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Sexual dimorphism in medulloblastoma features

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Aims: Male sex is a risk factor for medulloblastoma (MB), and is also a negative predictor for clinical outcome. The aim of this study was to assess sex differences in tumour biological features and hormone receptor profiles in a cohort of MB patients.

Methods and results: Sixty-four MBs and five normal cerebella were included in the study. Cell proliferation (Ki67), apoptosis (cleaved caspase-3) and microvessel density (CD31) were evaluated in tumours by immunohistochemistry. Tissues were analysed for oestrogen receptor (ER)α, ERβ1, ERβ2, ERβ5 and androgen receptor (AR) expression. The results demonstrated sex-specific features in MBs, with tumours from females showing a higher apoptosis/proliferation ratio and less tumour vascularization than tumours from males. MBs were negative for ERα and AR, but expressed ERβ isoforms at similar levels between the sexes. Altogether, these findings indicate that signalling mechanisms that control cell turnover and angiogenesis operate more efficiently in females than in males. The lack of sex differences in the hormone receptor profiles suggests that circulating oestrogens could be the major determinants of the sexual dimorphism observed in MB features.

Conclusions: Here, we provide molecular support for epidemiological data showing sex differences in MB incidence and outcome, completely defining the hormone receptor profile of the tumours.

Keywords: angiogenesis, apoptosis, brain cancer, clinical, ERβ, oestrogen receptor, sex

Introduction

Medulloblastoma (MB), a cerebellar embryonal tumour, accounts for ~20% of all childhood primary central nervous system tumours, whereas in adults the disease represents only 1% of primary brain tumours.[1,2] The effect of sex on the course of MB has been previously shown to be significant in epidemiological studies,[3,4] with clinical data suggesting that sex may be an important determinant of MB outcome, and females having a better prognosis.[5–7] Despite these findings, little research has been conducted on the effects of hormones on MB development, as the cerebellum itself is a part of the brain that has long been ignored as a site of steroid hormone action. Only recently has evidence redefined the cerebellum as a target of oestradiol signalling, notably showing the importance of oestrogen-driven pathways in cerebellar development, the modulation of synaptic neurotransmission, and cerebellar diseases, including cancer.[8]

Oestrogens exert their action through two oestrogen receptors, i.e. oestrogen receptor (ER)α and ERβ, that are encoded by separate genes. These two receptors are ligand-dependent transcription factors, and

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share both structural and functional homology. Several ERβ isoforms have been reported so far. ERβ1 is the wild-type receptor encoded by exons 1–8. ERβ2–ERβ5, although they share exons 1–7 with ERβ1, show sequences that are distinctive from that encoded by exon 8, a condition affecting their ability to activate ligand-dependent transcription. Thus, ERβ1 is thought to be the only fully functional isoform able to bind ligand, whereas ERβ2, ERβ4, and ERβ5 may control oestrogen activity through dimerization with ERβ1 or ERα.[9,10] Notably, however, some authors have suggested that ERβ2, ERβ4 and ERβ5 might directly participate in gene regulation, possibly through ligand-independent transcriptional properties.[11,12] Along with regulating oestrogen-responsive gene expression, oestrogens also exert non-genomic actions.[13]

Both ERα and ERβ are expressed in the cerebellum, and their expression varies with age.[8,14,15] During the early stages of cerebellar development, ERα is mostly confined to Purkinje cells, being involved in dendritic growth and synapse formation; in the adult, the protein is confined primarily to granule cells, albeit at very low concentrations. ERβ expression is also modulated by age, being higher in developing cerebellar neurons and glia during differentiation processes (after completion of mitosis), and then decreasing in mature cells. In the adult, ERβ is mainly expressed in Purkinje cells and in the granule cell layer.[8,14,15] No data are available on ERβ splice variant expression and function in cerebellar physiology and pathology.

In this context, over the last few years we have been investigating the role of oestrogens in MB tumorigenesis and progression. Using the murine heterozygous Patched1 knock-out mice (*Ptch1*$^{+/-}$, a preclinical model of radiation-induced MB), we showed that hormone deficiency induced by ovariectomy increased the susceptibility to MB development, whereas oestrogen replacement, possibly through ERβ-dependent signalling, restored tumour incidence to the values observed in control females.[16,17] More recently, we demonstrated that D283 Med xenografts grew significantly less in nude female mice than in males, the latter also showing undifferentiated tumours.[18] In keeping with our results, Belcher *et al.*[19] also reported significantly more rapid D283 Med xenograft growth in intact males than in females. Notwithstanding this, they observed robust stimulation in both cell culture and xenografts following oestradiol treatment, an effect reversed by the anti-oestrogen drug Faslodex. Therefore, their conclusion pointed to a stimulatory effect of oestrogens on MB development, via ERβ-dependent mechanisms (which is opposite to our hypothesis).

