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Expression of Peroxisome Proliferator-Activated Receptor Alpha (PPARα) in Non-Somatotroph Pituitary Tumours and the Effects of PPARα Agonists on MMQ Cells

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ABSTRACT

Peroxisome proliferator-activated receptor alpha (PPARα) has been involved in the regulation of somatotroph tumour cells and may be targeted by different drugs, some of them are in current clinical use. The aim of this study was to investigate the expression of PPARα in additional phenotypes of pituitary adenomas (PA), the relationship between PPARα and its potential molecular partner aryl hydrocarbon receptor interacting protein (AIP) in these tumours, and the effects of PPARα agonists on lactotroph cells. Seventy-five human PA – 57 non-functioning (NFPA) and 18 prolactinomas (PRL-PA) – were characterised for PPARα and AIP expression by real time RT-PCR and/or immunohistochemistry (IHC), and the effects of fenofibrate and WY 14 643 on MMQ cells were studied in vitro. PPARα was expressed in a majority of PA. PPARα immunostaining was observed in 93.7 % PRL-PA vs. 60.6 % NFPA ($p = 0.016$), the opposite being found for AIP (83.3 % in NFPA vs. 43.7 % in PRL-PA, $p = 0.003$). PPARα expression was unrelated to gonadotroph differentiation in NFPA, but positively correlated with tumour volume in PRL-PA. Both drugs significantly reduced MMQ cell growth at high concentrations (100–200 µM). At the same time, despite modest stimulating effects on PRL secretion were observed, these were overcome by the reduction in cell number. In conclusion, PPARα is commonly expressed by PRL-PA and NFPA, regardless of AIP, and may represent a new target of PPARα agonists.

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Introduction

With a clinical prevalence approaching 1/1000, pituitary adenomas (PA) are frequent endocrine tumours, with prolactinomas (PRL-PA) and clinically non-functioning (NFPA) accounting for 50–55 % and 20–25 % of the cases, respectively [1]. We previously reported the expression of peroxisome proliferator-activated receptor α (PPARα) in somatotropinomas, as a potential molecular partner of the aryl hydrocarbon receptor interacting protein (AIP) [2], encoded by the best characterised predisposing gene for acromegaly [3]. Fenofibrate (FF), a PPARα agonist, was found to modulate proliferation, apoptosis and hormone secretion in GH_3 cells [4]. Experimental data in *PPARα*$^{-/-}$ mice indicate that fasting increases PRL and gonadotropin secretion in a PPARα-dependent manner [5], whereas PPARα agonists increase PRL mRNA in GH_4C_1 cells [6] but reduce βFSH and βLH transcription in LβT2 cells [7]. Whether PPARα is expressed in PRL-PA and in NFPA, most of which are of gonadotroph origin, is currently unknown.

PPARs are a family of nuclear receptors involved in metabolic diseases, cancer, and inflammation [8, 9] and are therapeutic targets for drugs of clinical use or still under development [10, 11]. PPARα is expressed in human PA and its agonist rosiglitazone exerts anti-proliferative and anti-secretory effects on pituitary cell lines [12–14]. PPARα is expressed by a variety of neoplasia and, despite carcinogenic effects on the rodent liver, PPARα agonists generally exert anti-tumourigenic effects and have been proposed in several human malignancies [15, 16]. Anti-proliferative and pro-apoptotic effects of FF were also observed in GH_3 cells [4].

This study aimed to evaluate the expression of PPARα in PRL-PA and NFPA and its relationship with AIP expression, which may be paradoxically overexpressed in NFPA [17, 18]. The effects of two PPARα agonists on a lactotroph cell line were also studied.

Material and Methods

Patients and tumours

Surgical samples from 75 PA patients operated on for medical reasons (57 NFPA and 18 PRL-PA) were studied. Most were collected during the 2010–2016 period at the Neuromed Institute (Pozzilli, Italy), archive material from a subset of PRL-PA operated on in Liège (Belgium) was also included. In all cases diagnostic immunohistochemistry (IHC) for all pituitary hormones was performed. PRL-PA were defined by pre-operative hyperprolactinemia and PRL immunopositivity. NFPA were diagnosed in the absence of bioclinical evidence for acromegaly or hypercortisolism and classified into gonadotroph PA (GnPA, n = 38) (immunostaining for FSH and/or LH) and null cell PA (nc-PA, n = 19) (negative for all pituitary hormones). All NFPA and most PRL-PA (12/18) were macroadenomas (maximal diameter > 10 mm). Invasiveness towards surrounding structures was defined by pre-operative magnetic resonance imaging and intra-operative findings. The Ki67 index of proliferation was determined by MIB1 immunostaining [19]. Among PRL-PA, 6 (5 macro- and 1 microadenoma) had received cabergoline pre-operatively and all were resistant, as previously defined [20]. The study was perfomed according to the guidelines of the Declaration of Helsinski and approved by the Ethical committee at the Neuromed Institute (Pozzilli, Italy). Written informed consent was obtained from the patients, except for a minority of archive paraffin-embedded material from patients lost to follow-up.

