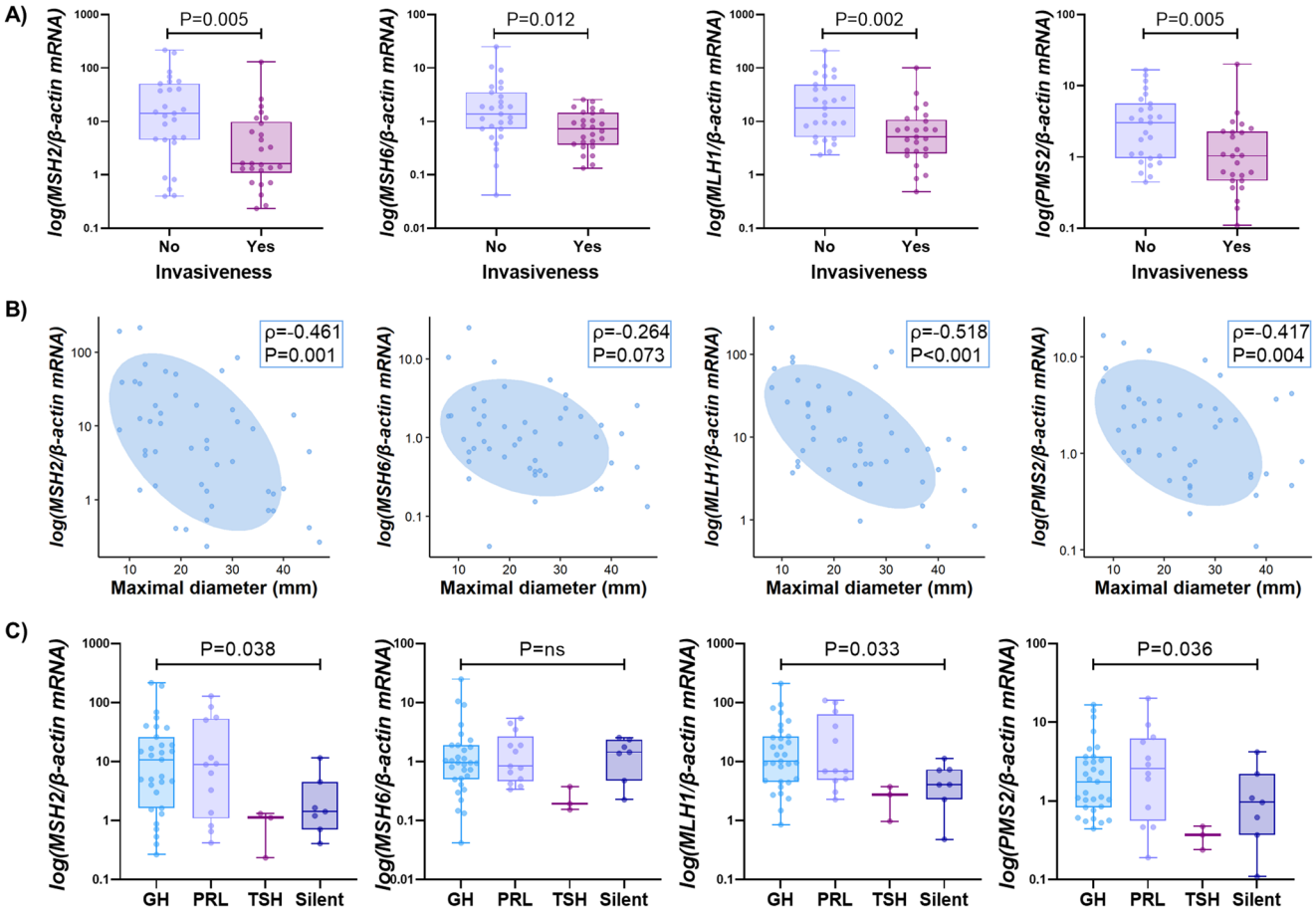

Fig. 2 Expression of *MMR* genes in 54 PitNETs derived from the PIT1 lineage. *Legend:* Data were obtained by RT-qPCR. ns non-significant. Box plots represent median values and 10th-90th percentiles. (**A**) Significantly lower *MMR* transcripts were found in invasive *vs* non-invasive tumors. (**B**) A significant negative correlation between *MSH2, MLH1* and *PMS2* expression and tumor maximal diameter was observed. (**C**) *MMR* transcripts according to patient pre-operative functional status (GH: acromegaly, PRL: prolactinoma, TSH: central hyperthyroidism, Silent: no bio-clinical evidence of hormone hypersecretion)

SF1/Gonadotroph PitNETs *MMR* transcripts were similar in male and female tumors. A significantly higher expression of *PMS2* was observed in giant tumors (P=0.011 *vs* non giant) and in invasive cases (P=0.015 *vs* non-invasive). Similar non-significant trends were observed towards a higher expression of *MSH2/MSH6/MLH1* in invasive tumors and *MLH1* in giant tumors. *MSH6* expression was significantly higher in FSH/LH-IR *vs* non-IR cases (P<0.001).