Thus, whereas there is ample evidence overall for the potential influence of ER signalling on MB growth, further research is undoubtedly needed. To gain insights into this issue, the present study sought to evaluate the occurrence of sexual dimorphism in biological MB features in a cohort of male and female patients. Overall, the findings presented here are in line with current clinical evidence showing a considerable sex bias in the pathogenesis of MB.

Materials and methods

PATIENTS

This retrospective study included normal cerebellar tissues and MB samples obtained from the Department of Pathology, School of Medicine, Catholic University of the Sacred Heart Rome, Italy. MBs were histologically classified according to the 2007 World Health Organization classification.[20] The study involved 39 males and 25 females, with median ages of 10 years (range, 1–54 years) and 9 years (range, 1–50 years), respectively. Patient characteristics are summarized in Table 1. Clinical information was obtained from the existing medical records in accord with institutional guidelines, and all data were managed by the use of anonymous numerical codes. The study was approved by the local institutional review board.

IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis was carried out as previously reported.[21] The following antibodies were used: anti-ERα (clone 6F11; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100), anti-ERβ1 (clone PPG5/10; Dako, Glostrup, Denmark; dilution 1:50), anti-ERβ2 (clone 57/3; Serotec, Oxford, UK; dilution 1:100), anti-ERβ5 (clone 5/25; Serotec;

Table 1. Clinicopathological features of the overall series

Characteristics	All	Males	Females
Cases, no. (%)	64	39 (61)	25 (39)
Median age in years (range)	10 (1–54)	10 (1–54)	9 (1–50)
Histotype, no. (%)			
Desmoplastic	11 (17.2)	5 (12.8)	6 (24)
Classic	35 (54.7)	22 (56.4)	13 (52)
Large-cell anaplastic	18 (28.1)	12 (30.8)	6 (24)

dilution 1:100), anti-androgen receptor (AR) (clone AR441; Abcam, Cambridge, UK; dilution 1:50), anti-Ki67 (clone MIB-1, M7240; Dako; dilution 1:50), anti-cleaved caspase-3 (CC3) (clone 5A1E; Cell Signaling Technology, Leiden, The Netherlands; dilution 1:100), and anti-CD31 (ready-to use, clone JC70A; Dako). The specificity of the antibodies against ERβ isoforms has been validated in neutralization studies using specific peptides for ERβ1, ERβ2, and ERβ5 (data not shown). Also, these antibodies have been widely used in clinical studies by our group and other groups.[21–29]

EVALUATION OF IMMUNOHISTOCHEMICAL DATA

Hormone receptor status was semiquantitatively assessed as previously reported.[21,30] Briefly, an immunohistochemical receptor score (IRS) (maximum value of 12) was calculated by multiplying the two factors obtained following categorization of the percentage of stained cells (0, negative; 1, 1–10%; 2, 11–33%; 3, 34–66%; and 4, 67–100%) and the intensity of staining (1, weak staining; 2, moderate staining; and 3, strong staining). Ki67 expression and CC3 expression were evaluated by considering the number of cells showing immunoreactivity in a minimum of 500 histologically identified neoplastic cells. For the quantitative analysis of microvessel density (MVD), CD31-positive intratumoral microvessels were counted blindly under a microscope field [×400 objective magnification; high-power field (HPF) area of 0.24 mm^2]. More than five tumour areas per section were evaluated. MVD was expressed as mean number of vessel per HPF. Immunohistochemical assessment was carried out by two investigators blinded to group.

STATISTICAL ANALYSIS

Hormone receptor score data were analysed for homogeneity of variance with Bartelett's test. If the group variance appeared to be homogeneous, a parametric ANOVA was used, followed by Tukey's test. If the variances were heterogeneous, log or reciprocal transformations were performed in an attempt to stabilize the variances. If the variances remained heterogeneous, a non-parametric test such as the Kruskall–Wallis test, followed by Dunn's multiple comparison test, was used. The remaining data were analysed for homogeneity of variance with an *F*-test. If the variances were heterogeneous, log or reciprocal transformations were performed in an attempt to stabilize the variances, followed by Student's *t*-test. If the variances remained heterogeneous, a non-parametric test such as the Mann–Whitney *U*-test was used. Data are reported as mean ± standard error of the mean. *P*-values are for two-sided tests; *P*-values of ≤0.05 were considered to be statistically significant. All statistical analyses were performed with GRAPHPAD PRISM5 (GraphPad, San Diego, CA, USA).