Gene expression analysis

Surgical biopsies were collected in RNA later solution (Ambion®, distributed by Life Technologies, Monza, Italy) and frozen at –80 °C until use. Total RNA was extracted by EuroGOLD TriFast™ (Euroclone, Pero, Italy). After DNAse treatment (Qiagen), 1 µg RNA was reverse-transcribed with Euroscript MLV (Euroclone, Pero, Italy) according to the manufacturer's instructions. Preliminary RT-PCR amplification of GADPH was performed for cDNA quality control, including PCR on DNAse-treated RNA to exclude the presence of genomic DNA. Preliminary screening for Tpit, and for Pit-1 in NFPA, was done by RT-PCR to exclude samples contamination by normal pituitary tissue [21]. Gene expression analysis was then performed by real time RT-PCR and corrected for β-actin expression, using a Taqman methodology on an Applied Biosystems 7500 Fast Real-Time PCR and ready-to-use gene expression assays (Applied Biosystems, Life Technologies, Monza, Italy): Hs00947536_m1 (PPARα), Hs00610222_m1 (AIP), Hs00174919_m1 (βFSH), Hs00751207_m1 (βLH), Hs00610436_m1 (Steroidogenic Factor 1, SF1), Hs00765553_m1 (Cyclin D1), Hs00234387_m1 (Caspase 3) and Hs_99999903 (β-actin). All experiments were run at least in duplicate. RNA extracted from six post-mortem normal pituitaries (NP) were used to define PPARα/AIP gene down- or upregulation (< 10° percentile and > 90° percentiles, respectively). In MMQ cells, PRL gene expression was corrected for Cyclophilin B expression, which was unaltered by PPARα agonists, using rat-specific assays: Rn00561791_m1 (PRL) and Rn03302274_m1 (Cyclophilin B) (Applied Biosystems, Life Technologies, Monza, Italy).

Immunohistochemistry (IHC)

Immunohistochemistry for PPARα and AIP was performed on paraffin-embedded sections of PA with a polyclonal rabbit anti-PPARα antibody (PA1-822 A, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) and a mouse monoclonal anti-AIP (clone 35-2, NOVUS Biologicals LLC, Littleton, CO, USA), using experimental conditions, positive/negative controls and semiquantative evaluation as previously reported [4]. Briefly, PPAR cytoplasmic and nuclear staining – PPARα(c) and PPARα(n) (range 0–3 each) – were summed to obtain a total PPARα score – PPARα(t) (range 0–6) – and AIP staining was quoted according to intensity and expression pattern (range 0–6) [4]. In both cases, immunopositivity was defined by a score > 2. Immunofluorescence (IF) was performed to co-localise PPARα and FSH in a normal post-mortem pituitary as previously described [4], using a mouse monoclonal anti-FSH antibody (cloneA-7, 52332, Santa-Cruz Biotechnology, Santa Cruz, CA, USA).

In vitro studies

MMQ cells – a kindly gift from A. Spada, University of Milan (Italy) – were grown in suspension in RPMI1640 supplemented with 7.5 % horse serum, 2.5 % calf serum, glutamine 1 %, penicillin (100UI/ml), and streptomycin (100 mg/ml) in a humidified atmosphere at 37 °C with 5 % CO_2, and subsequently plated at a 10^6 density in 25 cm^2 flask. FF (sc-204751, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and WY (c7081, Sigma-Aldrich, USA) were used at 50–

200 µM for 48 h. Cells were counted with a Burker chamber and viability assessed by Trypan blue 0.5 % exclusion (Euroclone, Pero, Italy). PPARα expression was confirmed by western blot analysis on MMQ protein extracts as reported [4]. At each experimental point, cellular cDNA was obtained from total RNA as reported hitherto and cell culture media was collected and stored at –80 °C until PRL measurement by ELISA with a rat-specific assay (A05101, SPI-BIO, Bertin Pharma, Montigny-le-Bretonneux, France). The detection limit was 0.2 ng/ml, and cross-reactivity between PRL and GH was < 1 %. All experiments were performed in duplicate and repeated at least twice.