TPIT/Corticotroph PitNETs No significant differences were observed according to patient's demographics or macro-

scopic tumor characteristics. However, *MSH2* and *PMS2* transcripts were significantly lower in functioning *vs* silent corticotroph tumors (P=0.002 and P=0.030, respectively), with a similar non-significant trend for *MSH6* (P=0.095) (Fig. 3). No hotspot *USP8* or *USP48* mutation was found.

Immunostaining for MMR Proteins

Semi-quantitative immunohistochemical data obtained in 46 PitNETs (24 PIT1, 13 SF1, 9 TPIT) are summarized in Fig. 4. Overall, the correlation between gene and protein expression was significant only for PMS2 (r=0.37, P=0.013).

A normal immunostaining score was observed in a majority of cases (n=25, 54.3%).

As illustrated in Fig. 5 and detailed in Table 3, MMRd (score 0) was observed in a single case (2.2%) and near global loss (score 1) in 5 cases (10.9%). Among these, very low MSH6-IR (score 0-1) was the most prevalent abnormality, including global loss in an invasive silent lactotroph tumor (P1) and near global loss in two tumors – one invasive SF1 (S1) and one recurrent functioning TPIT (T1) –. Near global loss also involved PMS2 – two functioning TPIT tumors (T2, T3) – and MSH2 – one invasive lactotroph tumor (P2) –, but not MLH1. Heterogeneous staining for additional MMR proteins was observed in two cases (P1, T2). Heterogenous staining for one or more MMR protein was also observed in 15 tumors (32.6%) involving MSH6 (n=10), MSH2 (n=5), MLH1 (n=4) and/or PMS2 (n=3) across PitNETs subgroups (9 PIT1, 5 SF1, 1 TPIT) (Fig.4).

To approach the bio-clinical significance of very low MMR-IR (scores 0-1), we came back to individual patients and tumors characteristics, including personal and/or familial history of neoplasia and *MMR* sequencing in five cases (Table 3). None of these patients had associated neoplasia. Two young patients (P1, P2) had invasive lactotroph PitNETs: (P1) had a familial history of cancer and a large silent tumor, which subsequently massively recurred; (P2) was operated on because of a slow-growing functional tumor resistant to dopamine-agonists. None had genetic evidence of LS. Patient (S1) had a large invasive gonadotroph tumor, which subsequently recurred, but no familial history of cancer or genetic evidence of LS. Patients with TPIT tumors (T1-3) had Cushing's disease. Somatic tDNA characterization was obtained in 2 cases (T1, T2). Patient (T1) had an unremarkable familial history of cancer, but somatic LOH at *MSH2* and *MSH6* loci with a class 3 *MSH3* variant (NM_002439.5:c178delinsCCCGCAGCGC) were observed. Patient (T2) had a strong but genetically unexplained familial history of cancer. He underwent surgery for the 4th time for an invasive recurrent tumor, which subsequently progressed despite repeated radiotherapy and TMZ, leading to patient's death 10 years later. No somatic *MMR* mutation was found, but a likely pathogenic *TP53* mutation (NM_000546.6 994-2A>G) and LOH at the *MSH2, MSH6* and *TP53* loci were observed. None of these tumors had fully methylated promoters for the corresponding genes or hotspot mutations in *SF3B1* (P1, P2) or *USP8/48* (T1, T2, T3). MMR-IHC was subsequently performed on archive material from two previous surgeries from (T2), revealing a progressive decrease in MMR-IR, from normal scores to intermediate and low scores – data obtained at the first available surgery are shown as (*T2**) in Fig. 5–. Conversely, no longitudinal change in MMR-IR was observed in three other recurrent tumors compared as paired samples (two gonadotroph – including S1 – and one somatolactotroph).