Results

PROLIFERATION AND APOPTOSIS

Analyses of cell turnover (programmed cell death and proliferation) have been reported to provide insights into tumour doubling time, prognosis, and treatment response. To determine whether cell turnover was indeed different between male and female MB patients, we carried out immunohistochemical analyses on tumour sections to evaluate proliferation (Ki67) and apoptotic rate (CC3) in our cohort (Figure 1). Tumour proliferation did not appear to be significantly different between the sexes (39.2 ± 3.0 and 41.3 ± 4.4 in males and females, respectively). Conversely, significantly higher CC3 levels were detected in females than in males (16.4 ± 4.0 and 5.7 ± 1.5, respectively, $P < 0.05$).

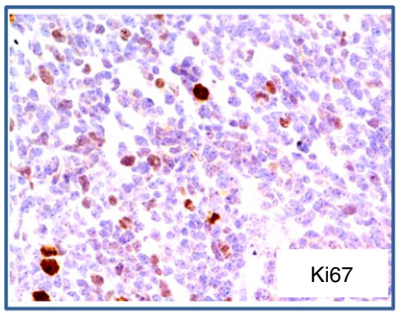
We then evaluated the ratio between CC3 and Ki67 values for each patient, as the imbalance between apoptosis and cell proliferation may contribute to tumorigenesis, and it is actually considered to be one of the hallmarks of cancer. Notably, we found that the CC3/Ki67 ratio was significantly higher in females than in males (0.37 ± 0.08 and 0.13 ± 0.02, respectively, $P < 0.01$), suggesting sex-specific biological features in MBs.

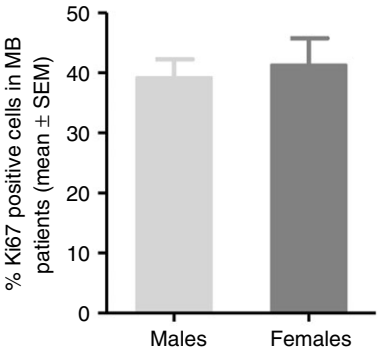
MVD

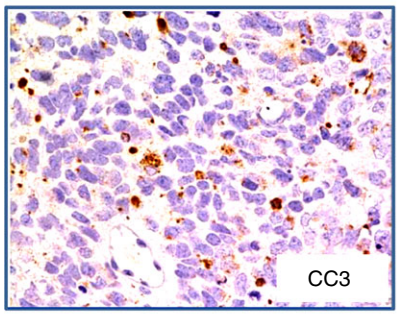
Angiogenesis has been shown to be required for tumour growth and metastasis, and to be correlated with prognosis and survival for many tumours, including MBs.[31,32] We thus assessed MVD in tumours by using CD31, a specific and sensitive endothelial marker for formalin-fixed paraffin-embedded tissues.[33] The results demonstrated significantly lower intratumoral MVD in females than in males (14.4 ± 2.2 and 20.2 ± 1.7 vessels/HPF, respectively, $P < 0.05$; Figure 2).

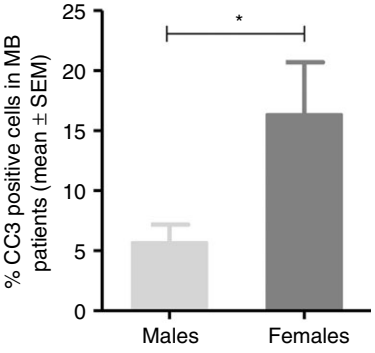
HORMONE RECEPTOR PROFILE OF NORMAL HUMAN CEREBELLUM

Table 2 and Figure 3 show the hormone receptor expression pattern in the normal human cerebella of five subjects (two males and three females). All tissues