Statistical analysis

Statistical analysis was performed using the JMP 11.0 software for PC (SAS Institute, Cary, NC, USA). Continuous data from patients and PA are expressed in median (range) and analysed by Mann-Whitney U test for 2 groups-analysis, Kruskall–Wallis for multiple comparisons and Spearman test for correlation studies. Categorical values were compared by the Chi-2 test. Data from in vitro cell culture experiments are expressed as mean (± SD) and analysed by ANOVA. A p-value < 0.05 was considered significant.

Results

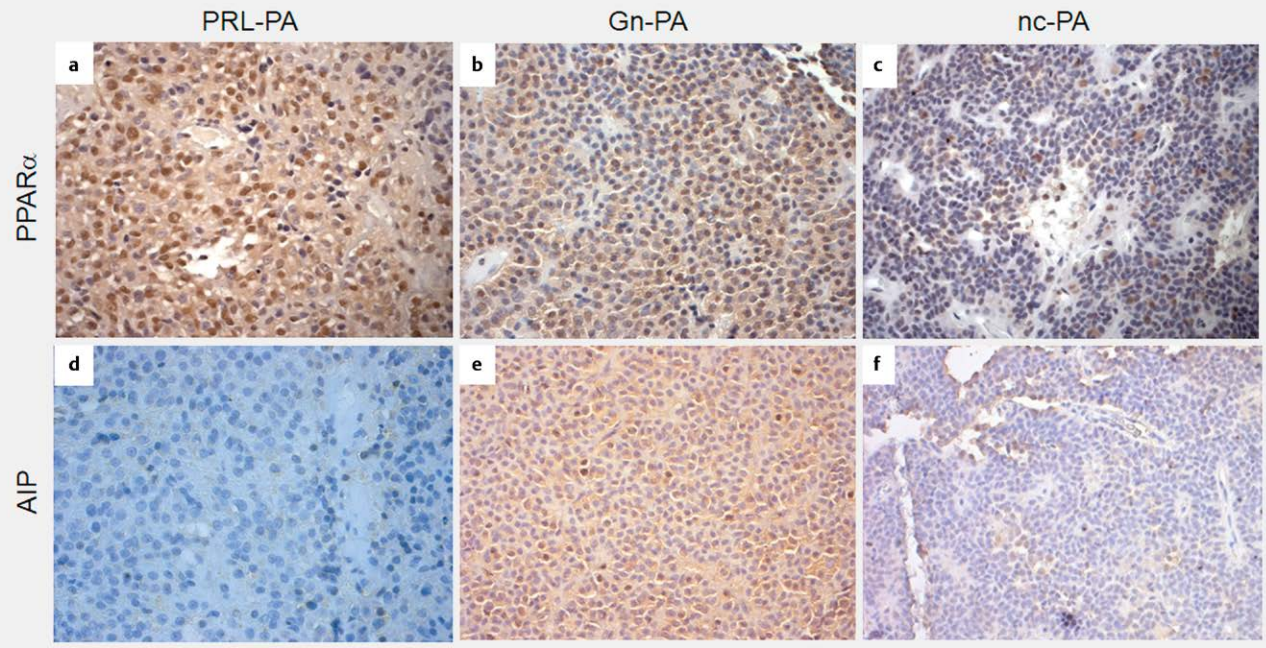
PPARα and AIP expression in PA

Data are summarised in ▶ **Table 1** and representative examples of AIP and PPARα immunostaining are shown in ▶ **Fig. 1**. As compared to NP, downregulation of PPARα gene expression was observed in 26/66 cases (39.4 %) (20 % in PRL-PA vs 43.6 % in NFPA, p ns). Accordingly, PPARα mRNA tended to be higher in PRL-PA (p = 0.065 vs. NFPA) (▶ **Fig. 2a**), PPARα immunopositivity was observed in all but one PRL-PA (93.7 % vs. 60.6 % in NFPA, $\chi^2 = 6.9$, p = 0.016) and the PPARα(t) score was significantly higher in PRL-PA (p = 0.044 vs. NFPA) (▶ **Fig. 2b**), due to a higher cytoplasmic score (p = 0.013 vs. NFPA) (▶ **Fig. 2b-d**). In contrast, the AIP score was significantly lower in PRL-PA (p = 0.0005 vs. NFPA) (▶ **Fig. 2e**) and AIP immunopositivity was significantly more frequent in NFPA (83.3 % vs. 43.7 % in PRL-PA, p = 0.003). Accordingly, AIP mRNA tended to be higher in NFPA (p = 0.093 vs PRL-PA) (▶ **Fig. 2f**), with a frequent AIP gene upregulation in this subgroup (42.1 % vs. 10 % in PRL-PA, p = 0.053). AIP expression was unremarkable in resistant PRL-PA with variable AIP mRNA and immunostaining (range 0–3).