Potential Role of *MMR* Genes Promoter Methylation on Gene Expression

RT-qPCR data were analysed according to *MSH2/6* and *MLH1* genes promoter methylation in 96 PitNETs (46 PIT1, 36 SF1, 14 TPIT) and 2 NP (Supplemental Fig.1). Both NP had a fully unmethylated *MLH1* and a partially methylated *MSH6* pattern, but differential *MSH2* patterns (one U, one U/M). A fully methylated *MSH2* pattern was found in 2 PIT1 macrotumors (2.1% overall, 4.3% PIT1), both operated in young patients – (P1) and one non-invasive somatotroph– and displaying low *MSH2* transcripts. A fully methylated *MLH1* pattern was observed in 2 invasive SF1 tumors (2.1%

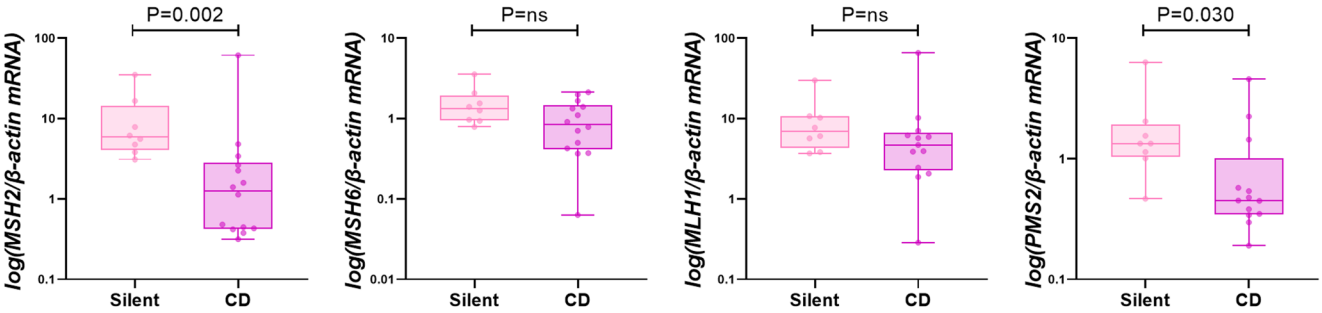

Fig. 3 Expression of *MMR* genes in 22 corticotroph PitNETs according to pre-operative functional status. *Legend:* Data were obtained by RT-qPCR. Box plots represent median values and 10th-90th percentiles. CD Cushing's disease, Silent no pre-operative hypercortisolism, ns non-significant