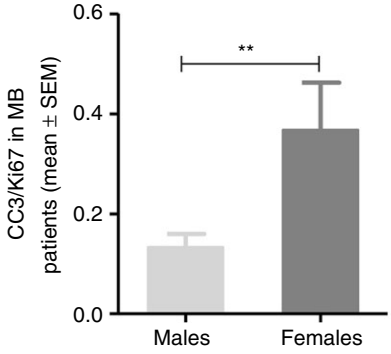
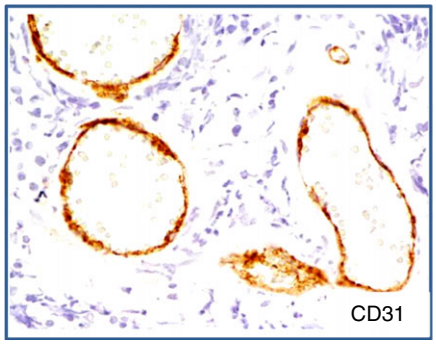


Figure 1. Immunostaining for Ki67 did not differ between male and female medulloblastomas (MBs). Immunostaining for cleaved caspase-3 (CC3) was significantly higher in females than in males (*$P < 0.05$). All results are expressed as the mean ± standard error of the mean; representative stained sections are shown. The cell apoptosis/proliferation ratio was significantly higher in female than in male MBs (**$P < 0.01$).



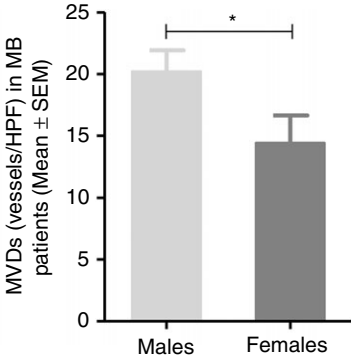


Figure 2. Immunostaining of medulloblastoma (MB) microvessels with the anti-CD31 antibody. The mean number of vessels per high-power field was significantly lower in female than in male MBs (*$P < 0.05$). All results are expressed as the mean ± standard error of the mean. MVD, microvessel density.

were negative for ERα, ERβ1, and AR. Conversely, ERβ2 was expressed in the nuclei of Purkinje cells and sporadically in granule neurons and molecular layer cells. High levels of staining were observed with anti-ERβ5 antibody in Purkinje cells, granule neurons, and molecular layer cells.

Table 2. Hormone receptor expression in normal human cerebella

Case	Sex	Age (years)	ERα	ERβ1	ERβ2	ERβ5	AR
1	M	39	NEG	NEG	+P, ±G, ±ML	+P, +G, +ML	NEG
2	M	57	NEG	NEG	+P, ±G, ±ML	+P, +G, +ML	NEG
3	F	52	NEG	NEG	+P, ±G, ±ML	+P, +G, +ML	NEG
4	F	57	NEG	NEG	+P, ±G, ±ML	+P, +G, +ML	NEG
5	F	78	NEG	NEG	+P, ±G, ±ML	+P, +G, +ML	NEG

AR, Androgen receptor; ER, Oestrogen receptor; F, Female; G, Granule neurons; M, Male; ML, Molecular layer; NEG, Negative staining in all cerebella layers; P, Purkinje cells; ±, Low positive staining in <10% of cells; +, Strong intensity in most cells.

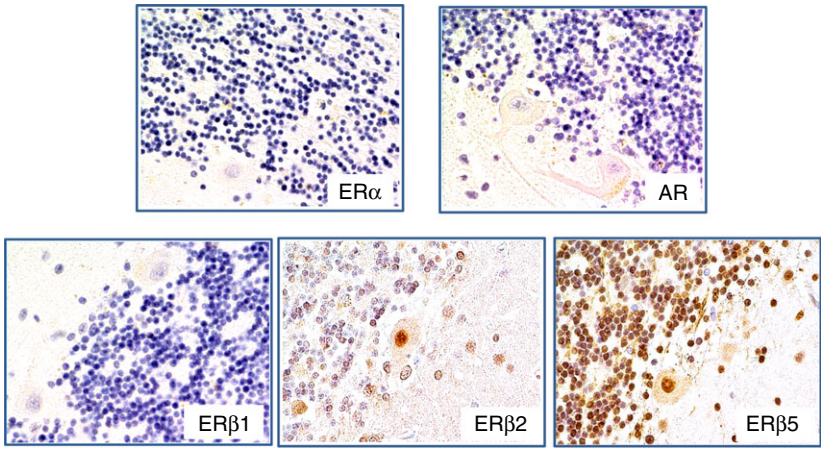


Figure 3. Representative images for oestrogen receptor (ER)α, ERβ1, ERβ2, ERβ5 and androgen receptor (AR) immunostaining in normal cerebellar tissues.