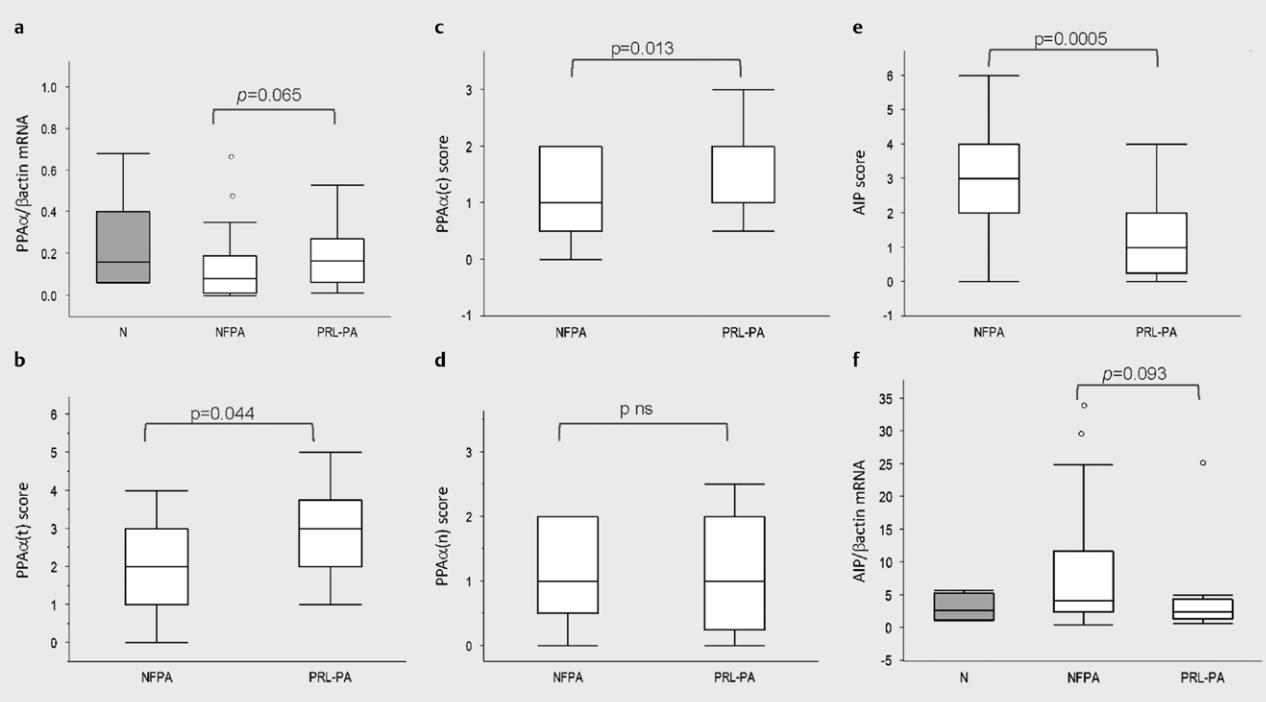
▶ **Table 1** Clinical characteristics and summary of AIP and PPARα expression in pituitary adenomas.

	NFPA	PRL-PA	p
I. Clinical data			
(n)	(57)	(18)	
Gender	22 F, 35 M	12 F, 6 M	**0.037**
Age (median) [range]	58.0 years [16–76]	40.5 years [16–63]	**0.0001**
Macroadenoma (%)	57/57 (100 %)	12/18 (66.7 %)	ns
Max diameter (median) [range]	2.65 cm [1.4–6.0]	2.25 cm [0.5–4.8]	0.076
Invasive tumours (%) *	33/55 (60.0 %)	12/18 (66.7 %)	ns
Recurrent tumours (%)	12/57 (21.0 %)	2/18 (11.0 %)	ns
High Ki-67 (≥ 3 %) (%) *	16/48 (33.3 %)	10/16 (62.5 %)	**0.040**
II.AIP and PPARα expression			
AIP/β-actin mRNA	4.157 [0.042–33.920]	2.427 [0.636–25.140]	
Median [range] (n)	(57)	(10)	0.091
AIP immunopositivity (%) *	35/42 (83.3 %)	7/16 (43.7 %)	**0.003**
AIP-IHC (score)	3.0 [0–6]	1.0 [0–4]	**0.0005**
PPARα/β-actin mRNA	0.081 [0.001–0.665]	0.168 [0.013–0.527]	
Median [range] (n)	(50)	(10)	0.065
PPARα immunopositivity (%) *	20/33 (60.6 %)	15/16 (93.7 %)	**0.016**
PPARα– IHC (scores)			
– PPARα (t)	2.0 [0.0–4.0]	3.0 [1.0–5]	**0.044**
– PPARα (c)	1.0 [0.0–2.0]	1.0 [0.5–3.0]	**0.012**
– PPARα (n)	1.0 [0.0–2.0]	1.0 [0.0–2.5]	ns
– PPARα (% positive nuclei)	21 [0–55]	23 [1–57]	ns

AIP: Aryl hydrocarbon receptor interacting protein; PPARα: Peroxisome proliferator activated receptor alpha; IHC: Immunohistochemistry; PPARα scores: (t) total, (c) cytoplasmic, (n) nuclear; n: Number of studied cases; * Note that some cases were missing due to unavailable data. Significant p-values are in bold and values approaching significance are in italics.