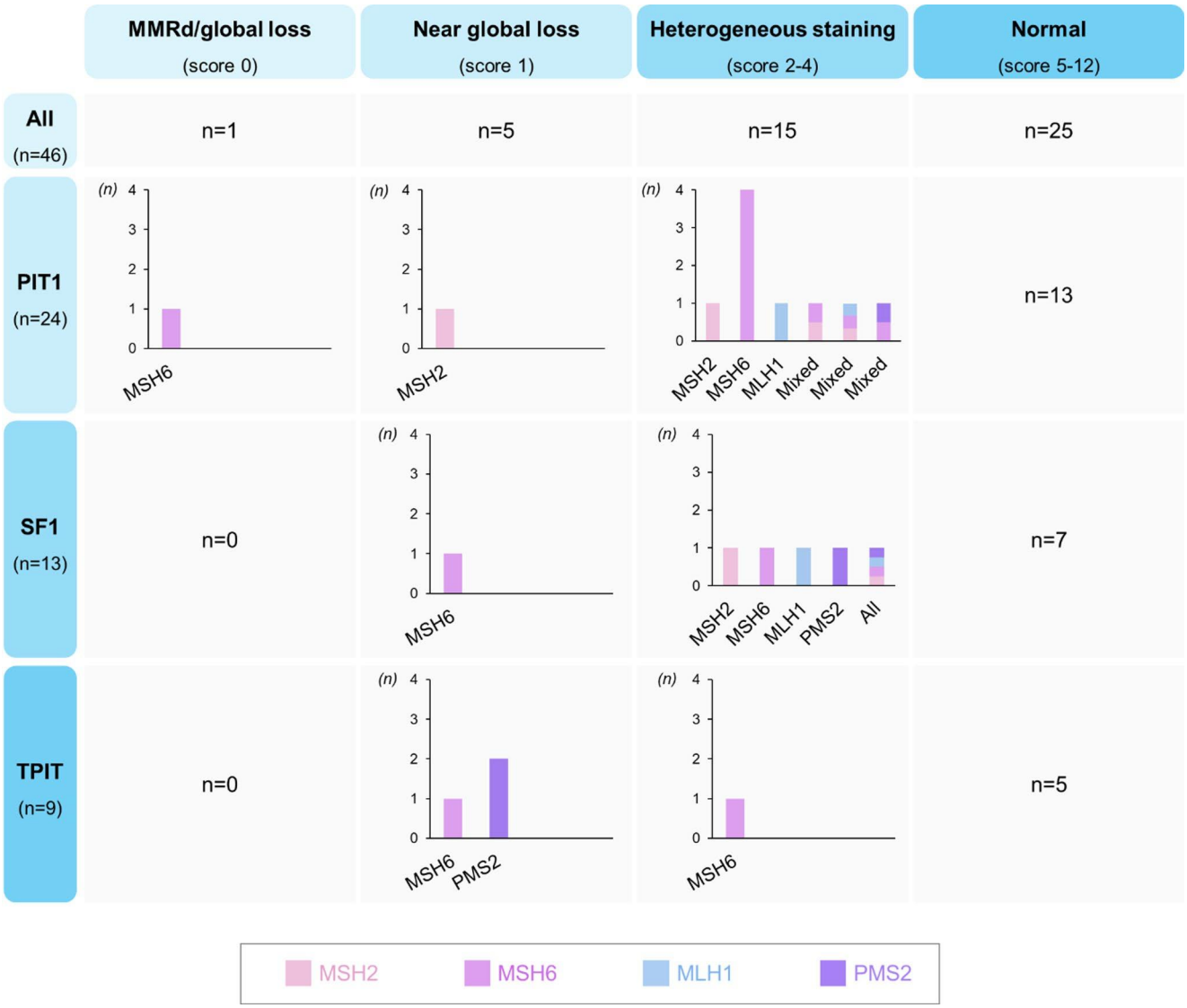

Fig. 4 Semi-quantitative MMR-IHC in 46 PitNETs according to their lineage of origin. *Legend*: IHC immunohistochemistry, MMRd MMR deficiency. The semi-quantitative score ranged from 0 to 12. The number of studied cases is indicated for each subgroup. Heterogeneous immunostaining could involve more than one MMR protein, as illustrated by different colours on individual cases

overall, 5.6% SF1) but *MLH1* expression was maintained. In the remaining cases, a partially methylated pattern was observed for *MSH6* (n=63, 65.6%), *MLH1* (n=48, 50%) and *MSH2* (n=31, 32.3%) promoters. However, no significant correlation with the corresponding gene expression was found, except a non-significant trend for higher *MSH2* transcripts in fully unmethylated samples.

Discussion

This study is the first to report and analyze the expression of the four key MMR components in a large series of PitNETs. Different patterns of *MMR* gene expression were observed according to tumor lineage of origin, which is consistent with different biological and molecular characteristics [1, 29–31]. Variations in *MMR* gene expression among PitNETs also emerged from previous transcriptomics analysis [29]. Using antibodies approved for the screening of LS in solid neoplasia, MMRd, strictly defined as a global loss of tumor immunostaining in any MMR protein, was quite uncommon in this series (1/46, 2.2%). However, near global loss (score 1) or heterogenous MMR-IR (scores 2-4) were not uncommonly observed (10.9% and 32.6%, respectively). Of note, this has been reported by semi-quantitative immunohistochemistry in other solid neoplasia [19, 32] and there is increasing interest towards the biological and genetic significance of partial MMR loss [33].

Overall, the significant correlations observed between *MMR* transcripts suggest a harmonic, albeit variable,