HORMONE RECEPTOR PROFILE OF MBS

Figure 4 shows representative images of hormone receptor tumour staining and levels of expression according to sex. All tumours were negative for ERα, and only three male patients of 64 were positive for AR. On the other hand, nuclear ERβs were detected in all patients, although with different expression levels. ERβ1 was the isoform that was least represented, with IRS values of 2.8 ± 0.4 and 2.0 ± 0.4 in males and females, respectively. ERβ2 was expressed at the highest levels in both sexes, with IRS values of 9.3 ± 0.5 and 9.4 ± 0.6. Intermediate levels were detected for ERβ5 in our cohort, with IRS values of 7.0 ± 0.5 and 6.9 ± 0.7. ERβ2 and ERβ5 levels showed a significant positive correlation in both sexes ($P < 0.01$). Overall, the distribution of hormone receptor immunopositivity was not different between male and female patients.

Discussion

In line with epidemiological data showing a male preponderance of MB in both adult and paediatric populations, along with a worse prognosis for males than for females, our previous studies in preclinical mouse models of MB suggested that endogenous oestrogens have a protective role against MB development.[16–18] Here, to gain insights into this issue, we examined cell turnover and MVD in MB clinical samples as biologically relevant markers of differences in outcome between the sexes.

The results demonstrated sex-specific biological features in MBs, with tumours from females showing a higher apoptosis/proliferation ratio, a finding suggesting that the mechanisms involved in cell turnover control operate more efficiently in females than in males. More importantly, although only limited differences were observed between sexes in the basal apoptotic levels, these may indeed be sufficient to cause different rates of response to treatment (radiation and/or drug-induced cytotoxicity), thus ultimately determining a different clinical outcome. Our data also showed decreased tumour vascularization in females as compared with males, in keeping with the concept that inhibition of angiogenesis can contribute to reduced cell proliferation and increased apoptosis.

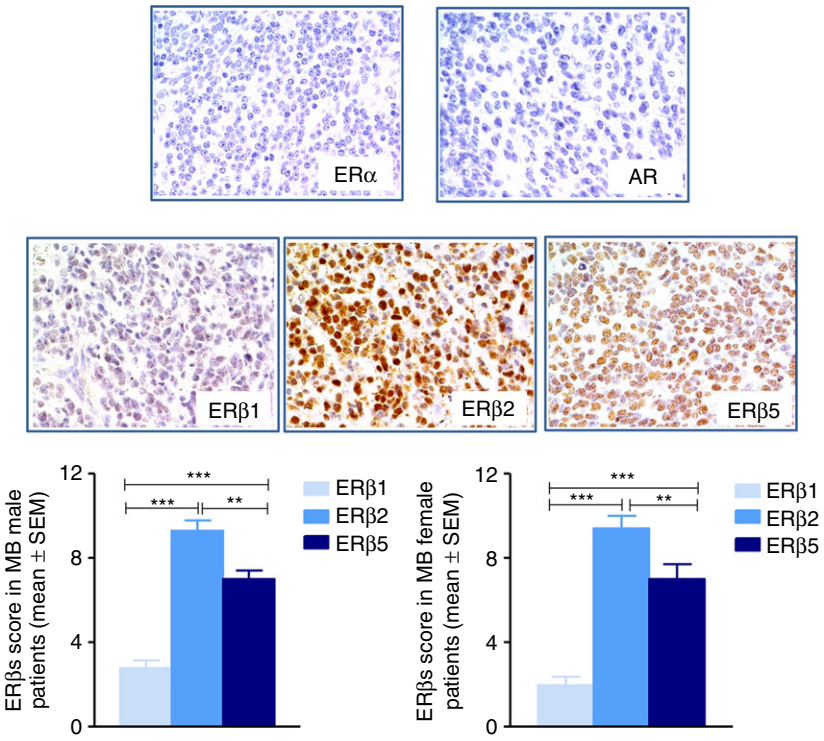


Figure 4. Representative images for oestrogen receptor (ER)α, ERβ1, ERβ2, ERβ5 and androgen receptor (AR) immunostaining in medulloblastoma patients, and a bar chart showing the distribution of ERβ isoforms stratified by sex. All results are expressed as the mean ± standard error of the mean; $^{**}P < 0.01$ and $^{***}P < 0.001$.

As far as we know, the results described here are the first to give molecular support to epidemiological data on sex differences in MB risk and prognosis.