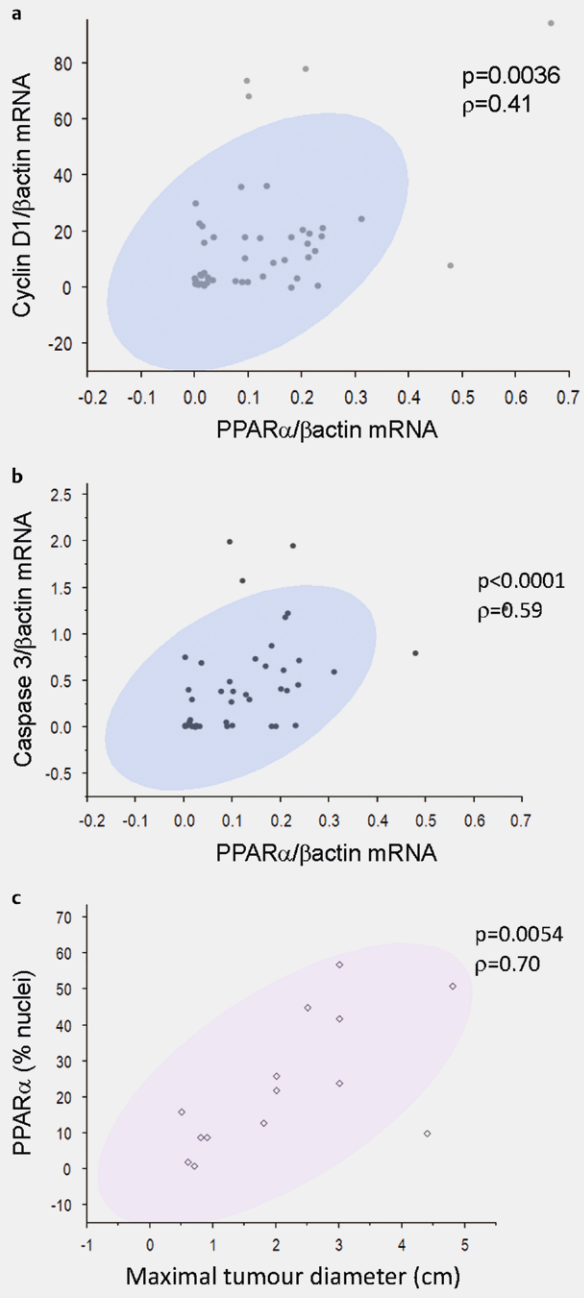
▶ **Fig. 1** Examples of PPARα and AIP immunostaining in PA: PRL-PA **a** PPARα score = 4 (nuclei 62 %) and **b** AIP score = 0; Gn-PA **C** PPARα score = 3 (nuclei 38 %), **d** AIP score = 3; nc-PA **e** PPARα score = 2 (nuclei 17 %), **f** AIP score = 1 (magnification × 400).



▶ **Fig. 2** Expression of PPARα (**a**–**d**) and AIP (**e**–**f**) in PA. Normal pituitary (N) is shown for gene expression only (grey boxes). NFPA: Non-functioning pituitary adenomas; PRL-PA: PRL-secreting adenomas. PPARα(t), (c), and (n): total, cytoplasmic and nuclear PPARα immunostaining scores. AIP staining was cytoplasmic. Significant differences between NFPA and PRL-PA are shown (in italics: non-significant trends). p = ns: Not significant.

PPARα expression in NFPA is independent from gonadotroph differentiation

PPARα could be observed by IF in normal gonadotrophs (**Fig. 1S**). However, PPARα gene and protein expression was similar in Gn-PA and nc-PA and no correlation was found between PPARα and βFSH, βLH or SF1 mRNA. Although PPARα expression was not significantly influenced by tumour characteristics or Ki67, a significant correlation was found between PPARα and Caspase 3 ($\rho = 0.59$, $p < 0.0001$) and Cyclin



▶ **Fig. 3** Correlations between PPARα and markers of cell turnover in PA. In NFPA: correlation between PPARα and Cyclin D1 **a** and Caspase 3 **b** mRNA. In PRL-PA: correlation between the percentage of PPARα immunopositive nuclei and maximal tumour diameter **c**. Spearman correlations coefficients (ρ), p-values and ellipses of density are shown for each set of data.