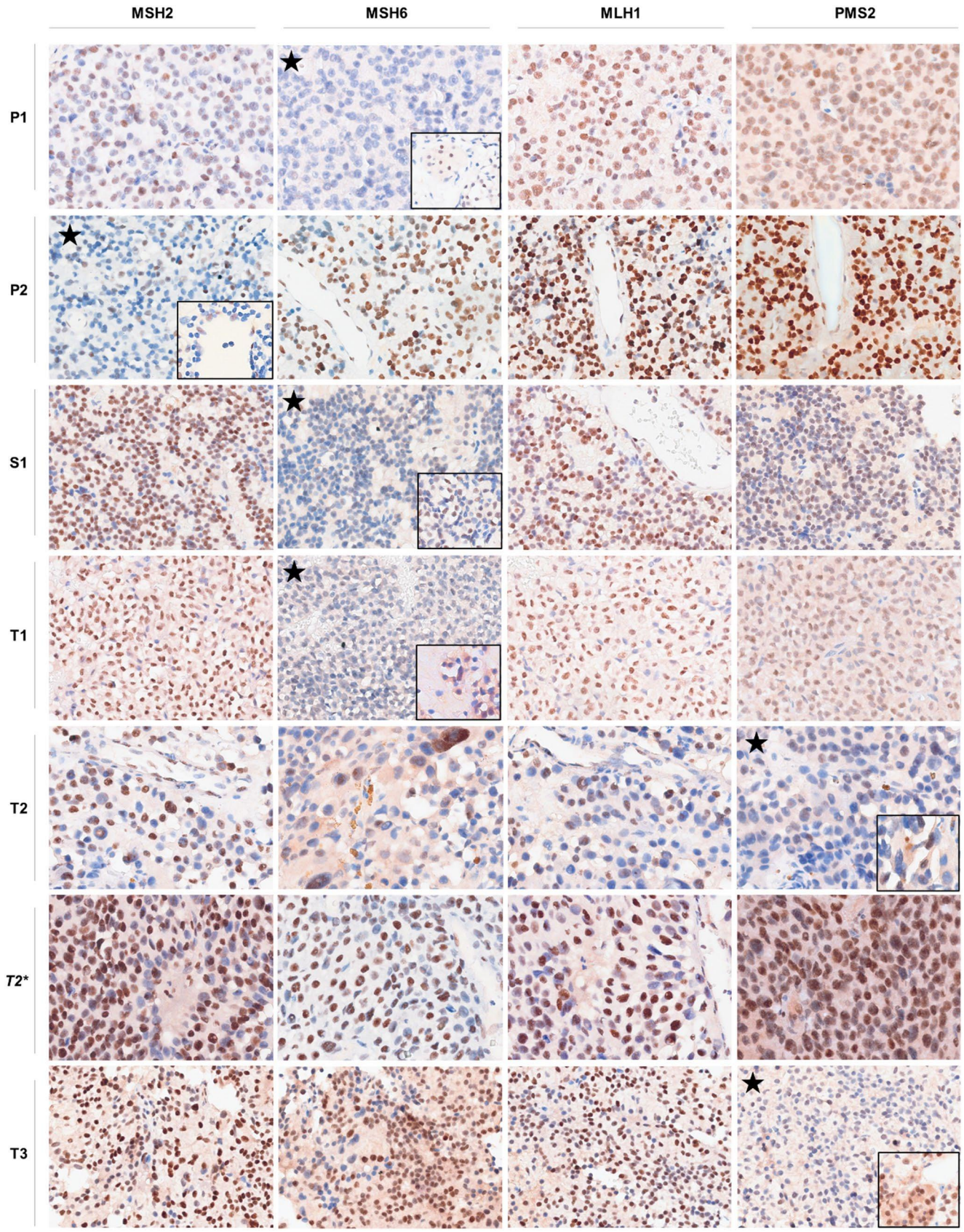
MSH2
MSH6
MLH1
PMS2
P1
P2
S1
T1
T2
T2*
T3

Fig. 5 Immunohistochemical evidence of MMR global or near global loss in six PitNETs. *Legend:* Global loss of MSH6 (P1), defining MMR deficiency, and near global loss of MSH2 (P2), MSH6 (S1, T1) or PMS2 (T2, T3) are indicated with a black star. (*T2**) shows retained MMR protein expression at first surgery from case (T2). Inserts show immunopositive control cells – endocrine cells in adjacent pituitary fragments and/or endothelial cells. Pictures are taken at x400 magnification

activation of the MMR machinery. Genes encoding the main heterodimer components (*MSH2*, *MLH1*) were expressed more than their relative partners (*MSH6* and *PMS2*, respectively), not excluding additional unexplored secondary components (*PMS1, MSH3, MLH3)* [34]. The higher *MSH2/MLH1* gene expression did not translate into a stronger nuclear immunostaining of the corresponding proteins, possibly due to the sensitivity of RT-qPCR and/or reflecting differences in mRNA or protein stability. Compared with NP, *MMR* genes expression was normal or high in most tumors. Accordingly, diffuse nuclear MMR immunoreactivity was observed on NP fragments (where present on pathological samples) and in a large majority of PitNETs. Nonetheless, some tumors had reduced MMR-IR, including global or partial MMR loss [35]. Of note, this study may not reflect the true prevalence of MMR defects in PitNETs due to selection bias: 1) the under-representation of lactotroph tumors – most of which are treated medically –, 2) the high proportion of large/invasive tumors due to neurosurgical recruitment, 3) cases selection for immunohistochemical studies – enriched with samples with low/normal transcriptional levels –. However, the very low prevalence of MMRd echoes data obtained in some extra-pituitary NETs [35].