The mechanisms underlying the role of sex in the pathophysiology of MB are not yet fully clarified, and to shed light on this we examined the sex steroid hormone receptor profiles of clinical MB samples, along with normal cerebella. Notably, whereas previous studies on clinical specimens have examined the expression of total ERβ, here we describe, for the first time, the ERβ isoform profiles of both tumours and normal tissues. Such a detailed characterization is indeed important, as there is evidence showing that the relative receptor expression levels in target cells may determine diverse cell responses. The results demonstrated that adult normal cerebellar tissues were almost completely negative for ERα, ERβ1, and AR, but showed immunostaining for ERβ2 and ERβ5. Conversely, MB samples showed positivity for three ERβ isoforms. Overall, our findings are in line with previous preclinical and clinical findings from our group and other groups,[16–19,34,35] indicating that only ERβ is expressed at detectable levels in normal cerebellum and MB samples, although limited references have also reported low expression of ERα and/or AR.[19,35] Notably, tumour levels of the different ERβ isoforms were not significantly different between the sexes. However, we observed an increase in ERβ1 expression from non-neoplastic cerebellum to MB samples, an increase that might occur as a self-protective mechanism against tumour proliferation. On the basis of these findings, and taking into account our previous preclinical results, we can speculate that circulating oestrogen levels are major determinants of the sexual dimorphism observed in MB features, possibly via ERβ1-mediated ligand-dependent mechanisms. Mechanistic studies do indeed support an antiproliferative and proapoptotic role of ERβ1, at least in some tumours,[10] and suggest a role for ERβ in mediating cell death in oestrogen-sensitive granule cell precursors,[14] the cells that are thought to give rise to MBs.[36,37] Also, as heterodimerization of ERβ5 with ERβ1 has been reported to significantly enhance the overall ligand-dependent activity,[38] sex-specific involvement of this ERβ isoform cannot be ruled out. Finally, other ligand-dependent mechanisms, such as tissue modulation of specific cofactors or changes in the dynamics of ER transcription complex assembly, could also contribute to the final effect observed.[39,40]

In line with our hypothesis on the role of circulating oestrogen in MB outcome, Curran *et al.*[7] actually reported that females with MB have a survival advantage only in older children (>3 years) and in adults. This suggests the existence of an interaction between sex and age with regard to outcome, possibly because of hormonal differences, among other factors. Indeed, endogenous oestrogen concentrations gradually rise in girls from infancy to late prepuberty,[41] and thus

significant differences between females and males only exist in children aged ≥4 years; in children aged <3 years, mean serum oestradiol levels are similar between sexes.[42]

Further evidence from studies in a larger cohort of patients should be sought to validate our hypothesis. Moreover, further stratification of patients by age could provide information on additional sex-specific features, such as differences in the tumour hormone receptor profile.

It is worth noting that, during the past few years, large-scale genomic and gene expression profile studies of primary MBs have revealed the existence of four distinct molecular subgroups: WNT, SHH, group 3, and group 4. MB subtypes differ in terms of genetics and clinical features, such as patient demographics and outcome, with a male preponderance being reported for all but the WNT group.[43] Specifically, whereas the male/female incidence ratio is almost 1:1 for the WNT subtype, for the SHH, group 3 and group 4 subtypes it increases to 1.5:1, 2:1, and 3:1, respectively.[43] Unfortunately, owing to the limited number of cases after stratification by sex, we were unable to perform a further molecular subgroup analysis in the present study. This could have added important information, as it is conceivable that such disease heterogeneity may result in intrinsic differences in the way in which oestrogens operate in the different molecular MB subgroups, ultimately controlling malignant progression. In this regard, it is interesting to note that our earlier preclinical studies did indeed show a protective oestrogen effect in both the SHH and group 4 mouse models of MB, in keeping with epidemiological data.[16–18,44] However, to fully uncover hidden elements of pathobiology in the molecular subtype-specific sex effects, an integrative multilevel and transdisciplinary research approach, involving molecular cell biology, preclinical and clinical studies, needs to be developed.

Collectively, our findings lend credence to the hypothesis that hormones have a significant impact on MB incidence and outcome, suggesting that the elucidation of the factors and pathways driving malignant progression could lead to the development of more targeted therapies and better risk stratification. Indeed, clinical trials on new treatments for MBs have shown limited efficacy to date.[45]

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Author contributions

G. F. Zannoni and D. Gallo conceived the study. G. F. Zannoni, A. Ciucci, G. Marucci and D. Gallo designed the study and analysed the data. A. Ciucci, D. Travaglia and E. Stigliano performed the experiments. G. F. Zannoni and D. Gallo wrote the manuscript. M. P. Foschini and G. Scambia provided conceptual advice. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest related to this work.

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