D1 (ρ = 0.41, p = 0.0036) mRNA (▶ **Fig. 3a, b**). This was confirmed in Gn-PA (ρ = 0.65, p < 0.0001 for Caspase 3 and ρ = 0.45, p = 0.01 and CyclinD1), with a similar trend in nc-PA (ρ = 0.45, p = 0.067 for Caspase 3 and ρ = 0.44, p = 0.073 for Cyclin D1) (data not shown).

PPARα expression in prolactinomas

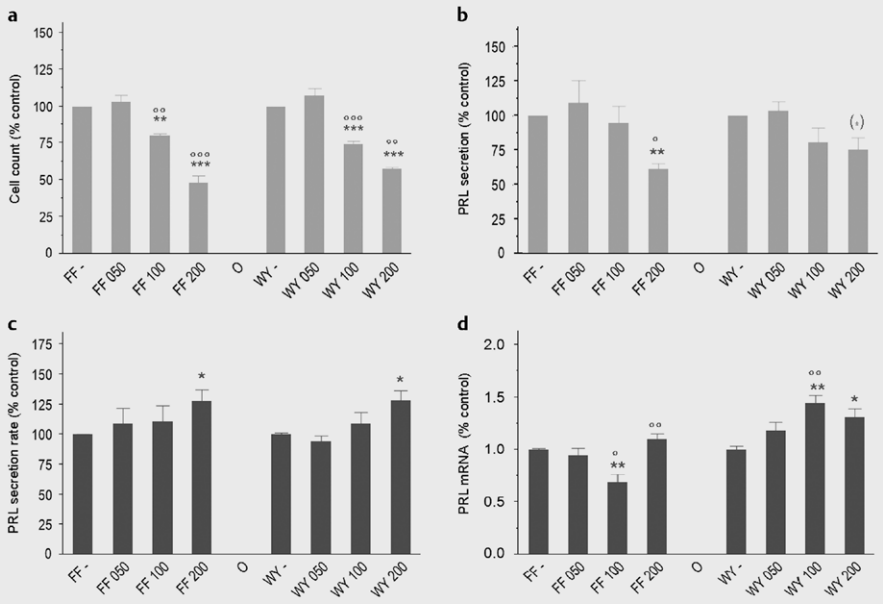
No significant difference was found between invasive and non-invasive PRL-PA, except a trend towards a higher percentage of positive nuclei in invasive cases ($\chi^2 = 3.4$, p = 0.065 vs. non-invasive). However, PPARα(t) and the percentage of positive nuclei significantly increased with tumour size (ρ = 0.64, p = 0.0147 and ρ = 0.70, p = 0.0054, respectively) (▶ **Fig. 3c**), but not with Ki67. Resistant prolactinomas also displayed PPAR immunopositivity [PPARα(t) range: 2–4] with a variable percentage of positive nuclei (range 10–57 %).

Effects of fenofibrate and Wyeth 14 653 on MMQ cells in vitro

After preliminary experiments showed no effect of FF and WY up to 50 µM (data not shown), concentrations up to 200 µM (▶ **Fig. 4**) revealed a dose-dependent reduction in cell growth at 100–200 µM (100 µM vs. control: p = 0.0013 for FF, p < 0.0001 for WY; 200 µM vs. control: p < 0.0001 for both) (▶ **Fig. 4a)**. A modest dose-dependent decrease in PRL secretion was also observed at 200 µM, reaching significance with FF (p = 0.011 vs. control, p = 0.025 vs. 100 µM) and approaching significance with WY (p = 0.069) (▶ **Fig. 4b**). However, once corrected for cell number, PRL secretion was slightly but significantly increased at 200 µM for either drug (p < 0.05 vs control) (▶ **Fig. 4c**), indicating that the overall decrease in PRL secretion mainly reflected the reduction in cell number. Noteworthy, PRL transcription was found to be significantly reduced by FF at 100 µM (p = 0.0003 vs. control, p = 0.02 vs. 50 µM and p = 0.001 vs. 200 µM) but increased by WY (p = 0.0003 and p = 0.01 vs. control at 100 µM and 200 µM, respectively) (▶ **Fig. 4d**).