We found gonadotroph/SF1 PitNETs to display normal/high *MMR* transcripts, with trends towards an increased expression in invasive and/or giant tumors reaching statistical significance for *PMS2*. Noteworthy, SF1 PitNETs are genetically more stable than PIT1 or TPIT PitNETs [29] and poorly represented among ag/met cases [2, 36]. The lower expression of *MMR* genes in hormone-negative cases may reflect the molecular heterogeneity of gonadotroph PitNETs [37, 38], but most had clearly detectable transcripts and diffuse nuclear MMR-IR. Despite a fully methylated *MLH1*

Table 3 Patients and PitNETs characteristics in the presence of global or near global loss of MMR-IR

ID (Sex, Age)	PitNET characteristics	Follow-up	Associated neoplasia	Familial history of malignancies	MMR-IR (scores *)	*MMR* sequencing	*MMR* genes promoter methylation
P1 (M, 16)	G (40 mm), Invasive, Silent PRL	Recurrence (24 months) TS (x2) RT	No	Father pancreatic cancer^{m} (53 yrs), brother glioma (18 yrs), paternal uncle colon cancer (59 yrs)	*Global loss* MSH6 (0;0) *Heterogeneous* MSH2 (1;4) PMS2 (1;4)	gDNA *MMR* – wt	*MSH6-M/U* *MSH2-M* *MLH1-U*
P2 (F, 21)	Ma (12 mm), Invasive, PRL	Resistant to CAB (3 mg/week) RT	No	None	*Near global loss* MSH2 (1;1)	gDNA *MMR* – wt	*MSH2-M/U* *MSH6*-M/U *MLH1*-U
S1 (M, 45)	Ma (30 mm), Invasive, NF FSH/LH-IR	Recurrence (54 months) TS (x2)+RT	No	None	*Near global loss* MSH6 (1;1)	gDNA *MMR* – wt	*MSH6-M/U* *MSH2-M/U* *MLH1*-U
T1 (F, 36)	Ma (23 mm), Non-invasive, ACTH (CD)	Post-operative remission (TS)	No	Father bronchial cancer (71 yrs, rf)	*Near global loss* MSH6 (1;1)	tDNA *MMR* - wt *MSH3* - (*a*) *MSH2/MSH6* – LOH	*MSH6* -M/U *MSH2-U* *MLH1*-U
T2 (M, 57)	Ma (35 mm), Invasive, ACTH (CD), Recurrence (4th TS)	RT (x2)+TMZ (x2) Deceased from progression (67 yrs-old)	No	Father bronchial cancer (74 yrs, rf), Sister^{1} thyroid cancer (69 yrs, #), Sister^{2} colon cancer (73 yrs), daughter of Sister^{2} bilateral breast and colon cancer (~40 yrs), maternal cousin hepatic cancer (70 yrs, rf)	*Near global loss* PMS2 (1;1) *Heterogeneous* MSH6 (2;1) MLH1 (1;2) MSH2 (2;2)	tDNA *MMR* – wt *MSH2/MSH6* –LOH *Additional TP53 - (b), LOH at the TP53 locus*	*MSH2* -U *MSH6-M/U* *MLH1*-U
T3 (F, 61)	m (7), Non-invasive, ACTH (CD)	Post-operative remission (TS)	N/A	N/A	*Near global loss* PMS2 (1;1)	N/A	N/A

Legend: ID Identification Number, m microtumor, Ma macrotumor, G giant tumors, CD Cushing's disease, NF non-functioning, CAB cabergoline, TS transsphenoidal surgery, RT radiotherapy, # died from disease, cancer^{m} metastatic cancer, rf known lifestyle risk factors, IR immunoreactivity, Global loss defined MMR deficiency, *details of the semiquantitative immunohistochemical score are indicated within brackets for near global loss or heterogeneous staining (intensity, percentage of positive cells, which product gives the global score), gDNA genomic DNA, tDNA tumor DNA, (a) Variant of Uncertain Significance (class 3), (b) Likely Pathogenic (class 4) see text for details, TMZ temozolomide, LOH loss of heterozygosity, wt wild-type, M fully methylated, U fully unmethylated, M/U partially methylated, N/A not available

pattern in two cases, *MLH1* gene and protein expression were maintained. Near global loss of MSH6-IR was found in a single invasive FSH/LH-IR case, in the absence of germline mutation. Noteworthy, this tumor subsequently recurred massively after nearly four years of stable residual disease. The lineage of origin of a metastatic "null cell" PitNET displaying loss of MSH6-IR [5] and of a "non-functioning" PitNET in the setting of LS due to a *PMS2* mutation [12] was unknown. Taken together, these data suggest a marginal role for MMR defects in gonadotroph PitNETs.

Conversely, MMRd was consistently documented in ag/met corticotroph PitNETs due to somatic events [7–10, 36] or germline mutations [11–13]. For example, a somatic *MSH6* mutation was reported in a liver metastasis but not in the primary tumor [7] and combined *MSH2/MSH6* mutations were part of a hypermutated phenotype in an orbital localization [9]. LS patients had germline *MSH2* mutations [12, 13] or a large *MLH1* germline deletion with somatic LOH at the *MLH1* locus and *MSH6* mutation [11]. Among clinically non-functioning PitNETs, silent corticotroph tumors were previously reported to express lower *MSH2/MSH6* transcripts than gonadotroph and silent PIT1 tumors [16]. While extending these to the TPIT subgroup, our study also reports pre-operative hypercortisolism to be associated with reduced *MMR* transcripts, reaching significance for *MSH2* and *PMS2*. Because dexamethasone *in vitro* reduces *MMR* expression in adipose cells through an epigenetic mechanism involving *miR*-155 [39, 40], the potential role of hypercortisolism in *MMR* regulation in corticotroph cells would deserve further investigation. A near global loss of MMR-IR was also observed in three functioning TPIT tumors: two non-invasive and a recurring invasive case which subsequently developed an aggressive and fatal behaviour. This latter case – previously reported with alternative telomere lengthening (ALT) [41] and oncogenic splicing of TrkA [42] – displayed LOH at the *MSH2/MSH6* loci, with a likely pathogenic *TP53* mutation, additional LOH at *TP53* locus, but no *USP8/48* mutation, a molecular pattern associated with genomic instability and adverse outcome in corticotroph PitNETs [43, 44]. In this case, the study of samples taken during prior surgeries showed that partial MMR loss was acquired during progression. This tumor subsequently showed resistance to TMZ, which is consistent with a permissive role of MMR for TMZ efficacy [6]. LOH at the *MSH2/MSH6* loci was also documented in a non-invasive, yet not recurring, functioning TPIT tumor. We were unable to disclose any effect of *MMR* gene promoter methylation or *USP8/USP48* mutations (which could be recognized by the MMR machinery) on MMR expression, but this would be better addressed in larger series including a higher proportion of microadenomas [45]. Taken together, these data support a role for MMR defects in corticotroph PitNETs beyond the spectrum of ag/met or LS tumors.

PitNETs issued from the PIT1 lineage include different clinical and pathological phenotypes, and we found PIT1 PitNETs to display the highest variability in MMR expression. In contrast with SF1 PitNETs, an inverse relationship was found in this group between tumor size and *MMR* transcripts, with a significantly lower expression in invasive *vs* non-invasive tumors. Such data encourage a further characterization of MMR-IR according to tumor size and invasiveness within the different hormone-secreting subtypes. Yet, a couple of lactotroph PitNETs have been associated with either a germline *MSH6* mutation [12] or loss of MSH6-IR during malignant progression [46]. Herein, we report global loss of MSH6 (MMRd) or near global loss of MSH2-IR in two young-onset invasive lactotroph tumors. The former subsequently required re-operation for a massive regrowth, and the latter was operated on because of uncontrolled hyperprolactinemia with slow tumor progression on high-dose cabergoline. Genetic screening for LS was negative in both patients and no somatic *SF3B1* mutation was found – but these are rare in non-metastatic cases [25, 47] –. Of note, isolated loss of MSH2 must always be confirmed as it is expected to destabilize MSH6, but has been occasionally reported [48]. A silent metastatic PitNET, already reported with diffuse MSH2/MSH6-IR and a remarkable response to pembrolizumab [49], showed heterogeneous MLH1-IR (this study), and heterogeneous MMR-IR was observed in an additional subset of PIT1 PitNETs. We found *MSH2* gene promoter methylation to be occasionally associated with low *MSH2* transcripts whereas somatotroph tumors harbouring a *GNAS1* mutation (all non-invasive) had normal/high *MMR* expression. Overall, these data suggest a role for MMR defects in some PIT1 tumors, in particular invasive lactotroph PitNETs.