Discussion

This study extends the normal pituitary expression of PPARα to gonadotrophs and provides the first evidence for PPARα expression in NFPA and PRL-PA, with a potential tumour down-regulation [4],thisstudy]. Despite a higher total PPARα score in PRL-PA, its nuclear expression was similar in NFPA, PRL-PA and GH-PA [4],thisstudy]. This suggests that PPARα may exert transcriptional effects in a variety of PA, as in different pituitary cell lines [4, 6, 7]. Most NFPA are of gonadotroph origin [22], as indicated by gonadotropins staining in Gn-PA and SF1 expression in nc-PA [23]. No correlation was found between PPARα and βFSH, βLH or SF1 mRNA, suggesting that PPARα expression is independent from their gonadotroph differentiation. However, since gonadotropins are regulated by Gn-RH – which receptor can be modulated by PPARs [7] – and sex steroids – which receptors are expressed in PA [24, 25], – the potential effects of PPARα on NFPA should be best approached on primary cultures in vitro. The relationship between PPARα and PRL mRNA could not be addressed owing to the small number of PRL-PA available for molecular analysis, due to the limited role of surgery in prolactinomas [26]. Sumanasekera et al. described a cytoplasmic AIP/hsp90/PPARα complex and an inhibition of PPARα transcriptional activity by AIP [2]. In this study, cytoplasmic PPARα immunostaining in PRL-PA contrasted in most cases with a low AIP expression, suggesting alternative mechanisms of PPARα stabilization [27–29]. We subsequently analysed PPARα expression according to tumour behaviour. In NFPA, a significant correlation was found between PPARα and Cyclin D1, which is frequently overexpressed in such tumours [30], and Caspase 3, a marker of apoptosis, suggesting a preferential expression in tumours with a high cel-



▶ **Fig. 4** Effects of PPARα agonists on MMQ cells. Cells were treated with fenofibrate (FF) and WY 14 463 (WY) at 50, 100, and 200 µM for 48 h. Legend: * $p<0.05$, * * $p<0.005$, and * * * $p<0.0001$ vs. control cells; ° $p<0.05$, °° $p<0.005$, and °°° $p<0.0001$ vs. the lower drug concentration. (*) indicates a trend approaching significance. Results are shown as the mean ± SE of experiments performed in duplicate (three experiments for cell count and two for PRL concentration and mRNA). A significant dose-dependent reduction in cell count was observed at 100 and 200 µM for each drug **a**. A decrease in PRL secretion was observed at 200 µM with either drugs, only approaching significance on WY ($p=0.069$ vs. control). However, a small but significant increase in PRL secretion corrected for cell number (10^6 cells) was observed at 200 µM for each drug **c**, with a significant decrease in PRL mRNA with FF at 100 µM, contrasting with a significant increase during WY treatment at 100–200 µM.

lular turnover. As NFPA are slowly growing [31] and reliable markers of aggressiveness are still lacking in clinical practice [32], molecular markers may be more accurate in the identification of the less quiescent cases. PPARα expression was also found to increase with tumour size in PRL-PA and was observed in tumours resistant to cabergoline. This suggests that NFPA with high cell turnover and large/resistant PRL-PA may be potential target for PPARα agonists.

We therefore evaluated the effects of two PPARα agonists on MMQ cells. Both drugs significantly inhibit cell proliferation, albeit at higher concentrations than in GH_3 cells (100–200 µM vs. 25–50 µM), and moderately reduced PRL secretion, this latter reaching significance with FF only. This appeared to reflect the reduction in cell number, as PRL secretion was slightly increased at 200 µM when corrected for cell number. Although some leakage from dying cells could not be excluded, PRL transcription was also found to be moderately stimulated by WY. These findings are reminiscent of those reported in GH_4C_1 cells under similar conditions of WY treatment, where the increase in PRL gene transcription was found to be mediated by an interaction between PPARα and Pit-1 [6], no binding site for PPARα being identified in the PRL gene promoter yet [6, 33]. Of note, WY has a peculiar bipartite binding to PPARα, with the second binding site accounting for specific properties of this drug [34]. In contrast, a modest decrease in PRL transcription by FF was already reported in GH_3 cells [12], suggesting a different interaction with the PRL transcriptional machinery [33]. In GH_3 cells, PRL secretion was likely to be maintained despite transcriptional down-regulation through a non-genomic effect of FF on hormone release [4, 32]. FF also induced insulin release from an insulinoma cell line through an inhibition of K_{ATP} and K_v channels [35]. A similar mechanism may be present in lactotroph cells, as the activation of K channels is involved in the inhibition of PRL release by dopamine [36]. Finally, both drugs have a modest dual PPARα/γ agonist activity [37], which might contribute to their effects on pituitary tumour cells [12].