The mechanisms leading to an impaired MMR expression in PitNETs, where present, should be further elucidated. The best characterized cause of MMR defects is the presence of germline mutations. Identifying a LS setting is highly relevant for the management of affected patients and familial counselling [4]. Despite occasional reports of LS-related PitNETs [13, 16, 46], PitNETs were quite uncommon in the Swedish LS register (0.3%) [12], not excluding under-reporting in oncological patients. In our study, the absence of *MMR* mutations in 5/6 of PitNETs displaying global or near global MMR loss appear to further support a rare association between LS and PitNETs. Nonetheless, germline *MMR* mutations were incidentally found by NGS in patients with unselected PitNETs [50], also involving *MSH3* [29, 51, 52]. These latter and several variants in key *MMR* genes, especially *MSH6* [34] are of uncertain significance, which complicates genetic counselling. In addition, if IHC is a recognized screening

tool in common LS neoplasia [52], some issues remain about the significance of heterogeneous MMR-IR, which was not rarely encountered in our study. In a series of 38 carriers of pathogenic *MMR* mutations (mainly *MLH1* and *MSH2*), only 8 had global loss of the corresponding protein and cut-offs based on semi-quantitative scoring of heterogeneous staining were proposed to enhance screening sensitivity [19]. Thus, the potential predictive value of MMR-IHC should be further assessed in PitNETs, in which intermediate MMR scores seem more common than in LS malignancies [32] and somatic alterations are likely to prevail over inherited defects. We suggest that, based on the increased prevalence of neoplasia reported in PitNETs patients beyond acromegaly [53], further attention to individual/familial oncological history may help considering a potential LS setting. MMR defects can also occur at a somatic level. Unless functional methylation sites, which may be cell-specific, were under-recognized by MS-PCR, our data suggest a limited role for *MMR* gene promoter methylation. *MMR* genes were not recognized among the main differentially methylated genes in PitNETs, not excluding potential variations according to their lineage of origin [54]. Conversely, chromosome loss involving *MMR* gene loci – 2p (*MSH2* and *MSH6*), 3p (*MLH1*) and 7p (*PMS2*) –, are not uncommon [29], and we observed LOH at *MSH2/6* loci in two corticotroph tumors. Unrecognized epigenetic changes, microRNAs, splicing variants, as well as the potential impact of the steroid milieu on *MMR* gene transcription [33] can also be hypothesized, as suggested herein for hypercortisolism.

Another issue is the potential impact of MMR defects on PitNETs behaviour. In colon-rectal NETs, MMRd was restricted to neuroendocrine carcinomas [35]. In our series, out of 6 PitNETs with very low MMR-IR, 3 recurred and one became aggressive, so that 2 patients were re-operated and 4 required radiotherapy. Longitudinal studies on unselected PitNETs would be useful to clarify this aspect. In ag/met PitNETs, the evaluation of a full MMR-IHC panel would help understanding its potential role in clinical management, since MMRd may limit the efficacy of TMZ [6] while theoretically enhancing the response to immune checkpoint inhibitors (ICI). In LS-neoplasia, this has been attributed to the high tumor mutational burden (TMB) determining the synthesis of neo-antigens [55, 56]. Recent guidelines also suggest a trial with ICI in TMZ-resistant ag/met PitNETs with MMRd or high TMB [57]. With a few exceptions [7], TMB is modest or low in ag/met PitNETs, with no clear impact on ICI efficacy [49]. Conversely, *PDL1* transcripts have been positively correlated with *MSH6/2* expression and *MSH2/6* knockout reduced *PDL1* expression in pituitary cell lines [16]. Additional functions of MMR proteins beyond DNA repair are under active investigation [33] and may be relevant in PitNETs.

In conclusion, this study reports a reduced MMR expression in a subset of PitNETs – mainly among PIT1 and TPIT tumors –, extending beyond the spectrum of rare ag/met or LS-related cases. Germline *MMR* mutations are likely to be uncommon, but attention to personal/familial oncological history may help guiding genetic evaluation. Further studies are needed to better define the mechanisms of MMR defects in PitNETs, the prognostic significance of MMR-IHC, and their potential implications for personalized follow-up and management.

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Author Contributions F.C and M.L.J.R designed and organized the study, elaborated final results and wrote the main manuscript text. F.C also performed molecular experiments on tumor samples with F.M., M.M. and L.E. F.G and A.A performed immunohistochemical studies and ensured proper sample collection and characterization, F.G. also provided MMR-IHC scoring. T.F. and D.D.A collected clinical data with V.E. and R.M who operated the patients. T.F. and D.D.A also contributed to scientific content with A.I. A.D.P. and S.G performed genetic counselling and NGS sequencing. All the authors provided multidisciplinary scientific expertise and comments, reviewed and approved the manuscript.

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Data Availability Raw data are available upon reasonable request at the Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, and at the Neuromed IRCCS, Pozzilli, Italy.

Declarations

Ethical Approval The study was performed according to the guidelines of the Declaration of Helsinki and approved by the Internal Review Board of the Neuromed IRCCS (Pozzilli, Italy)

Competing interests The authors declare no competing interests.

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