In conclusion, NFPA and PRL-PA often express PPARα regardless of AIP. As PPARα agonists may exert anti-proliferative effects in lactotroph cells, with potential drug-related differences in their endocrine effects, studies on primary cultures of PA would be of interest. Indeed, PPARα agonists might offer some new perspectives in NFPA – for which no therapy is currently recommended, – or in resistant PRL-PA. Due to the increasing interest in the development of new PPARα agonists in the fields of metabolism, oncology, inflammation and neuroprotection [9–11, 38–40], their potential impact on pituitary function and/or on common PA should also be considered.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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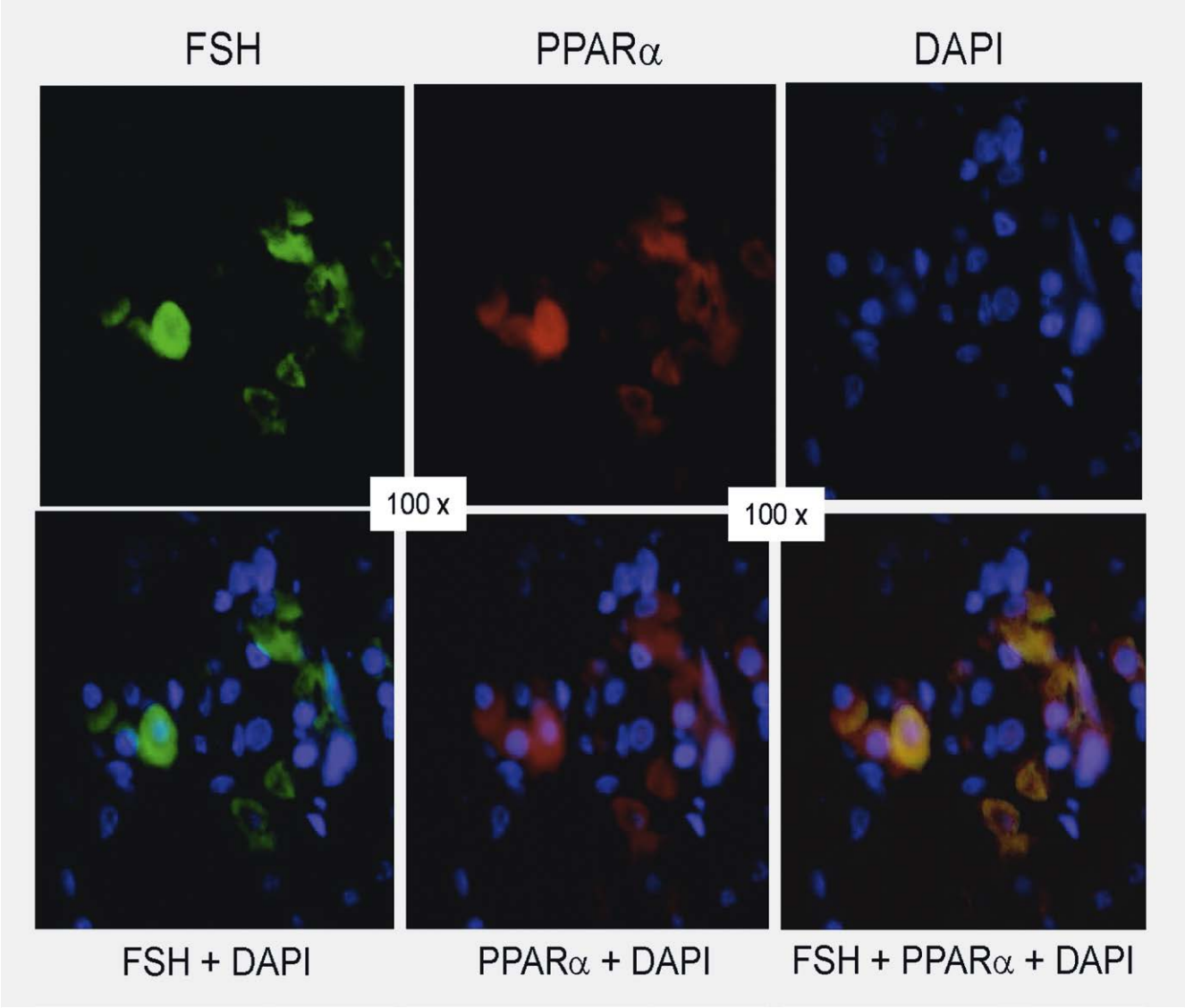
Thieme

Supplementary Material



▶ **Fig. 1S** Co-localization of PPARα and FSH in the normal pituitary (pars tuberalis) by immunofluorescence. Upper panel: FSH (green), PPARα (red), nuclei are stained by DAPI (blue). Lower panel: FSH and PPARα-positive cells and co-localization of both (orange).

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